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Tumour necrosis factor receptor-associated periodic syndrome (TRAPS). Identification of novel *TNFRSF1A* mutations and intracellular signalling defects.

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Academic dissertation

To be publicly discussed with the permission of the Faculty of Medicine, University of Helsinki, in the Biomedicum Lecture Hall 3, Haartmaninkatu 8, Helsinki, on the 29th of August 2008, at 12 noon.

Helsinki 2008

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ISBN 978-952-92-4226-9 (paperback)
ISBN 978-952-10-4848-7 (PDF)
http://ethesis.helsinki.fi
Helsinki University Press
Helsinki 2008

To Mikko, Amos and Aino

To my family

LIST OF PUBLICATIONS

The thesis is based on the following original communications, referred to in the text as Roman numerals:

I. Nevala H*, Karenko L*, Stjernberg S*, Raatikainen M, Suomalainen H, Lagerstedt A, Rauta J, McDermott MF, Peterson P, Pettersson T, Ranki A. A novel mutation in the third extracellular domain of the tumor necrosis factor receptor 1 in a Finnish family with autosomal-dominant recurrent fever. Arthritis Rheum 2002;46:1061-6.
* equal contribution.

II. Stjernberg-Salmela S, Pettersson T, Karenko L, Blazevic V, Nevala H, Pitkänen S, Peterson P, Ranki A. A novel tumour necrosis factor receptor mutation in a Finnish family with periodic fever syndrome. Scand J Rheumatol 2004;33:140-4.

III. Stjernberg-Salmela S, Kivisaari A, Puolakkainen P, Färkkilä M, Kemppainen E, Ranki A, Pettersson T. Intra-abdominal abscess in a patient with tumour necrosis factor receptor-associated periodic fever syndrome. J Intern Med 2006;259:209-13.

IV. Stjernberg-Salmela S, Ranki A, Karenko L, Siitonen S, Mustonen H, Puolakkainen P, Sarna S, Pettersson T, Repo H. Low TNF-induced NF-κB and p38 phosphorylation levels in leukocytes in tumour necrosis factor receptor-associated periodic syndrome (TRAPS). Submitted.

In addition this thesis contains some previously unpublished data.

ABBREVIATIONS

AA= amyloid A
AP-1= activation protein-1
APAF= apoptotic protease activating factor 1
ARTS1= aminopeptidase regulator of TNFR1 shedding
ASC= apoptosis-associated speck-like protein with a caspase recruitment domain
ASK-1= apoptosis signal-regulating kinase -1
ATF-2= activating transcription factor-2
AUC= area under the curve
BRE= stress-responsive protein highly expressed in the brain and reproductive organs
CAPS= cryopyrin-associated periodic syndromes
CARD= caspase recruitment domain
CARD15= caspase recruitment domain-15 gene= *NOD2*
CATERPILLER= CARD-transcription enhancer, R-binding, pyrin, lots of leucine repeats
CD2BP1= CD2 binding protein-1= PSTPIP1
CHOP= C/EBP-homologous protein
cIAP= cellular inhibitor of apoptosis protein
CIAS1= cold-induced autoinflammatory syndrome-1 gene
CINCA= chronic infantile neurological cutaneous and articular syndrome= NOMID= neonatal-onset multisystem inflammatory disorder
CRD1= first cystein rich domain= PLAD= preligand assembly domain
CRP=C-reactive protein
DD= death domain
DED= death effector domain
ELISA= enzyme-linked immunosorbent assay
Elk-1= Eph-like kinase-1
EOS= early-onset sarcoidosis
ER= endoplasmic reticulum
ESR= mean erythrocyte sedimentation rate
eTNFR1= exosome-associated tumour necrosis factor receptor type 1
FACS= fluorescence-activated cell sorter
FADD= fas-associated death domain
FCAS= familial cold autoinflammatory syndrome= FCU= familial cold urticaria
FHF= familial Hibernian fever
FMF= familial Mediterranean fever
FOH= farnesol
GOOH= geranylgeraniol
GTP= guanosine triphosphate
HIDS= hyperimmunoglobulinemia D with periodic fever syndrome
HMG-CoA = 3-hydroxy-3-metylglutaryl-CoA
Hsp90= heat shock protein-90
IFNγ= interferon gamma
Ig= immunoglobulin
IκB= inhibitor of kappa B
IKK= inhibitor of kappa B (IκB) kinase; α, β, γ
IL-1β= interleukin-1 beta
sJIA = systemic juvenile idiopathic arthritis
JNK= c-Jun N-terminal protein kinase= SAPK= stress-activated protein kinase
LPS= lipopolysaccharide

LRR= leucine-rich repeat
MA= mevalonic aciduria
MAPK= mitogen-activated protein kinase
MEFV= Mediterranean fever gene
MEKK= mitogen-activated protein kinase (MAPK) kinase (MEK) kinase= MAPKKK
MDP= muramyl dipeptide
MFI= mean fluorescence intensity
MIM= Mendelian inheritance of Man
MK= mevalonate kinase
MKD= mevalonate kinase deficiency
MVK= the gene coding for mevalonate kinase
MWS= Muckle-Wells syndrome
NACHT= neuronal apoptosis inhibitor protein, CIITA, HET-E, and TP-1= NOD= nucleotide-binding oligomerization domain
NAD= NACHT-associated domain
NALP3= cryopyrin= protein containing a NACHT domain, a leucine-rich repeat and a PYRIN domain
NEMO= NF-κB essential modulator= inhibitor of kappa B (IκB) kinase (IKK) γ
NF-κB= nuclear factor kappa B
NIK= NF-κB-inducing kinase
NOD-LRR= nucleotide-binding oligomerization domain-leucine-rich repeat
NSAIDs= nonsteroidal anti-inflammatory drugs
PAPA= pyogenic sterile arthritis, pyoderma gangrenosum and acne
PBS= phosphate buffered saline
PCR= polymerase chain reaction
PFAPA= periodic fever, aphthous stomatitis, pharyngitis, and adenitis syndrome
PMA= phorbol myristate acetate
PSTPIP1= proline serine threonine phosphatase-interacting protein 1= CD2BP1
PTP-HSCF= protein tyrosine phosphatase hematopoietic stem cell fraction
PTP-PEST= protein tyrosine phosphatases with a PEST
PYD= pyrin domain
PYPAF1= pyrin domain containing APAF-like protein
RFU= relative fluorescence units
RICK= receptor-interacting protein (RIP)-like interacting CARD kinase
RIP= receptor-interacting protein
SAA= serum amyloid A (AA)
SAPK= stress-activated protein kinase= JNK= c-Jun N-terminal protein kinase
SODD= silencer of death domain (DD)
SPRY= SPIa and ryanodine receptor
SREBPs= sterol regulatory element binding proteins
TAK= transforming growth factor (TGF) activated kinase
TGF-β= transforming growth factor-β
TLR2= Toll-like receptor 2
sTNFR1= soluble tumour necrosis factor receptor type 1
TNFα= tumour necrosis factor alpha
TNFRSF1A= TNFR1= CD120a= p55TNFR= tumour necrosis factor (TNF) receptor type 1
TNFRSF1B= TNFR2= CD120b= p75TNFR= tumour necrosis factor (TNF) receptor type 2
TRADD= tumour necrosis factor (TNF)-associated death domain
TRAF= tumour necrosis factor (TNF)-associated factors
TRAPS= tumour necrosis factor (TNF) receptor-associated periodic syndrome

WASP= Wiscott-Aldrich syndrome protein
WT= wild-type

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ABSTRACT

The systemic autoinflammatory disorders are a group of rare diseases characterized by periodically recurring episodes of acute inflammation and a rise in serum acute phase proteins, but with no signs of autoimmunity. At present eight hereditary syndromes are categorized as autoinflammatory, although the definition has also occasionally been extended to other inflammatory disorders, such as Crohn's disease. One of the autoinflammatory disorders is the autosomally dominantly inherited tumour necrosis factor receptor-associated periodic syndrome (TRAPS), which is caused by mutations in the gene encoding the tumour necrosis factor type 1 receptor (TNFRSF1A). In patients of Nordic descent, cases of TRAPS and of three other hereditary fevers, hyperimmunoglobulinemia D with periodic fever syndrome (HIDS), chronic infantile neurologic, cutaneous and articular syndrome (CINCA) and familial cold autoinflammatory syndrome (FCAS), have been reported, TRAPS being the most common of the four. Clinical characteristics of TRAPS are recurrent attacks of high spiking fever, associated with inflammation of serosal membranes and joints, myalgia, migratory rash and conjunctivitis or periorbital cellulitis. Systemic AA amyloidosis may occur as a sequel of the systemic inflammation.

The aim of this study was to investigate the genetic background of hereditary periodically occurring fever syndromes in Finnish patients, to explore the reliability of determining serum concentrations of soluble TNFRSF1A and metalloproteinase-induced TNFRSF1A shedding as helpful tools in differential diagnostics, as well as to study intracellular NF-κB signalling in an attempt to widen the knowledge of the pathomechanisms underlying TRAPS.

Genomic sequencing revealed two novel *TNFRSF1A* mutations, F112I and C73R, in two Finnish families. F112I was the first *TNFRSF1A* mutation to be reported in the third extracellular cysteine-rich domain of the gene and C73R was the third novel mutation to be reported in a Finnish family, with only one other *TNFRSF1A* mutation having been reported in the Nordic countries. We also presented a differential diagnostic problem in a TRAPS patient, emphasizing for the clinician the importance of differential diagnostic vigiliance in dealing with rare hereditary disorders. The underlying genetic disease of the patient both served as a misleading factor, which possibly postponed arrival at the correct diagnosis, but may also have predisposed to the pathologic condition, which led to a critical state of the patient. Using a method of flow cytometric analysis modified for the use on fresh whole blood, we studied intracellular signalling pathways in three Finnish TRAPS families with the F112I, C73R and the previously reported C88Y mutations. Evaluation of TNF-induced phosphorylation of NF-κB and p38, revealed low phosphorylation profiles in nine out of ten TRAPS patients in comparison to healthy control subjects.

This study shows that TRAPS is a diagnostic possibility in patients of Nordic descent, with symptoms of periodically recurring fever and inflammation of the serosa and joints. In particular in the case of a family history of febrile episodes, the possibility of TRAPS should be considered, if an etiology of autoimmune or infectious nature is excluded. The discovery of three different mutations in a population as small as the Finnish, reinforces the notion that the extracellular domain of *TNFRSF1A* is prone to be mutated at the entire stretch of its cysteine-rich domains and not only at a limited number of sites, suggesting the absence of a founder effect in TRAPS. This study also

demonstrates the challenges of clinical work in differentiating the symptoms of rare genetic disorders from those of other pathologic conditions and presents the possibility of an autoinflammatory disorder as being the underlying cause of severe clinical complications. Furthermore, functional studies of fresh blood leukocytes show that TRAPS is often associated with a low NF-κB and p38 phosphorylation profile, although low phosphorylation levels are not a requirement for the development of TRAPS. The aberrant signalling phenotype would suggest that the hyperinflammatory phenotype of TRAPS is the result of compensatory NF-κB-mediated regulatory mechanisms triggered by a deficiency of the innate immune response.

TABLE OF CONTENTS:

page

page

4. INTRODUCTION

The systemic autoinflammatory disorders are a group of hereditary diseases, characterized by periodically recurring inflammatory attacks, without any obvious external stimuli. A defining characteristic of these diseases is the absence of autoantibody formation or antigen-specific T-cell activation, which distinguishes the autoinflammatory disorders from the autoimmune diseases (McDermott et al., 1999; Galon et al., 2000; Hull et al., 2002a; Hull et al., 2003). Common features in all autoinflammatory disorders are recurring attacks of high fever, accompanied by inflammation of serosal membranes, muscular and articular involvement and a rise in the mean erythrocyte sedimentation rate (ESR) and serum acute phase proteins C-reactive protein (CRP) and serum amyloid A (SAA). In the medical literature, four hereditary periodic fever syndromes, identifed as autoinflammatory disorders, had been described by the end of the 1990s: Familial Mediterranean fever (FMF), hyperimmunoglobulinemia D with periodic fever syndrome (HIDS), tumour necrosis factor receptor-associated periodic syndrome (TRAPS) and Muckle-Wells syndrome (MWS) (Drenth et al., 2000). FMF and HIDS are inherited autosomally recessively, whereas TRAPS and MWS are inherited dominantly in the autosome. Subsequently, four additional autoinflammatory disorders have been added to the list: familial cold autoinflammatory syndrome (FCAS), formerly known as familial cold urticaria (FCU), chronic infantile neurological cutaneous and articular (CINCA) syndrome, also known as neonatal-onset multisystem inflammatory disorder (NOMID), pyogenic sterile arthritis in combination with pyoderma gangrenosum and acne (PAPA) and Blau syndrome or familial granulomatous arthritis (Hull et al., 2003; Stojanov and Kastner, 2005; Brydges and Kastner, 2006). The latter four disorders all have an autosomally dominant mode of inheritance. The term autoinflammatory disorder has also recently been extended to describe a number of other diseases, some of which are non-hereditary. The underlying causes of the systemic autoinflammatory disorders are mutations in six different genes, five of which code for proteins involved in the inflammatory reaction and one for an enzyme involved in the isoprenoid biosynthesis pathway. The pathomechanisms of the disorders, however, are still largely unknown.

In patients of European descent, dominantly inherited periodic fever was reported in 1964 (Nilsson and Floderus, 1964) and in 1968 (Bergman and Warmenius, 1968) in Swedish patients, in 1982 in a Scottish/Irish family (Williamson et al., 1982) and in 1985 (Välimäki et al., 1985), as well as in 1992 (Karenko et al., 1992), in a Finnish family. The genetic background of one dominantly inherited periodic fever syndrome was established in 1999, when an international research consortium localized the genetic defect to chromosome 12p13, to the gene coding for the tumour necrosis factor (TNF) receptor type 1 (TNFRSF1A) (McDermott et al., 1999), giving rise to the name TRAPS. The original work revealed six different *TNFRSF1A* missense mutations in seven different families, one of which was Finnish. Subsequently, the genetic background of the four other dominantly inherited systemic autoinflammatory syndromes and HIDS have been revealed, in addition to the previously discovered gene responsible for FMF.

The aims of this study were to determine the genetic background in Finnish families with hereditary periodic fever, to evaluate the reliability of clinical analyses as differential diagnostic methods in TRAPS and to study the pathomechanism of TRAPS, using functional analyses to evaluate intracellular nuclear factor kappa B (NF-κB) signalling. The methods used in this thesis include mutation detection by polymerase chain reaction (PCR) and sequence analysis, analysis by fluorescence-activated cell sorter (FACS) to evaluate shedding of TNFRSF1A from the cell surface and to determine intracellular levels of phosphorylated NF-κB and p38, enzyme-linked immunosorbent assay (ELISA) for the determination of soluble TNFRSF1A, immunohistochemistry to visualize intracellular phosphorylated NF-κB (pNF-κB) and p38 (pp38)

levels, electrophoretic mobility shift assay (EMSA) for the determination of pNF-κB activity, as well as a critical analysis of the clinical phenotypes associated with TRAPS.

5. REVIEW OF THE LITERATURE

5.1. The systemic autoinflammatory disorders

The systemic autoinflammatory disorders are a group of diseases, the unifying features of which are recurrent episodes of fever and inflammation without apparent external causes, as well as the absence of antigen-specific T-cell activation or high-titer autoantibody production (McDermott et al, 1999; Galon et al., 2000; Hull et al., 2002a; Hull et al., 2003). The systemic autoinflammatory disorders include familial Mediterranean fever (FMF), hyperimmunoglobulinemia D with periodic fever syndrome (HIDS), tumour necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), Muckle-Wells syndrome (MWS) (Drenth et al., 2000), familial cold autoinflammatory syndrome (FCAS), formerly known as familial cold urticaria (FCU), chronic infantile neurological cutaneous and articular (CINCA) syndrome (also known as neonatal-onset multisystem inflammatory disorder, NOMID), pyogenic sterile arthritis in combination with pyoderma gangrenosum and acne (PAPA), and Blau syndrome or familiar granulomatous arthritis (Hull et al., 2003; Brydges and Kastner, 2006). Recently, the term autoinflammatory has also been used to describe inflammatory diseases such as Crohn's disease, Behçet syndrome, periodic fever with aphthous stomatitis, pharyngitis and cervical adenopathy (PFAPA) (Marshall et al., 1987; Stojanov and Kastner, 2005), as well as gout and Schnitzler syndrome, which are non-hereditary (Simon and van der Meer, 2007; De Koning et al., 2007). Systemic juvenile idiopathic arthritis (sJIA) and adult-onset Still's disease are also systemic inflammatory diseases, characterized by fever, rashes, and arthritis (Church et al, 2007). The etiology of both is unknown, but the clinical manifestations ressemble those of the autoinflammatory disorders, which has led to argumentations for the classification of sJIA as an autoinflammatory disorder (McGonagle et al., 2006).

The systemic autoinflammatory disorders present variations in their clinical phenotype, but share some common features: recurring attacks of fever, inflammation of serosal membranes, muscular and articular involvement and different types of rash. In between the febrile episodes, asymptomatic, subclinical attacks, accompanied by a rise in acute phase reactants, may occur (Tunça et al., 1999; Hull et al., 2002a; Bentancur et al., 2004; Köklü et al, 2005; Lachmann et al., 2006). The chronic inflammatory state in association with the diseases may result in systemic amyloidosis as a result of the accumulation of the acute phase protein serum amyloid A (SAA) in the tissues (Buxbaum, 2004). SAA production by hepatocytes is stimulated by TNF or IL-6 (McDermott et al., 1999). Systemic amyloidosis is a common complication of FMF, TRAPS, MWS, and CINCA/NOMID (Dodé et al., 2002b; Aganna et al., 2002; Stojanov and Kastner, 2005), although amyloidosis has also been reported in HIDS (Obici et al., 2004; D'Osualdo et al., 2005; Lachmann et al., 2006) and FCAS (Thornton et al., 2007; Hoffman et al., 2001). The genetic background of the eight hereditary periodic fevers has been solved as the genetic defect underlying the syndromes has been localized to six different genes, five of which code for proteins involved in the inflammatory reaction and one for an enzyme involved in the cholesterol metabolism pathway (International FMF Consortium, 1997; French FMF Consortium, 1997; Houten et al., 1999; Drenth et al., 1999; McDermott et al., 1999; Cuisset et al., 1999; Hoffman et al., 2001; Feldmann et al., 2002; Miceli-Richard et al., 2001; Wang et al., 2002; Wise et al., 2002).

Table 1. The clinical characteristics of the main systemic autoinflammatory disorders.

Autoinflammatory disorder	Gene affected	Main clinical characteristics	Duration of the attacks	Differential diagnostic trait	Therapy	References
TRAPS	*TNFRSF1A* 12p13	Fever, abdominal pain, myositis, arthralgia, conjunctivitis, periorbital edema, peritonitis, pleuritis, migratory erythema	Several days to months	Periorbital edema, muscular pain, migratory rash	Corticosteroids; etanercept; anakinra	Brydges and Kastner, 2006; Kimberley et al, 2007; Hull et al., 2002a; Simon et al., 2004
FMF	*MEFV* 16p13	Fever, abdominal pain or peritonitis (85%), mono-articular arthritis, myalgia, erysipe-loid erythema, amyloidosis	2-3 days	Ethnic background (Arabic, Turkish, Armenian or non-Ashkenazi Jewish)	Colchicine; a possible response to anakinra, etanercept, infliximab, anakinra or prazosin	Aksentijevich et al., 1999; Drenth et al., 2001; Tunça et al., 1999; Nakamura et al., 2007
HIDS	*MVK* 12q24	Fever, abdominal pain, oligo-articular arthritis, maculo-papular erythema, lymphadenopathy	3-8 days	Lymphadenopathy; an increase in concentrations of mevalonic acid in the urine during attacks, often a rise in serum Ig D concentrations	NSAIDs; a possible response to corticosteroids, etanercept or anakinra	Drenth et al., 1994; Drenth et al., 1999; Houten et al., 1999; Arkwright et al., 2002; Stojanov et al., 2004b; Cailliez et al., 2006; Farasat S et al., 2008
MWS	*CIAS1* 1q44	Fever, arthralgia, sometimes arthritis, lymphadenopathy, sensori-neural deafness, amyloidosis (25%)	2-3 days	Sensori-neural deafness	A possible response to anakinra; corticosteroids, NSAIDs	Aganna et al., 2002a; Dodé et al., 2002; Hawkins et al., 2004; Stojanov and Kastner 2005; Alexander et al., 2005; Rynne et al., 2006; Aksentijevich et al., 2007; Farasat S et al., 2008
FCAS/FCU	*CIAS1* 1q44	Fever, urticaria-like rash, arthralgia, sometimes arthritis, headache, conjunctivitis	1-2 days	Attacks triggered by exposure to cold	NSAIDs; a possible response to corticosteroids or anakinra	Hoffman et al., 2004; Stojanov and Kastner, 2005; Aksentijevich et al., 2007; Farasat et al. 2008
CINCA/ NOMID	*CIAS1* 1q44	Fever, migratory rash, chronic aseptic meningitis, ocular changes, mental retardation	Conti-nuous	Neurological manifestations, deficient cartilage growth and other involvement of the joints	No established therapy; a possible response to anakinra, high-dose corticosteroids or a combination of corticosteroids and metotrexate	Prieur et al., 1981; Hoffman et al. 2001; Feldmann et al., 2002; Aksentijevich et al., 2005; Hill et al., 2007; Farasat et al., 2008

TNFRSF1A= tumour necrosis factor receptor type 1; *MEFV*= Mediterranean fever gene; *MVK*= mevalonate kinase gene; *CIAS1*= cold-induced autoinflammatory syndrome-1 gene; TRAPS= tumour necrosis factor receptor-associated periodic syndrome; FMF= familial Mediterranean fever; HIDS= hyperimmunoglobulinemia D with periodic fever syndrome; IgD= immunoglobulin D; MWS= Muckle-Wells syndrome; FCAS= familial cold autoinflammatory syndrome= FCU= familial cold urticaria; CINCA= chronic infantile neurological cutaneous and articular syndrome= NOMID= neonatal-onset multisystem inflammatory disorder. NSAIDs= nonsteroidal anti-inflammatory drugs. The table is adapted from Pettersson et al., 2006 and Farasat S et al., 2008.

5.1.1. Tumour necrosis factor receptor-associated periodic syndrome (TRAPS)

The most common of the dominantly inherited systemic autoinflammatory disorders is tumour necrosis factor receptor-associated periodic syndrome (TRAPS, Mendelian inheritance of Man (MIM) 142680), originally reported as familial Hibernian fever (FHF) in a Scottish (Williamson et al., 1982). TRAPS was initially described in families of European ancestry, but has subsequently been reported in various ethnic groups (Hull et al., 2002a**;** Trost et al., 2005; Tchernitchko et al., 2005). Typical symptoms in TRAPS are recurrent attacks of high fever and serosal inflammation in the form of abdominal and muscular pain, arthralgia, conjunctivitis or periorbital edema, pleurisy, and possibly migratory erythema, often located on the limbs (Brydges and Kastner, 2006; Kimberley et al., 2007). Manifestations, such as fasciitis, sacroiliitis and pericarditis have also been reported (Trost et al., 2005; Hull et al., 2002b). During the attacks, an acute phase response is observed, as indicated by a rise in the levels of ESR, CRP and SAA, as well as neutrophilia, thrombocytosis (Dodé et al., 2003; Stojanov and Kastner, 2005), and sometimes increased levels of IgA and IgD (Aganna et al., 2003; Rudofsky et al., 2006). An acute phase response may be observed also in between attacks, indicating the presence of a subclinical inflammatory state (Hull et al., 2002a). The attacks appear to occur without a clear external stimulus, although physical strain, heavy meals, hormonal changes and minor infections have been reported as predisposing factors (Karenko et al., 1992; Kriegel et al., 2003). The duration of the inflammatory attacks is longer than in FMF, HIDS, MWS or FCAS, often enduring over a week to months (Galon et al., 2000; Hull et al., 2002a; Brydges and Kastner, 2006). The symptoms usually appear in childhood, a recent study reporting an average age of onset as low as 3 years of age (Kimberley et al., 2007). Systemic AA amyloidosis develops in 10-25% of TRAPS patients (Galon et al., 2000; Aksentijevich et al., 2001; Drenth et al., 2001a).

The genetic defect underlying TRAPS was established as mutations in *TNFRSF1A*, located on chromosome 12p13 and coding for the TNF type 1 receptor (TNFRSF1A, CD120a, p55, TNFR1) (McDermott et al., 1999). To date more than 80 *TNFRSF1A* mutations have been reported, all mutations affecting the extracellular region of the transmembrane protein, the majority being located in exons 2-4 and 6, in the first, second and third cysteine-rich domains (INFEVERS database. URL: http//www.fmf.ign.cnrs.fr/infevers) (Figure 1). Mainly point mutations occur, with only four short deletions, one longer deletion of 27 bp and one insertion reported. Mutations affecting cysteine residues and thus preventing the formation of disulphide bonds have been associated with a more severe clinical picture, with an increased risk for amyloidosis, and a higher penetrance of the disease, than non-cysteine mutations (Aksentijevich et al., 2001). Certain *TNFRSF1A* mutations, in particular R92Q and P46L, have been shown to occur also in the healthy control population (Aksentijevich et al., 2001; D'Osualdo et al., 2006; Ravet et al., 2006; Tchernitchko et al., 2005), lending support to the theory that they may be polymorphisms providing some biologic advantage, rather than disease-causing mutations. The R92Q mutation does not appear to greatly affect TNFRSF1A activity, as cell surface expression of the receptor and TNF binding remain nearly intact (Huggins et al., 2004; Lobito et al., 2006; Rebelo et al., 2006; Todd et al., 2007). The R92Q mutation has also been associated with an increased risk for other disorders, such as rheumatoid arthritis or multiple sclerosis, but recent data decline any linkage of the mutation and these diseases (Dieude et al., 2007; Jenne et al., 2007). In some patients with periodic fever, mutations in both *TNFRSF1A* and *MEFV* have been detected (Booth et al., 2001; Aganna et al., 2004; Stojanov et al., 2004a).

The inflammatory episodes in TRAPS usually respond well to courses of glucocorticoids and non-steroidal anti-inflammatory drugs (NSAIDs), and in addition the TNFRSF1B:Fc fusion protein etanercept, has been proven to be effective in alleviating inflammatory symptoms (Hull et al.,

2002a; Hull et al., 2003) and in some cases, even in reducing the formation of amyloid in TRAPS patients with nephrotic syndrome (Drewe et al., 2000; Drewe et al., 2004). The use of etanercept has been reported to permit a reduction of glucocorticoid and nonsteroidal anti-inflammatory drug doses, although some TRAPS patients have symptoms resistant to etanercept (Church et al., 2006) and a rise in TNF-concentrations in the plasma has been observed in TRAPS patients on etanercept (Nowlan et al., 2006). One report describes the successful use of human interleukin-1 (IL-1) receptor antagonist anakinra in treating the inflammatory symptoms in a TRAPS patient (Simon et al., 2004).

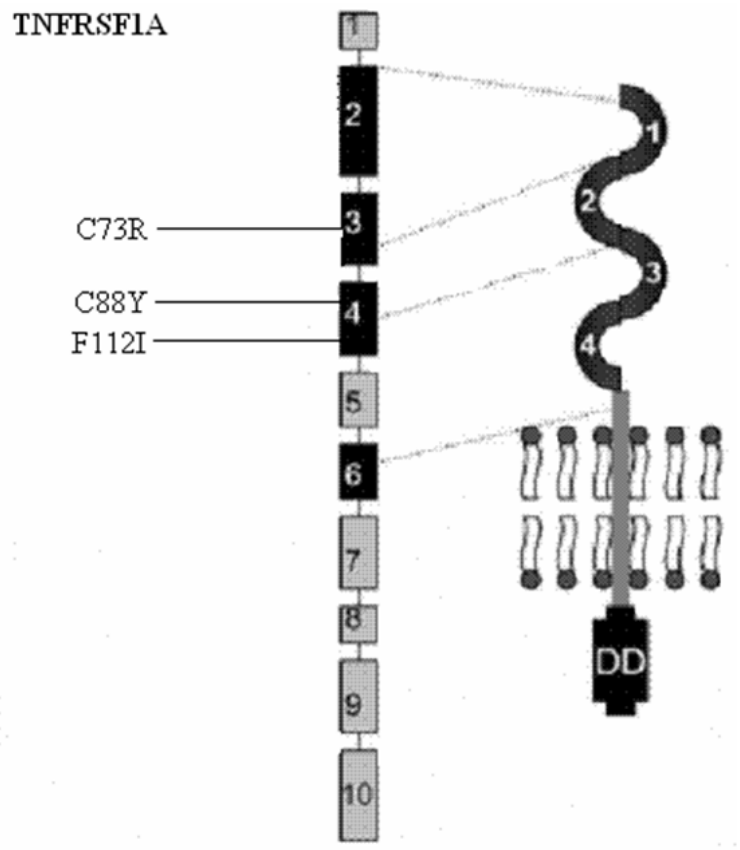

Figure 1. A schematic representation of the ten exons and the four cysteine-rich extracellular domains of TNFRSF1A showing the sites of the three *TNFRSF1A* mutations detected in the Finnish families. Exons 2-4 and 6, where most mutations are located, are represented with black. The figure is adapted from the figure presented by Stojanov and Kastner, 2005. DD= death domain

5.1.2. Familial Mediterranean fever (FMF)

Familial Mediterranean fever (FMF, MIM 249100) is the most common and thoroughly studied of the systemic autoinflammatory disorders. FMF has an autosomal recessive mode of inheritance and is common in populations originating around the Mediterranean Sea, in particular in non-Ashkenazi Jews, Armenians, Turks, and Arabs (Aksentijevich et al., 1999; Gershoni-Baruk et al., 2001), although the disease has been reported also in other populations (Shinozaki et al., 2002; Kotone-Miyahara et al., 2004). Typical symptoms in FMF include periodic attacks of fever and abdominal

pain lasting for 2-3 days. Other symptoms include arthralgia, pleurisy, myalgia, cutaneous involvement in the form of erysipeloid erythema, and in rare cases, pericarditis (Drenth et al., 2001). During the attacks, an acute phase response with an elevated ESR and increased levels of CRP, fibrinogen, interleukin 2 receptor (IL-2r), IL-6, IL-8, TNFα and interferon gamma (IFNγ) can be seen (Gang et al., 1999; Direskeneli et al., 1999; Baykal et al., 2003; Bagci et al., 2004; Köklü et al., 2005). The most severe complication of FMF is the development of systemic AA amyloidosis, which may occur prior to the occurrence of febrile episodes. This complication is believed to be due to the presence of asymptomatic subclinical attacks (Tunça et al., 1999; Hull et al., 2002a; Bentancur et al., 2004; Köklü et al, 2005; Korkmaz et al., 2002; Dzova et al., 2003; Notarnicola et al., 2002).

The genetic defect associated with FMF was established in 1997, as the causative gene was located to chromosome 16p13.3 (The International FMF consortium. 1997; The French FMF consortium. 1997). The gene, subsequently named the Mediterranean fever gene (*MEFV*), codes for pyrin (also known as marenostrin), a 781 amino acid protein expressed in granulocytes, dendritic cells, fibroblasts and activated monocytes (Centola et al., 2000; Matzner et al., 2000; Diaz et al, 2004). Pyrin plays a central role in regulating inflammation and apoptosis, through inhibition of the pro-inflammatory cytokine IL-1β and possibly of the transcription factor NF-κB (Chae et al., 2003; Chae et al., 2006; Dowds et al., 2003; Masumoto et al., 2003; Richards et al., 2001; Stehlik et al., 2002). At present, 117 different *MEFV* mutations have been reported, most mutations affecting exons 2, 3, 5 and 10 of the gene (INFEVERS database. URL: http//fmf.igh.cnrs.fr/infevers. Touitou, et al., 2004; Dodé et al., 2000b; Touitou et al., 2001). Despite FMF mainly being inherited recessively in the autosome, a third of all symptomatic FMF patients only possess a single *MEFV* mutation (Kastner et al., 2005; Kastner DL, 2005; Stojanov and Kastner, 2005) and FMF with an autosomal dominant mode of inheritance, in addition to the absence of a genetic defect in *TNFRSF1A* or *CIAS1*, has been reported (Aldea et al, 2004; Booth et al., 2000). The *MEFV* gene contains numerous polymorphisms, and some of them, such as E148Q, exist also in a large number of the Middle Eastern and Chinese control populations (Aksentijevich et al., 1999; Booth et al., 2001). E148Q homozygosity, however, has been reported to cause FMF, with a typical clinical picture (Topaloglu et al., 2005), and the presence of the E148Q variant has been associated with an increased risk for AA amyloidosis in certain chronic inflammatory disorders, such as rheumatoid arthritis, Crohn's disease and juvenile idiopathic arthritis (Aganna et al., 2004). The M694V *MEFV* mutation has been reported to be associated with an increased risk for systemic amyloidosis, especially in Jewish, Arab and Armenian FMF patients homozygous for this mutation (Pras et al., 1982; Cazeneuve et al., 1999).

Colchicine is effective in alleviating or preventing the febrile attacks in association with FMF, and has been shown to prevent the development of amyloidosis in FMF patients. In some patients with symptoms resistant to colchicine, the IL-1 receptor antagonist anakinra, TNF-inhibitors (etanercept, infliximab or thalidomide) or the catecholamine antagonist prazosin have been successfully used (Nakamura et al., 2007; Calligaris et al. 2007; Bhat et al., 2007). .

5.1.3. The cryopyrin-associated periodic syndromes (CAPS)

The cryopyrin-associated periodic syndromes (CAPS) or cryopyrinopathies signify three dominantly inherited autoinflammatory disorders: Muckle-Wells syndrome (MWS, MIM 191900), familial cold autoinflammatory syndrome (FCAS or FCU, MIM 120100), and chronic infantile neurologic, cutaneous and articular syndrome (CINCA syndrome), also called neonatal-onset multisystem inflammatory disorder (NOMID, MIM 607115) (Aksentijevich et al., 2002). The

cryopyrinopathies share the same genetic background, but are regarded as separate diseases, due to distinct clinical phenotypes. All three disorders present with varying degrees of fever, urticaria-like rash, and arthritis, FCAS having the mildest and CINCA/NOMID the most severe clinical phenotype (Aksentijevich et al., 2007). Acute phase reactants are markedly increased. FCAS and MWS are characterized by periodically occurring inflammatory attacks, whereas in CINCA/NOMID the inflammatory state is more or less continuous (Aksentijevich et al., 2007). MWS is characterized by inflammatory episodes, starting in childhood, and progressive sensorineural loss of hearing. In about 25 per cent of the patients, systemic amyloidosis results as a sequel of the disease (Aganna et al., 2002a; Dodé et al., 2002c). In FCAS, the inflammatory attacks are triggered by exposure to cold and usually have durations of one day. The symptoms appear before the age of 6 months and include headache, arthralgia, sometimes conjunctivitis (Stojanov and Kastner, 2005), as well as a rash, which resembles urticaria, but lacks mast-cell infiltration (Hoffman et al., 2001). Sensorineural loss of hearing occurs also in NOMID/CINCA, but in addition, symptoms include fever and migratory rash, appearing during the neonatal period or in early infancy. The symptoms develop into neurological manifestations, in the form of chronic aseptic meningitis, increased intracranial pressure, cerebral atrophy, accompanied by cerebral ventricular dilatation, seizures, ocular changes, and mental retardation (Prieur et al., 1981; Hoffman et al., 2001; Feldmann et al., 2002). Major features of the disease are also deficient cartilage growth, overgrowth of the patella and the epiphyses of the long bones, as well as involvement of other joints (Prieur et al., 1981; Aksentijevich et al., 2005; Hill et al., 2007). 20 per cent of patients with NOMID/CINCA die at childhood (Prieur et al., 1987; Hashkes et al., 1997).

Mutations in a common gene, *CIAS1*, located on chromosome 1q44 were established as the underlying causes of MWS and FCAS (Hoffman et al., 2001), and soon thereafter of CINCA/NOMID, as well (Feldmann et al., 2002). *CIAS1* has 9 exons and codes for cryopyrin (also known as PYPAF1 (pyrin domain containing APAF-like protein) or NALP3 (NACHT domain-, leucine-rich repeat- and pyrin domain containing protein) and belonging to the NOD-LRR (or CATERPILLER, NALP, PYPAF) proteins (Tschopp et al., 2004). At present, 85 true mutations have been reported, most affecting exon 3 of *CIAS1* (INFEVERS database. URL: fmf.igh.cnrs.fr/infevers). Although the cryopyrinopathies are inherited autosomally dominantly, the occurrence of spontaneous mutations, especially in CINCA/NOMID, is common, and in a large number of symptomatic patients, no *CIAS1* mutation has been identified (Aksentijevich et al., 2007). Cryopyrin is expressed in monocytes, granulocytes and chondrocytes (Manji et al., 2002; Feldmann et al., 2002) and is involved in the regulation of the immune reaction, in response to external stimuli, through formation of a protein-complex, named the inflammasome (Martinon et al., 2002; Agostini et al., 2004). The inflammasome has been shown to activate caspase-1, leading to the processing and secretion of the pro-inflammatory cytokines IL-1β and IL-18 (Gumucio et al., 2002; Kanneganti et al., 2006; Mariathasan et al., 2006; Martinon et al., 2006; Sutterwala et al., 2006).

NSAIDs are commonly used to relieve articular pain associated with FCAS and MWS (Farasat et al., 2008) and the inflammatory attacks also respond well to corticosteroids at high doses (Ormerod et al., 1993). The inflammatory symptoms in CINCA may be alleviated by high doses of corticosteroids (Farasat et al., 2008) and according to one report, by a combination of corticosteroids and methotrexate (Stojanov et al., 2004c). A large number of reports show that treatment with the IL-1 receptor antagonist anakinra has alleviated symptoms and repressed the acute phase response (Matsubayashi et al., 2004; Hawkins et al., 2004a; Hawkins et al., 2004b; Seitz et al., 2005; Boschan et al., 2006; Goldbach-Mansky et al., 2006; Aksentijevich et al., 2007) in patients with all three forms of cryopyrinopathies. Anakinra has even improved visual (Alexander

et al., 2005) and hearing (Rynne et al., 2006) loss in MWS patients, as well as prevented the attacks induced by exposure to cold in FCAS (Hoffman et al., 2004).

5.1.4. Pyogenic sterile arthritis, pyoderma gangrenosum, and acne (PAPA syndrome)

Another inflammatory disorder with an autosomal dominant mode of inheritance was first described in 1997 (Lindor et al., 1997). The disorder is characterized by destructive inflammation of joints, skin and muscles, the symptoms appearing in childhood and recurring spontaneously or following minor trauma (Lindor et al. 1997; Wise et al., 2002). Neutrophil-rich, sterile infiltrates accumulate in the joints, and skin manifestations typically include pyoderma gangrenosum and cystic acne (McDermott et al., 2002; Shoham et al., 2003).

PAPA syndrome (MIM 604416) is caused by mutations in the gene coding for the CD2-binding protein 1 (CD2BP1), also known as the proline-serine-threonine phosphatase interactin protein 1 (PSTPIP1), located on chromosome 15q24 (Yeon et al., 2000; Wise et al., 2002). To date five *CD2BP1* mutations have been reported (INFEVERS database. URL: http//www.fmf.ign.cnrs.fr/infevers). CD2BP1 is expressed in neutrophils (Hull et al., 2003) and is involved in the adaptive immune response, by regulating T-cell activity (Li et al., 1998). CD2BP1 has also been shown to interact with pyrin (Shoham et al., 2003), thereby linking it to the innate immune system. An increase in the production of IL-1β, as well as enhanced binding of CD2BP1 to pyrin, in association with mutations of *CD2BP1* have also been reported (Shoham et al., 2003).

The periodic joint inflammations require surgical drainage or intra-articular administration of glucocorticoids (Lindor et al., 1997; Wise et al., 2000; Cortis et al., 2004; Wise et al., 2002; Tallon et al., 2006). A favourable response to the IL-1 receptor antagonist anakinra on reducing the pain and swelling associated with the sterile arthritis in PAPA (Dierselhuis et al., 2005), and an ameliorating effect of the anti-TNF antibody infliximab on pyoderma gangrenosum in one PAPA patient (Stichweh et al., 2005), have been reported. One PAPA patient with increased levels of TNF-α was successfully treated with etanercept (Cortis et al., 2004)

5.1.5. Blau syndrome

Blau syndrome (MIM 186580) is a granulomatous disorder, affecting the joints, the skin and the eyes (Blau et al., 1985). Blau syndrome is inherited dominantly in the autosome and typical symptoms are granulomatous arthritis associated with rash and uveitis, with joint contractures as a long-term complication of the disease (Blau et al., 1985). Interstitial pneumonitis and lymphadenitis in association with Blau syndrome have also recently been reported (Becker et al., 2007). Blau syndrome has a clinical phenotype very similar to early-onset sarcoidosis (EOS, OMIM 609464), which occurs sporadically (Becker et al., 2005).

Blau syndrome is caused by mutations in the gene located on chromosome 16q12.1 and coding for the NOD2 or CARD15 protein (Miceli-Richard et al., 2001), the same gene which has been shown to be associated with an increased risk for Crohn's disease (Hugot et al., 2001; Ogura et al, 2001) and later proved to be responsible for EOS (Kanazawa et al., 2005). NOD2 consists of ten C-terminal LRR repeats, a NACHT domain and two N-terminal CARD domains (Ogura et al, 2001). Mutations in the NACHT domain of *NOD2*, appear to cause Blau syndrome or EOS, whereas mutations affecting the LRR motif may predispose to Crohn's disease (Hugot et al., 2001; Ogura et al., 2001; Miceli-Richard et al., 2001; Kanazawa et al., 2005). At the time of writing, six missense mutations, all in exon 4 of *NOD2*, have been reported in Blau syndrome (INFEVERS database.

URL: http//www.fmf.ign.cnrs.fr/infevers). The NOD2 protein is expressed in the intestine in the Paneth cells and in activated intestinal cells, as well as in myeloid cells (Ogura et al., 2003; Lala et al., 2003; Eckmann et al., 2005). NOD2 recognizes bacterial muramyl dipeptide (MDP) (Girardin et al., 2003; Inohara et al., 2003) and through interactions with several proteins (Eckmann et al., 2005), is involved in activating IκB kinase and NF-κB and subsequently the transcription of proinflammatory cytokines (Abbot et al., 2004; Barnich et al., 2005). MDP-independent NF-κB activation has been reported in Blau syndrome-associated *NOD2* mutations (Chamaillard et al., 2003) and increased basal levels of activated NF-κB in EOS-associated *NOD2* mutations (Kanazawa et al., 2005). In animal models, mutations in the LRR motif of *NOD2*, associated with Crohn's disease, show increased MDP-induced NF-κB activation and IL-1β secretion (Maeda et al., 2005), and NOD2 knock-out mice an increase in NF-κB activation, mediated through the Toll-like receptor 2 (TLR2), as well as an increase in T helper cell type 1 activity (Watanabe et al., 2004).

The inflammatory attacks in association with Blau syndrome have traditionally been treated with courses of corticosteroids and methotrexate, although treatment with the TNF inhibitor infliximab has also been reported as efficient in alleviating the inflammatory symptoms (Milman et al., 2006; Becker et al, 2007).

5.1.6. Hyperimmunoglobulinemia D with periodic fever syndrome (HIDS)

Hyperimmunoglobulinemia D with periodic fever syndrome (HIDS, MIM 260920) is another recessively inherited autoinflammatory disorder, which occurs mainly in patients of western European descent, but has been reported also in Italian and Albanian patients (D'Osualdo et al., 2005). HIDS is characterized by periodic attacks of fever, lymphadenopathy, splenomegaly, abdominal pain, arthralgia and maculo-papular rash, as well as continuous high concentrations of immunoglobulin D in the serum of many patients (Drenth et al., 1994) and a moderate increase in the concentrations of mevalonic acid in the urine during the febrile episodes (Drenth et al, 1999; Frenkel et al., 2001; Kelley and Takada, 2002; Mandey et al., 2006). High levels of IgA in the serum are often detected (Haraldsson et al., 1992; Klasen et al., 2001). The attacks last from four to eight days, recur every four to eight weeks and may be triggered by vaccination, physical or emotional stress, minor infections or may occur without predisposing factors (Drenth et al., 1994). The occurrence of amyloidosis is rare (Obici et al., 2004; Lachmann et al., 2006; Siewert et al., 2006).

The underlying genetic cause of HIDS is a deficiency in mevalonate kinase (MK) activity, caused by mutations affecting the mevalonate kinase gene (*MVK)*, which is located on chromosome 12q24 (Drenth et al, 1999; Houten et al, 1999). To date more than 80 mutations have been reported (Infevers database. URL: http//fmf.igh.cnrs.fr/infevers). Most patients are compound heterozygotes for two different mutations (Mandey et al., 2006), one of which is often V377I (Cuisset et al, 2001; Houten et al., 2001). Homozygosity for V377I is associated with a mild phenotype (Simon et al., 2003). Three patients with a mutation in *TNFRSF1A* in addition to one or two *MVK* mutations have been reported, two of which presented with a clinical phenotype more severe than patients with *MVK* mutations alone (Stojanov and Kastner, 2005; Arkwright et al., 2002; Stojanov et al., 2004b; Hoffman et al., 2005).

MK is an enzyme involved in the isoprenoid or cholesterol biosynthesis pathway (Goldstein and Brown. 1990). Mutations in *MVK* cause a 1-10 per cent decrease in MK activity (Houten et al., 1999; Houten et al., 2001; Cuisset et al., 2001), although isoprenylation of proteins remain normal in HIDS (Houten 2003a; Houten et al., 2003b). The correlation between the deficiency in the

enzymatic activity of MK and the inflammatory and febrile attacks associated with HIDS is yet unclear. Elevated IL-1β secretion has been demonstrated in HIDS patients (Drenth et al., 1995; Drenth et al., 1996; Frenkel et al., 2002), suggesting an association of this proinflammatory cytokine with the pathogenesis of HIDS. Another hypothesis is that a transient decrease in isoprenoid production, resulting in a deficient prenylation of guanosine triphosphate (GTP)-binding intracellular signalling molecules may be involved (Sinensky, 2000; Frenkel et al., 2002; Houten et al., 2003a).

With the discovery of the causative gene of HIDS, a connection to another familiar disorder, mevalonic aciduria (MA) (Hoffmann et al., 1986), also caused by mutations in *MVK* (Hoffmann et al., 1993) was made. MA is associated with a more severe phenotype than HIDS, involving mental retardation, ataxia, dysmorphic features and cataracts in addition to the febrile episodes. The febrile attacks may include vomiting and diarrhea, which often lead to death at infancy (Hoffmann et al., 1993). In MA, the MK activity is practically inexistent and the concentration of mevalonic acid in the urine, plasma and tissues of the patients are clearly higher than in HIDS (Hoffmann et al., 1993; Hinson et al., 1997; Houten et al., 1999). Due to the common genetic background, HIDS and MA have been grouped under the heading mevalonate kinase deficiency (MKD), with varying severity in the clinical phenotype (Houten et al., 2003b). In some cases, the same mutation has been demonstrated in both HIDS and MA patients, as is the case for the H20P, I268T, V310M, A334T and L264F mutations (Mandey et al., 2006).

There is no specific treatment for HIDS, but NSAIDs have been used to relieve symptoms in association with mild attacks (Farasat et al., 2008) and a favourable effect on the symptoms has been reported using the TNFRSF1B:Fc fusion protein etanercept (Arkwright et al., 2002; Takada et al., 2003; Demirkaya et al., 2007). According to another report, the IL-1-receptor antagonist anakinra also proved to be effective in preventing febrile attacks in association with HIDS (Cailliez et al., 2006). Two patients with a *TNFRSF1A* mutation in addition to *MVK* mutations have been more responsive to corticosteroids (Stojanov et al., 2004b) and etanercept (Arkwright et al., 2002) than HIDS patients with *MVK* mutations alone.

5.2. Functions of the affected genes in relation to inflammation

5.2.1. TNFRSF1A

5.2.1.1. Tumour necrosis factor alpha (TNFα) and its receptors

Tumour necrosis factor alpha (TNFα) is a multipotent cytokine discovered initially as a factor inducing necrosis in tumours (Carswell et al., 1975) and produced by a variety of cells, including activated macrophages, monocytes, lymphocytes, polymorphonuclear leukocytes and endothelial cells, as a 26-kDa transmembrane protein, which is cleaved to produce a 17-kDa soluble protein (Vassalli, 1992; Idriss et al., 2000). TNFα is also involved in inducing apoptosis, cellular proliferation and differentiation, inflammatory reactions, tumour growth, viral replication, as well as in monitoring immune cells (McEwan, 2002). TNFα mediates its biological actions as a homotrimer of 157 amino acid subunits (Chen et al., 2002). Monomers of TNFα form homotrimers, either in soluble form (Hakoshima et al., 1988) or intracellularly, prior to migrating to the cell membrane as membrane bound TNFα (mTNFα) homotrimers (Tang et al., 1996). TNFα is capable of binding to a number of receptors, but two TNF-specific receptors have been identified; TNFRSF1A (TNFR1, p55TNFR, CD120a) and TNFRSF1B (TNFR2, p75TNFR, CD120b).

However, experimental data shows that the majority of the biological action of TNFα is mediated through TNFRSF1A (Chen et al., 2002; McEwan, 2002). TNFRSF1A and TNFRSF1B are transmembrane glycoproteins, each containing a transmembrane region and four extracellular cysteine-rich domains (CRDs), the first cysteine-rich domain (CRD1) being called the preligand assembly domain (PLAD), allowing non-covalent interaction between the receptors and formation of homotrimers at the cell surface (Chan et al., 2000; Siegel et al., 2000; Miki et al., 2002), which is necessary for signal transduction. CRD2 and CRD3 interact with trimeric TNFα (Lobito et al., 2006; Banner et al., 1993), whereas CRD4 does not appear to be necessary for TNFα-binding (Corcoran et al., 1994). The extracellular domains of TNFRSF1A and TNFRSF1B are 28 per cent homologous (Corcoran et al., 1994; Naismith et al., 1998). Soluble and mTNFα bind to TNFRSF1A with equal affinity, and mTNF binds to TNFRSF1B with a comparable affinity as to TNFRSF1A, whereas soluble TNFα binds poorly to TNFRSF1B (Grell et al., 1995). The two TNF receptors differ in their intracellular structure. TNFRSF1A, but not TNFRSF1B, contains an intracellular death domain (DD) (McEwan, 2002; Tartaglia et al., 1993a), involved in the induction of pro-apoptotic signalling. Despite the absence of a DD, TNFRSF1B is capable of initiating apoptosis, possibly directly (Heller et al., 1992) by binding TNFα or by initiating expression of mTNFα, which in turn binds to and activates TNFRSF1A (Weiss et al., 1998). By directly binding TNF TNFRSF1B causes an increase of the TNF concentration also in proximity of TNFRSF1A, thus passing on TNFα to TNFRSF1A, through which apoptotic pathways are initiated (Tartaglia et al., 1993b; Tartaglia et al., 1993c).

5.2.1.2. TNFα-induced and TNFRSF1A-mediated signalling

The binding of TNFα homotrimers to the second or third cysteine-rich domains (CRD2 or CRD3) in the extracellular domain of trimeric TNFRSF1A initiates several intracellular signalling cascades (Figure 2). In the resting cell, the inhibitory protein, silencer of death domain (SODD), binds to the DD, preventing initiation of apoptosis (Jiang et al., 1999). Another protein, which negatively regulates TNFα-mediated signalling, is the stress-responsive protein highly expressed in the brain and reproductive organs (BRE), which binds specifically to the juxtamembrane region of TNFRSF1A (Gu et al., 1998). Upon binding of TNFα, SODD is released, allowing the adaptor protein TNF receptor-associated death domain (TRADD) to bind to the DD of TNFRSF1A (Hsu et al., 1995). The recruitment of additional molecules, TNF receptor-associated factor (TRAF2) (Wajant et al., 2001; Rothe et al., 1994), receptor-interacting protein (RIP) (Hsu et al., 1996) and Fas-associated death domain (FADD) (Hsu et al., 1996) to TNFRSF1A has been considered to occur through the mediation of TRADD. More recent data, however, demonstrate that the binding of RIP and TRAF-2 to membrane bound TNFRSF1A may occur without the recruitment of TRADD to the intracellular domain of the receptor and that the recruitment of TRADD to TNFRSF1A necessitates the internalization of the receptor into the cytoplasm (Schneider-Brachert et al., 2004).

TRAF2 interacts with several downstream signalling molecules, including MAPK kinase (MEK) kinases (MEKKs). TRAF2 binds to cellular inhibitor of apoptosis protein-1 (cIAP-1) and cIAP-2 (Boldin et al., 1996), which in turn bind to and inhibit cell death proteases caspase 3 and 7 (Roy et al., 1997), thereby hindering apoptosis.

TRAF2 also interacts with the inhibitor of kappa B kinase (IKK) complex (Devin et al., 2000; Zhang et al., 2000) at its catalytic subunits IKKα and IKKβ (Devin et al., 2001). After recruitment of IKK to TRAF2, RIP binds to the catalytic subunit of IKK (IKKγ, also called NEMO), thus activating the IKK complex, possibly through interaction with a MAPK kinase kinase 3 (MEKK3)

(Devin et al., 2000; Devin et al., 2001; Yang et al., 2001) or transforming growth factor-β (TGF-β)-activated kinase (TAK1) (Takaesu et al., 2003; Sato et al., 2005; Shim et al., 2005). IKKβ of the activated IKK complex phosphorylates inhibitor of kappa B (IκB) at its serine residues Ser-32 and Ser-36, resulting in its degradation and in the release of the transcription factor NF-κB, allowing its translocation to the nucleus (Neumann et al., 2007). NF-κB induces expression of pro-inflammatory cytokines, such as TNFα, anti-apoptotic mediators, such as cIAPs and TRAF2, as well as proteins involved in cell adhesion and leukocyte activation and migration (Karin and Lin, 2002; Yeung et al., 2004). TNFα-mediated NF-κB activation also requires the binding of TRAF2 family member associated NF-κB activator (TANK) to NEMO of the IKK complex through a zinc finger motif (Bonif et al., 2006), as well as the phosphorylation of histone H3 by IKKα in the nucleus (Anest et al., 2003; Yamamoto et al, 2003). The TNFα-induced and TNFRSF1A-mediated NF-κB activation is regarded as the conventional or canonical pathway, but another, non-conventional pathway of NF-κB activation also exists (Neumann et al., 2007; Perkins ND, 2007). The non-conventional pathway necessitates phosphorylation by IKKα homodimers of the NF-κB subunit p100, to allow its degradation to p52, but is independent of IKKβ or NEMO (Hayden, et al., 2004).

The binding of RIP to TRAF2 and the recruitment of MAPK kinase kinases (MAPKKKs) to RIP also activates p38 mitogen-activated protein kinase (p38-MAPK) pathways (Yuasa et al., 1998). p38 is a member of the MAPK family, comprising also the ERK and the c-Jun NH_2-terminal kinase/stress-activated protein kinase (JNK/SAPK) subfamilies (Winston et al., 1997). p38 is known to activate several transcription factors, such as IκB, activating transcription factor (ATF)-2, Eph-like kinase (Elk)-1, and C/EBP-homologous protein (CHOP) (Raingeaud et al., 1995; Wang et al., 1996; Brunet et al.,1996). These transcription factors induce proinflammatory cytokine expression, possibly through activating protein-1 (AP-1) (Baud et al., 2001) or NF-κB (Winston et al., 1997) pathways. p38 has also been reported to be involved in post-transcriptional regulation of gene expression (Winzen et al., 1999; Lasa et al., 2000) and in the induction of apoptosis (Xia et al., 1995).

The interaction of TRAF-2 with MEKK1 (Baud et al., 1999) or alternatively the MAPK kinase kinase apoptosis signal-regulating kinase-1 (ASK-1) (Nishitoh et al., 1998; Hoeflich et al. 1999) in response to stimulus by TNFα induces activation of the JNK/SAPK pathway (Minden et al., 1994), by activating MAPK kinase (MKK)-7. MKK-7 activates JNK through phosphorylation of the threonine-residue Thr180, preferentially in combination with phosphorylation of the JNK tyrosine residue Tyr182 by MKK-4 (Tournier et al., 2001). The activated JNK kinases translocate into the nucleus, where they activate transcription factors, including ATF-2 and the AP-1 subunit c-Jun (Natoli et al., 1997; Gupta et al., 1995). Other functions of JNK include post-transcriptional regulation of gene expression (Winzen et al., 1999; Lasa et al., 2000), cell proliferation (Sabapathy et al., 1999) and induction of apoptosis (Xia et al., 1995).

The recruitment of FADD to TRADD initiates a proapoptotic pathway, as caspases-8 and -10 interact with the DD-domain of the TRADD/FADD complex through their death effector domains (DED) (Locksley et al., 2001; Boldin et al., 1996; Muzio et al., 1996; Vincenz et al., 1997). Caspases-8 and -10, in addition to caspases-2 and -9, activate the downstream procaspases-3, -6 and -7, which trigger the pro-apoptotic cascade (Denecker et al, 2001). A study using different cell lines transfected with internalization-deficient TNFRSF1A demonstrated that the recruitment of TRADD, FADD and caspase-8 to TNFRSF1A requires the endocytosis of the TNF-binding receptor (Schneider-Brachert et al., 2004).

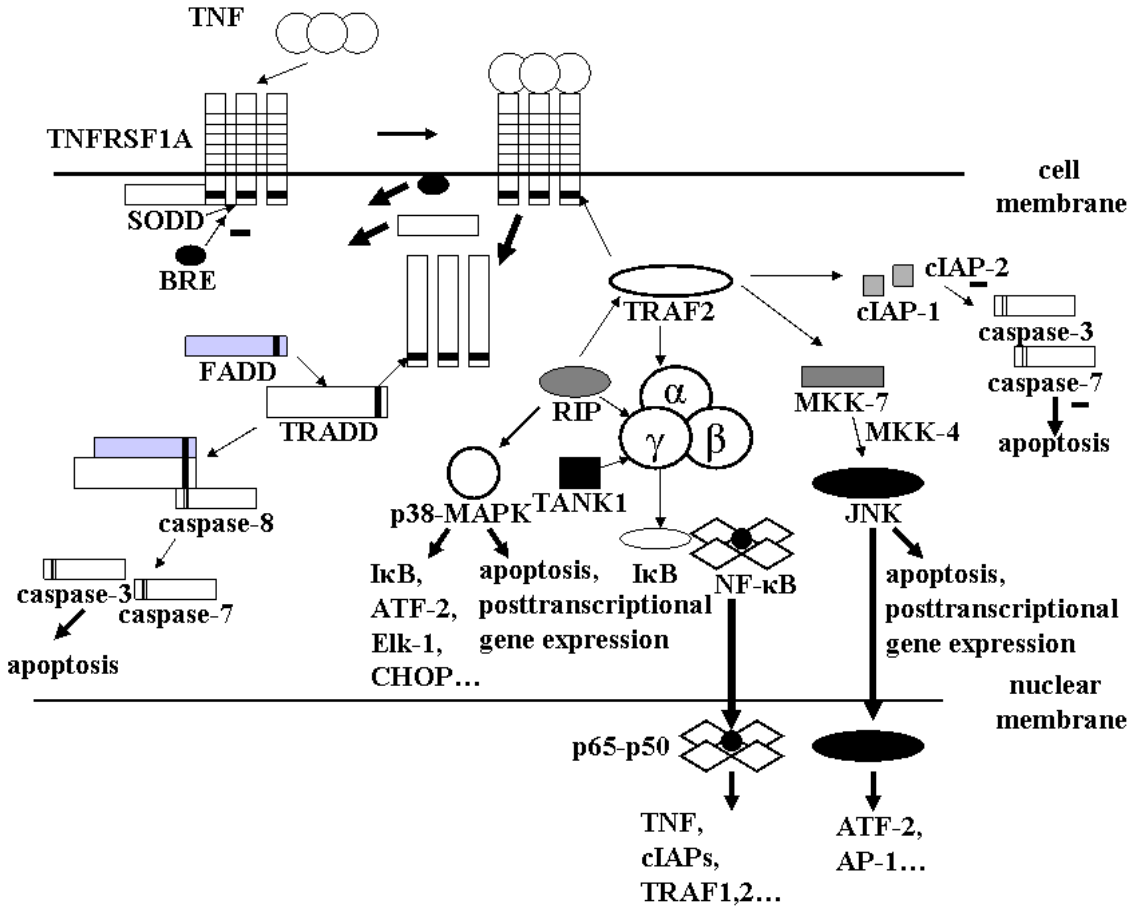

Figure 2. The main signalling pathways activated by TNFα-binding to TNFRSF1A. The binding of TNFα to TNFRSF1A causes the release of SODD (silencer of death domain) from the DD (death domain) of TNFRSF1A, allowing the binding of TRADD (TNF receptor-associated death domain) to the DD and subsequently the recruitment of adaptor proteins RIP (receptor-interacting protein), TRAF2 (TNF receptor-associated factor) and FADD (Fas-associated death domain). TRAF2 and RIP may also bind to TNFRSF1A directly. TRAF2 interacts with the IKK (inhibitor of kappa B kinase) complex, to which RIP is also recruited. As a result, IκB (inhibitor of kappa B) is phosphorylated allowing the release of NF-κB, which translocates to the nucleus to induce the expression of proinflammatory cytokines, including TNFα. Through its interaction with cIAP-1 and -2 (cellular inhibitor of apoptosis protein-1 and -2), TRAF2 promotes apoptosis. TRAF2 also interacts with MEKKs (MAPK kinase (MEK) kinase) to activate the JNK/SAPK (c-Jun NH_2-terminal kinase/stress-activated protein kinase) pathway, which stimulates transcription factors, cell proliferation, apoptosis and post-transcriptional regulation of gene expression. Furthermore, the interaction between RIP and TRAF2 and MAPKKKs (MAPK kinase kinases) activate p38-MAPK (p38 mitogen-activated protein kinase), which results in the activation of several transcription factors, IκB among others, which induce expression of proinflammatory cytokines, apoptosis and post-transcriptional regulation of gene expression. The figure is adapted from Stjernberg-Salmela et al., 2004.

5.2.1.3. TNFRSF1A in TRAPS

At present, the main mechanisms behind TRAPS are considered to be 1) reduced shedding of activated TNFRSF1A from the cell surface, 2) decreased cell-surface expression of TNFRSF1A, associated with 3) reduced TNFα-binding capacity and 4) intracellular retention of mutated TNFRSF1A, resulting in aberrant induction of apoptosis and transcription factor activation.

Following activation of TNFRSF1A, by binding of trimeric TNFα to the CRD2 or CRD3 of the receptor, ADAM17/TACE, a metalloprotease-disintegrin responsible also for cleaving TNF from the cell surface (Moss et al., 1997; Black et al., 1997; Reddy et al., 2000), cleave the transmembrane receptor at an extracellular region close to the cell membrane (Schall et al., 1990; Brakebush et al., 1994; Müllberg et al., 1995). As the receptor is released from the cell surface, the TNFα-induced signalling on the cell is disrupted and the soluble receptor may bind circulating TNFα, thus limiting the inflammatory reaction (Brydges and Kastner, 2006).

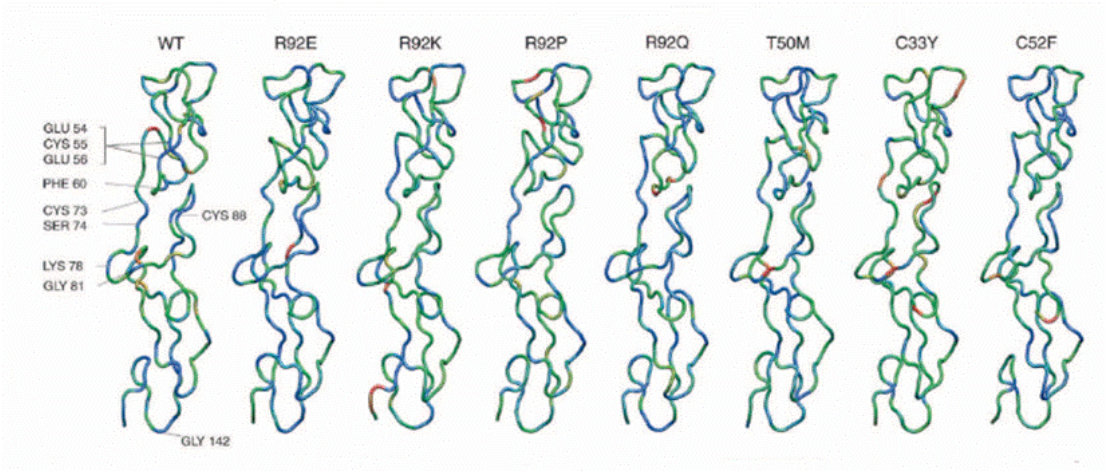

Figure 3. The structure of the extracellular domains of wild-type TNFRSF1A and the structural changes brought about by some TNFRSF1A mutations. The picture was published by Rebelo and co-workers, who produced models of TNFRSF1A mutants on which molecular dynamics simulations were performed (Rebelo et al., 2006). The colouring represents the average root-mean square distance of the residues in molecular dynamic simulations, with blue indicating the most stable residues, green indicating the residues with intermediate movement and red indicating the residues with the greatest movement during the simulation.

As all reported *TNFRSF1A* mutations in TRAPS affect the cysteine-rich extracellular domains, whereas no mutations have been reported in the transmembrane or intracellular regions, conformational changes, resulting in an inappropriately functioning receptor are believed to underlie the aberrant inflammatory reaction associated with TRAPS (Galon et al., 2000; Rebelo et al., 2006) (Figure 3). Low levels of soluble TNFRSF1A in the serum during the asymptomatic periods in between inflammatory attacks and only a minimal increase in sTNFRSF1A concentrations during inflammation was observed in the TRAPS patients initially studied (McDermott et al., 1999; Aganna et al., 2003).

Defective shedding of TNFRSF1A from the cell surface, following phorbol myristate acetate (PMA)-induced metalloprotease activation, leading to cleavage of the receptor, was also observed in association with the initially reported mutations (McDermott et al., 1999; Aksentijevich et al., 2001; Aganna et al., 2003; Xanthoulea et al., 2004). Reduced cleavage of the mutated receptor was

thought to result in a sustained TNF-mediated activation of the proinflammatory cascades and in a subsequent prolonged inflammatory reaction. The theory, referred to as the "shedding hypothesis" (Figure 4), was believed to explain, at least in part, the hyperinflammatory phenotype of TRAPS.

Subsequently, with an increasing number of novel *TNFRSF1A* mutations reported, normal receptor-shedding was observed in several mutations (Aksentijevich et al., 2001; Dodé et al, 2002; Aganna et al., 2003). A study by Huggins et al. (Huggins et al., 2004), using transfected HEK 293 cells, as well as leukocytes and dermal fibroblasts from TRAPS patients with the C33Y mutation, demonstrated a difference in TNFRSF1A shedding between cell types in the same form of TRAPS. Recent studies, using transfected HEK-293 cells, show that mutations affecting the CRD1, cause a decreased cell-surface expression of TNFRSF1A, with defective TNFα-binding, and an increased cytoplasmic retention of the misfolded receptor in the form of aggregates (Todd et al., 2004) (Figure 4). Signalling properties of the mutated TNFRSF1A in the cytoplasm remained intact, indicating the capacity of the receptor to function spontaneously, independent of ligand-binding. Other transfection experiments reinforce the results of increased intracellular retention of mutant TNFRSF1A, capable of maintaining signalling functions, despite reduced cell-surface expression and TNF-binding (Huggins et al., 2004; Rebelo et al., 2006; Todd et al., 2007).

Another study, using transfected cells and TNFRSF1A mutant "knock-in" mice for different mutations affecting the CRD1 and CRD2 (H22Y, C30S, C30R, C33G, C43S, T50M, C52F, C88R, R92P), showed that mutant TNFRSF1A is retained in the endoplasmic reticulum (ER), incapable of being secreted in soluble form or binding TNF (Lobito et al., 2006) (Figure 4). With the exception of the R92Q variant, mutant TNFRSF1A self-associate through disulphide bonds, whereas normal TNFRSF1A interactions occur noncovalently through the CRD1, but do not interact with or disrupt wild-type TNFRSF1A functions. The signalling functions of mutant TNFRSF1A are preserved, even though induction of apoptosis and NF-κB activation are reduced. The intracellular retention appeared to be more pronounced in the cysteine mutations.

An experiment using dermal fibroblasts from TRAPS patients with the C43S mutation demonstrated a reduction in TNF-induced apoptosis and NF-κB and AP-1 activation, in spite of which proinflammatory cytokine induction remained normal (Siebert et al., 2005). Based on the results, it was hypothesised that the reduction in apoptosis could result in prolonged cytokine production, leading to an exaggerated inflammatory reaction. On the other hand, a study using transfected 293T cells, report TNF-independent NF-κB activation and self-association of the TNFRSF1A in the case of the T50K TNFRSF1A mutation (Yousaf et al., 2005), suggesting that a sustained inflammatory phenotype does not necessitate direct interaction between TNF and TNFRSF1A.

The autoinflammation in TRAPS may thus result from changes in the structure of TNFRSF1A, resulting in reduced affinity for its ligand, a decrease in TNF-induced apoptosis, defective shedding of the receptor, or its misfolding and intracellular retention, leading to ligand-independent activation of the pro-inflammatory protein cascades. It is possible, however, that different TNFRSF1A mutations may involve different pathomechanisms, explaining the variety in the clinical phenotype and the experimental findings (Kimberley et al., 2007; Todd et al., 2007).

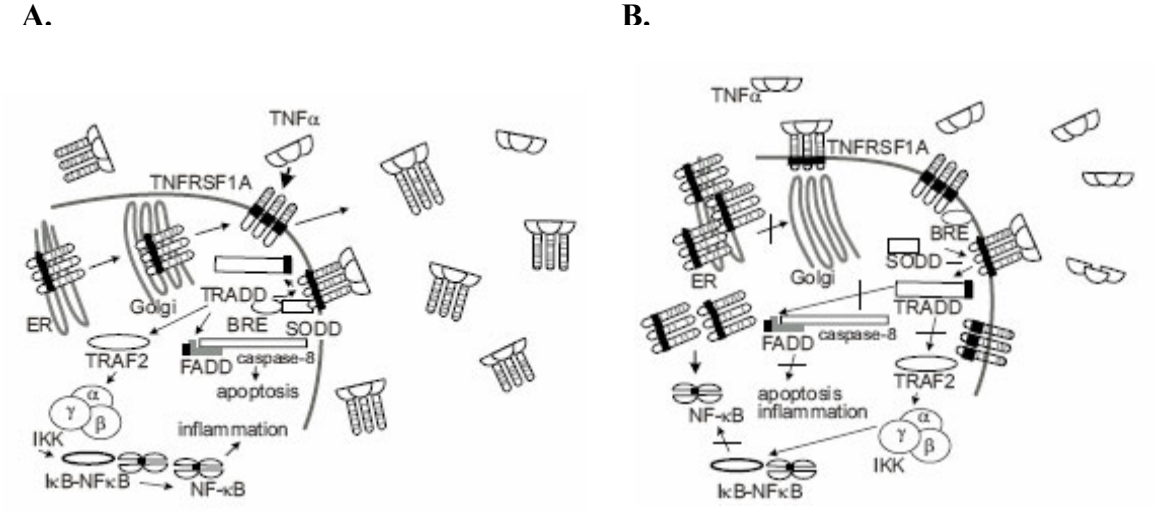

Figure 4. The assumed pathomechanisms behing TRAPS. The figure illustrates some of the intracellular signalling pathways activated by the interaction of TNFα with TNFRSF1A. Figure A represents the TNFα-induced intracellular protein cascades in a healthy individual. A large proportion of the transmembrane receptors are cleaved by metalloproteinases and released into soluble form, following binding of TNFα to the receptor. The soluble receptors compete with membrane-bound TNFRSF1A to bind TNF, thus limiting the proinflammatory effect induced by TNFα. Intracellularly, TNFRSF1A molecules are transported from the endoplasmic reticulum (ER) to the Golgi complex and subsequently to the cell membrane. The binding of TNFα to TNFRSF1A initiates signalling cascades inducing NF-κB activation and apoptosis. Figure B represents the TNF-induced signalling pathways in a patient with a TNFRSF1A mutation. A large proportion of the transmembrane receptors remain bound to the cell membrane, due to deficient cleavage. Subsequently, TNFα continues to induce its proinflammatory effects on TNFRSF1A, resulting in a proinflammatory phenotype. Mutant TNFRSF1A is retained intracellularly in the ER or as aggregates in the cytoplasm, causing TNFα-independent NF-κB activation. The transport of misfolded TNFRSF1A from the ER to the Golgi complex and to the cell membrane is inhibited, resulting in decreased TNFRSF1A cell surface expression and a subsequent impaired TNFα-induced activation of NF-κB and apoptosis. The figure is adapted from Stjernberg-Salmela et al., 2004, Stojanov and Kastner 2005 and Simon and van der Meer 2007.

5.2.2. The genes responsible for the other autoinflammatory disorders

5.2.2.1. *MEFV*

MEFV codes for the protein pyrin or marenostrin (International FMF consortium, 1997; French FMF consortium, 1997). Pyrin is a 781 amino acid protein, consisting of ten exons, and possessing four domains: a 92 amino acid N-terminal PYRIN domain (PYD) (Bertin et al., 1999; Martinon et al., 2001; Brydges and Kastner, 2006), a B-box zinc finger, a coiled-coil region and a C-terminal B30.2/rfp/SPIa and ryanodine receptor (SPRY)/domain (Vernet et al., 1993; Henry et al., 1998). Proteins sharing this structural motif are referred to as the CATERPILLER proteins (CARD-transcription enhancer, R-binding, pyrin, lots of leucine repeats) (Harton et al., 2002). The PYD belongs to the death domain superfamily (Fairbrother et al., 2001; Richards et al., 2001), in which the structure of six alpha helices, common to the protein family, permits the formation of electrostatic interactions with other proteins, although in PYD, the third alpha helix is unfolded

(Eliezer, 2003; Hiller et al., 2003). Pyrin interacts with the adaptor protein apoptosis-associated speck-like proteins with a CARD (ASC), which also contains an N-terminal PYD, through PYD-PYD interactions (Masumoto et al., 1999; Richards et al., 2001; Martinon et al., 2001; Dowds et al., 2003). The CARD of pyrin binds to procaspase-5 and the CARD of ASC binds to the CARD domain of procaspase-1 (Figure 5) (Martinon et al., 2002; Srinivasula et al., 2002).

Two different mechanisms behind the action of pyrin have been suggested (Simon and van der Meer, 2007) (Figure 5A and B). One suggested mechanism is that the binding of pyrin to ASC prevents ASC from interacting with cryopyrin in forming the NALP3 inflammasome (Chae et al., 2003), thereby inhibiting the activation of caspase-1 mediated by the cryopyrin inflammasome, and the subsequent activation and release of IL-1β. Chae and co-workers also demonstrated that pyrin is capable of binding pro-caspase-1 and caspase-1 independently of ASC, thereby inhibiting the caspase activity and the activation of IL-1β (Chae et al., 2006). Another recent study using transfected 293T cells shows that pyrin binds to caspase-1 and pro-IL-1β through its B30.2/rfp/SPRY domain (Papin et al., 2007), suggesting that pyrin has an inhibitory effect on the formation of active IL-1β. As most *MEFV* mutations affect the B30.2/rfp/SPRY domain, it has been proposed that these mutations disrupt binding of pyrin with other proteins (Simon and van der Meer, 2007; Goulielmos et al., 2006; Grütter et al., 2006; Woo et al., 2006), including the inhibitory effect of pyrin on inflammasome formation and IL-1β secretion, resulting in an inappropriate inflammatory phenotype (Stojanov and Kastner, 2005). An inhibitory effect of pyrin on inflammasome activity has been demonstrated in associated with two 30.2B/rfp/SPRY domain affecting *MEFV* mutations, M694V and M680I (Chae et al., 2006), using siRNA transfection of THP-1 cells, although this effect was not observed in the aforementioned transfection study (Papin et al., 2007).

Another mechanism of pyrin recently proposed, is that instead of interfering with inflammasome formation, wild-type pyrin is also capable of forming an inflammasome by interacting with ASC (Yu et al., 2006). The formation of the inflammasome results in caspase-1 activation by autocatalysis, as the procaspase-1 bound to ASC through CARD-CARD interactions interacts with another molecule of procaspase-1 (Chae et al., 2006). Caspase-1 cleaves the IL-1β precursor, to release the pro-inflammatory IL-1β in its active form (Srinivasula et al., 2002). The inflammasome theory designates pyrin a proinflammatory role, and the B30.2/rfp/SPRY domain has been suggested to be the site of binding of pathogens (Schaner et al., 2001; Yu et al., 2006), inducing formation of a pyrin-ASC-procaspase-1 inflammasome. Mutations affecting the *MEFV* B30.2/rfp/SPRY domain, might thus alter the binding capacity of the domain, resulting in an aberrantly strong interaction between pyrin and various pathogens and a subsequent exaggerated inflammatory reaction (Brydges and Kastner, 2006; Simon and van der Meer, 2007).

Some studies show a regulatory effect of pyrin on NF-κB activity, through interactions between ASC and the IKK complex, resulting in either NF-κB activation or inhibition (Stehlik et al., 2002; Dowds et al., 2003; Masumoto et al., 2003). The more recent study by Yu et al. (Yu et al., 2006), used transfected HEK293 cells to demonstrate that the interaction of pyrin, or cryopyrin, with ASC causes caspase-1 activation, but has no effect on NF-κB activity.

Pyrin may also affect apoptosis, although contradictory results have been reported. One study demonstrates a deficiency in apoptosis in pyrin-mutated mice (Chae et al., 2003), suggesting a proapoptotic role of pyrin, and thus enhanced inflammation as a result of prolonged cell survival in association with *MEFV* mutations. Several other studies, on the contrary, demonstrate an anti-apoptotic effect of wild type pyrin (Richards et al., 2001; Dowds et al., 2003; Masumoto et al., 2003).

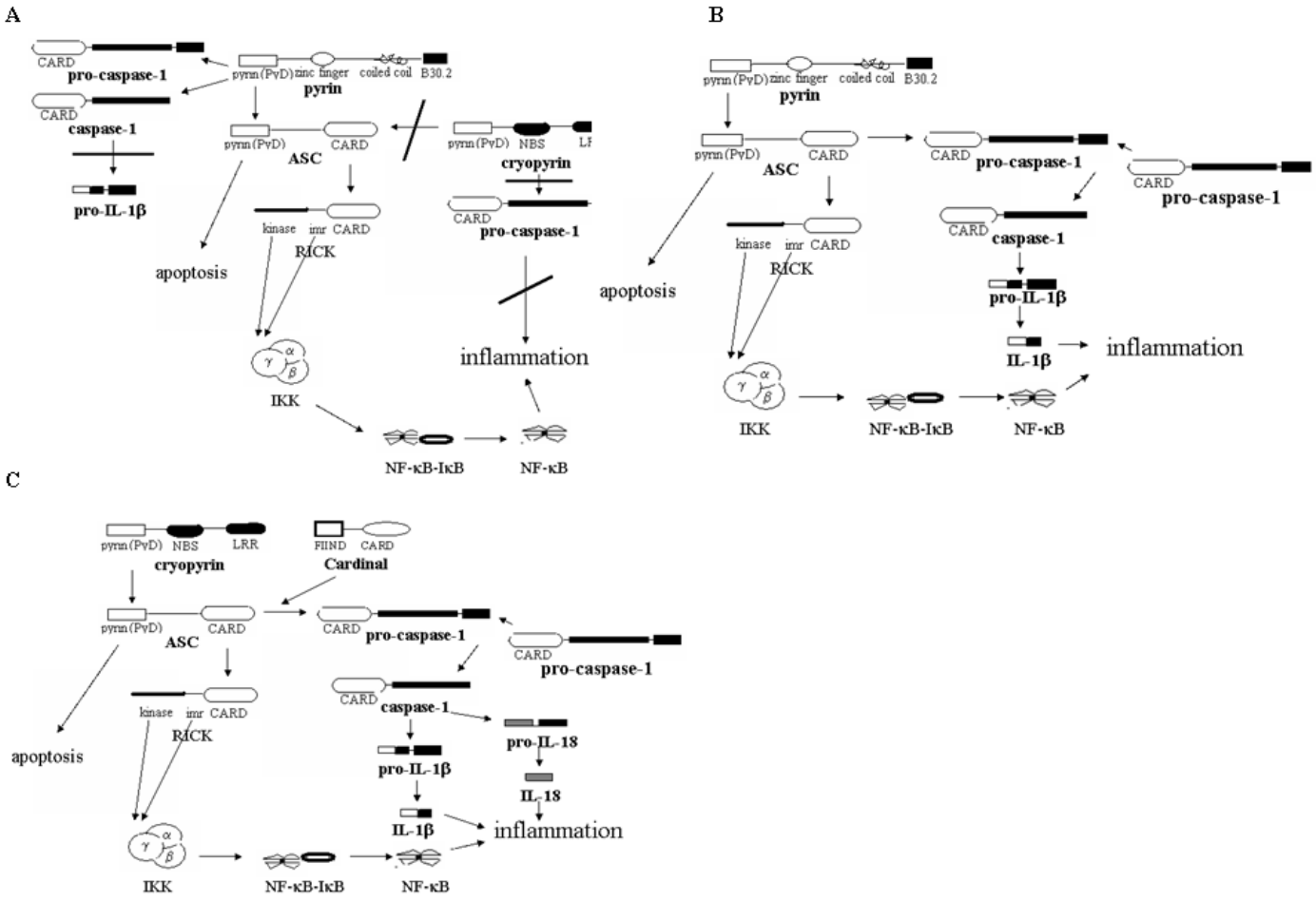

Figure 5. The pathways through which pyrin and cryopyrin are believed to regulate the inflammatory response. A) One theory suggests that the interaction of pyrin with ASC through PYD-PYD interactions, interferes with the interaction between ASC and cryopyrin, whereby the formation of the NALP3 inflammasome with subsequent pro-caspase 1-activation and IL-1β secretion is inhibited (Chae et al., 2003). Pyrin may also bind pro-caspase-1 and caspase-1 independently of ASC, disabling their activity and inhibiting pro-IL-1β processing (Chae et al., 2006). Mutations in B30.2/rfp/SPRY domain of *MEFV* would thereby alter the binding capacity of pyrin and thereby disrupt its regulatory effect on NALP3 inflammasome formation and IL-1β secretion (Stojanov and Kastner, 2005; Simon and van der Meer, 2007). B) Another theory suggests that pyrin is capable of forming an inflammasome of its own by interacting with ASC and pro-caspase-1 (Yu et al., 2006). The inflammasome induces pro-inflammatory reactions through caspase-1 activation (Srinivasula et al., 2002). Mutations in the B30.2/rfp/SPRY domain of *MEFV* would cause abnormally strong bonds between pyrin and pathogens, resulting in an enhanced pro-inflammatory phenotype (Brydges and Kastner, 2006; Simon and van der Meer, 2007). C) Cryopyrin binds to ASC through PYD-PYD interactions, and to Cardinal to form the NALP3 inflammasome (Agostini et al., 2004). The NALP3 inflammasome activates caspase-1, leading to IL-1β activation(Martinon et al., 2004; Mariathasan et al., 2006; Kanneganti et al., 2006; Martinon et al., 2006). Mutations in the NACHT domain of *CIAS1* are thought to disable the binding of the NACHT domain to the LRR stretch, thereby interfering with its autoregulatory effect on inflammasome formation and resulting in a hyperinflammatory reaction (Agostini et al., 2004). POP2 regulates NALP3 inflammasome formation through interactions with ASC (Dorfleutner et al., 2007). The effects of pyrin and cryopyrin on apoptosis and NF-κB activation remain unclear. The figures are modified from the picture published by Stjernberg-Salmela et al., 2004.

5.2.2.2. *CIAS1*

Another PYD containing protein, is cryopyrin, also known as NALP3 (Nacht domain-, leucine-rich repeat- and pyrin domain-containing protein) or PYPAF (pyrin domain-containing APAF-like protein), (Aganna et al., 2002a; Stehlik et al., 2004). Cryopyrin is encoded by *CIAS1*, consisting of nine exons. Being a member of the CATERPILLER protein family, cryopyrin is constituted of an N-terminal PYD, a NACHT (neuronal apoptosis inhibitor protein, CIITA, HET-E, and TP1) domain (Koonin et al., 2000) and a C-terminal LRR domain (Tschopp et al., 2003). The NACHT domain consists of 2-3 subdomains and a NACHT-associated domain (NAD) (Aksentijevich et al., 2007), and shares structural homology with the apoptotic protease activating factor 1 (APAF1) (Zou et al., 1997; Ting et al., 2006). APAF1 binds to cytochrome c to form an apoptosome, which binds to and activates procaspase 9 (Li et al., 1997; Ferraro et al., 2003), resulting in the activation of pro-apoptotic caspases-3 and -7 (Acehan et al., 2002). Cryopyrin interacts with ASC through PYD-PYD interactions and with another adaptor protein, Cardinal, to form an inflammasome (Agostini et al., 2004) (Figure 5C). Formation of the cryopyrin-inflammasome activates caspase-1 through pro-caspase-1 autocatalysis, with subsequent activation and release of IL-1β and IL-18, in response to bacterial peptidoglycans, antiviral compounds, ATP, and urate crystals (Martinon et al., 2004; Mariathasan et al., 2006; Kanneganti et al., 2006; Martinon et al. 2006).

The mutations associated with the cryopyrinopathies are mainly missense mutations in exon 3 of *CIAS1*, affecting the NACHT domain (Aksentijevich et al., 2007; INFEVERS database. URL: fmf.igh.cnrs.fr/infevers). It has been proposed that the LRR domains of unactivated cryopyrin may interact with its NACHT domain and exert an inhibitory effect on NACHT domain interactions with Cardinal (Agostini et al., 2004). Mutations in the NACHT-domain could thereby disrupt the autoinhibitory effect of the LRRs on inflammasome formation, resulting in an exaggerated inflammatory phenotype. Studies supporting this theory demonstrated high levels of IL-1β in monocytes of CINCA/NOMID patients (Aksentijevich I et al., 2002), as well as increased muramyl dipeptide (MDP)-triggered IL-1β secretion in a MWS patient (Martinon et al., 2004). Another study, using transfected cells, showed enhanced binding of mutant cryopyrin by its PYD to ASC, also in the absence of external stimuli, resulting in increased IL-1β release and NF-κB activation (Dowds et al., 2004). The effect of wild type cryopyrin on NF-κB activation is yet unclear, as different studies have demonstrated a stimulatory (Manji et al., 2002), an inhibitory (O'Connor et al., 2003), a variable (Stehlik et al., 2002), or no (Yu et al., 2006) effect of cryopyrin on the activation of NF-κB. It has also been hypothesized that *CIAS1* mutations may weaken the LRR-NACHT interaction in the cold, allowing inflammasome formation and IL-1β release in FCAS (Stojanov and Kastner, 2005).

The NALP3 inflammasome formation appears to be regulated by a cellular pyrin domain-only protein 2 (POP2), which binds to ASC and PYD-Nod like receptor 1 (PAN1) (Dorfleutner et al., 2007). POP2 also interferes with TNF-induced NF-κB nuclear trafficking and distribution, disrupting its transcriptional activity (Bedoya et al., 2007).

5.2.2.3. *CD2BP1*

CD2BP1 encodes for the CD2-binding protein (CD2BP1) (Li et al., 1998) or proline serine threonine phosphatase-interacting protein 1 (PSTPIP1) (Spencer et al., 1997; Wise et al., 2002). Three domains of PSTPIP1 have been characterized: an N-terminal Fer-CIP4 domain (Aspenström, 1997), a coiled-coil domain and a C-terminal SH3 domain (Shoham et al., 2003). Through the coiled-coil domain PSTPIP1 interacts with pyrin (Wise et al., 2002) or other proteins, such as

protein tyrosine phosphatases with a PEST (PTP-PESTs) domain (Spencer et al., 1997). The interaction with pyrin occurs at the pyrin B box zinc finger domain and necessitates also the PSTPIP1 SH3 domain (Shoham et al., 2003). The activity of PSTPIP1 appears to be regulated by the degree of phosphorylation of its tyrosine residue by a PTP-PEST named PTP-HSCF (PTP-hematopoietic stem cell fraction) (Spencer et al., 1997). PSTPIP1 is involved in the reorganisation of the actin cytoskeleton (Spencer et al., 1997; Fankhauser et al., 1995) and T-cell adhesion (Li et al., 1998; Badour et al., 2003), by mediating interactions between the T-cell receptor CD2 and the Wiscott-Aldrich syndrome protein (WASP) or between PTP-PESTs and WASP (Côté et al., 2002). The mutations associated with the PAPA syndrome affect the coiled-coil region of *CD2BP1* (Infevers), causing decreased interaction with PTP-PEST, leading to hyperphosphorylation of PSTPIP1 (Dowbenko et al., 1998; Cote et al., 2002; Wise et al. 2002) and increased binding to pyrin (Shoham et al., 2003). Transfection experiments also demonstrated an increase in IL-1β secretion, as well as an increase in IL-1β-induced IL-6 and IL-12p70 levels, and cultured peripheral blood mononuclear cells from a PAPA patient showed increased secretion of IL-1β (Shoham et al. 2003). Based on these findings, it has been hypothesized that the abnormal binding of mutant PSTPIP1 to pyrin prevents pyrin from binding other proteins, such as ASC, thus inhibiting the regulatory effect of pyrin on inflammation. It has also been proposed that PSPTPIP1 may affect T-cell mediated immune reactions, although mutations in PSPTPIP1 do not alter its interactions with CD2 or WASP (Wise et al., 2002).

5.2.2.4. *NOD2/CARD15*

NOD2/CARD15 (Miceli-Richard et al., 2001; Wang et al. 2002), encodes for NOD2, another protein in the CATERPILLER family (Ting et al., 2004; Ting et al 2006). NOD2 contains ten C-terminal LRR repeats, a NACHT domain and two N-terminal CARD domains (Ogura et al., 2001). NOD2 transcription is induced by TNF and IFN-γ in cultured cells and by inflammatory conditions in vivo (Rosenstiel et al., 2003). NOD2 is located in the cytoplasm and plays a role in the recognition of pathogenic components, in particular muramyl dipeptide (MDP), through its LRR domain (Girardin et al., 2003; Inohara et al., 2003). Through CARD-CARD interactions, NOD2 associates with RIP2/RICK protein kinase to activate IKK, by inducing IKK-γ ubiquitinylation at lysine 285, and subsequently NF-κB, which induces transcription of pro-inflammatory cytokines (Abbott et al., 2004; Eckmann et al., 2005). NOD2 may also induce NF-κB activation by interacting with other proteins, such as GRIM-19 in epithelial cells (Barnich et al. 2005), TGFβ-activated kinase 1 (TAK1) (Takaesu et al., 2003; Chen et al., 2004) in the presence of RIP2/RICK, or independently of RIP2/RICK (Girardin et al., 2003; Inohara et al., 2003). NOD2 also activates MAPK signalling pathways.

All *NOD2/CARD15* mutations associated with Blau syndrome, affect the NACHT-domain (INFEVERS database. URL: fmf.igh.cnrs.fr/infevers). An MDP-independent increase in the basal level NF-κB activity has been demonstrated in these NACHT-domain mutants (Chamaillard et al., 2003; Kanazawa et al., 2005), wherefore it has been proposed that the NACHT and LRR domains of unstimulated NOD2 could self-associate, as in the case of cryopyrin (Agostini et al., 2004), to prevent activation of the immune response, and that mutations in the NACHT domain could disrupt this self-association and its immunoregulatory effect.

5.2.2.5. *MVK*

Mevalonate kinase, an enzyme involved in the pathway of cholesterol and non-sterol isoprenoid biosynthesis, is encoded by *MVK*, located on chromosome 12q24 (Houten et al., 1999a; Drenth et al., 1999). Mevalonate kinase phosphorylates mevalonic acid in the pathway that produces cholesterol and isoprenoids, such as isoprenylated proteins, dolichol, isopentenyl transfer RNAs, ubiquinone 10 and heme A from acetyl-CoA (Figure 6) (Goldstein et al., 1990; Houten et al., 2003b; Brydges and Kastner, 2005; Simon and van der Meer, 2007). The isoprenoids have diverse functions in cellular processes, such as the isoprenylated guanylate-binding proteins, the expression of which is induced by LPS or IFN-γ (Nantais et al., 1996), or prenylated G-proteins involved in intracellular signalisation or organization of the cytoskeleton (Pendergast, 2000). 3-hydroxy-3-metylglutaryl-CoA (HMG-CoA) is derived from three molecules of acetyl-CoA in two steps, after which HMG-CoA is converted into mevalonic acid by HMG-CoA reductase, this reaction being the rate-limiting step of the pathway (Goldstein et al., 1990). Mevalonic acid is phosphorylated by mevalonate kinase, then by phosphomevalonate kinase, to yield 5-pyrophosphomevalonate. The activity of MVK is regulated at the transcriptional level by sterol regulatory element binding proteins (SREBPs) (Tanaka et al., 1990) and post-translationally by downstream intermediate products at the MVK ATP-binding site (Dorsey et al., 1968; Tanaka et al., 1990; Hinson et al, 1997).

In HIDS and MA patients, reduced mevalonate kinase activity (Hoffmann et al., 1993; Houten et al., 1999b; Cuisset et al., 2001), accompanied by an increased HMG-CoA reductase activity (Hoffmann et al., 1993; Kelley et al., 2001; Simon et al., 2004) have been demonstrated. In between febrile attacks, the impaired mevalonate kinase activity is thought to be compensated by an increase in mevalonic acid concentrations. An increase in temperature, as may occur as a result of physical exercise or fever, appears to further impair mevalonate kinase activity (Houten et al., 2002). It has been hypothesized that under such circumstances, the compensatory increase in intracellular mevalonic acid concentrations is insufficient for maintaining normal isoprenoid pathway function, due to mevalonic acid saturation, resulting in extracellular leakage of mevalonic acid. As a result, isoprenoid and cholesterol production decreases momentarily, followed by a reactive increase in HMG-CoA reductase and affecting possible anti-inflammatory cellular functions of the isoprenoids (Houten et al. 2003a; Houten et al., 2003b). Thus, the instantaneous deficiency in isoprenoids is hypothesized to cause the proinflammatory phenotype in HIDS and MA.

Increased levels of IL-1β have been demonstrated in culture of blood from HIDS patients drawn during febrile attacks or following stimulation with LPS (Drenth et al., 2005; Drenth et al., 2006), as well as an increase in IL-1β secretion in cultured cells from HIDS patients (Frenkel et al., 2002). IL-1β secretion was enhanced by treatment with statins, and decreased by farnesol (FOH), geranylgeraniol (GOOH), and mevalonic acid, suggesting that indeed the lack of isoprenoids and not an excess of mevalonic acid causes the inflammatory symptoms in HIDS. One report reveals that the inflammatory symptoms in three HIDS patients have successfully been treated with the IL-1a receptor antagonist anakinra (Bodar et al., 2006), supporting the proinflammatory role of IL-1β in mevalonic acid deficiencies.

The effect of statins have been shown to be either pro- (Frenkel et al., 2002; Coward et al. 2006; Montero et al., 2004) or anti-inflammatory (Jain et al., 2005; Nagashima et al., 2006), corresponding with variable clinical effects of statins in mevalonic acid deficiency (Hoffmann et al. 1993; Simon et al., 2004). Nonetheless, the exact mechanisms by which mutations in *MVK* cause inappropriate inflammation are yet unresolved.

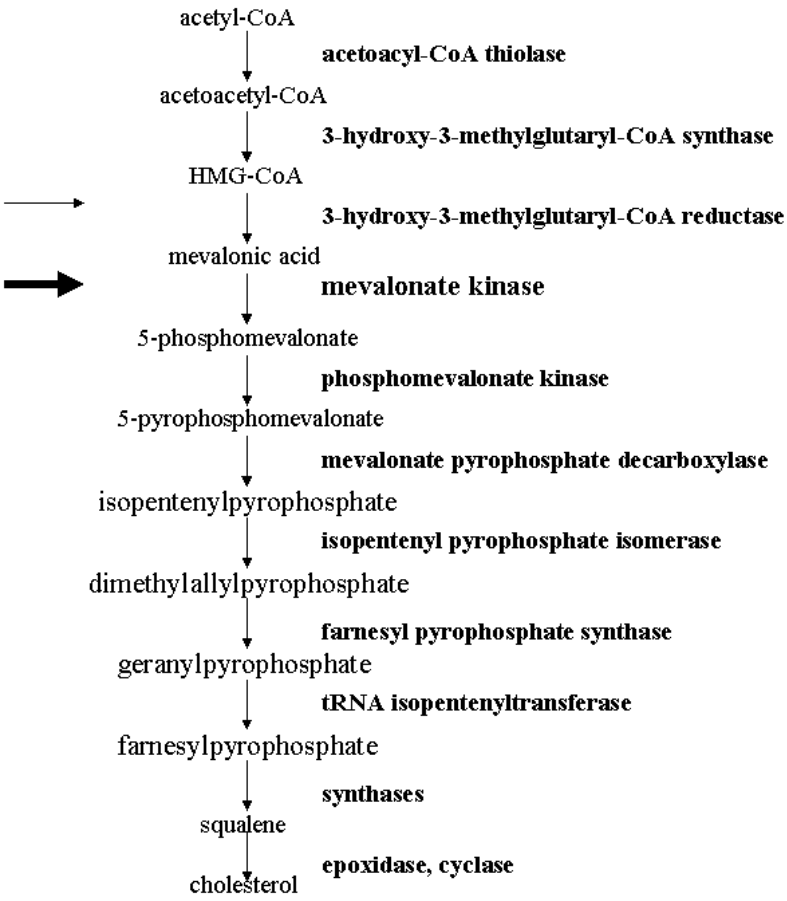

Figure 6. The pathway of isoprenoid biosynthesis. The thin arrow indicates the rate limiting step of the reaction, of which HMG-CoA reductase is the enzyme. The thick arrow indicates the step affected in HIDS and MA, where a deficiency in mevalonate kinase is caused by mutations of MVK. The figure is based on the figures published by Hoffmann et al., 1996, Frenkel et al., 2002 and Houten et al., 2003b.

6. AIMS OF THE STUDY

The entity of TRAPS was defined in 1999, when the underlying genetic defect was identified as missense mutations in the gene coding for the tumour necrosis factor receptor type 1 (TNFRSF1A) (McDermott et al., 1999). Six different missense mutations of *TNFRSF1A* were discovered in seven families, one of which was Finnish. This discovery led to the formation of the concept of autoinflammatory disorders.

The aims of this thesis were:

1) to establish the specific genetic cause of periodic fever in Finnish patients with a phenotype resembling TRAPS and a family history of febrile episodes, as well as to further delineate the clinical course of the disease,

2) to evaluate the clinical value of determining the soluble TNFRSF1A concentrations in serum of patients with TRAPS,

3) to investigate metalloproteinase-induced TNFRSF1A shedding in leukocytes from patients with TRAPS and

4) to study intracellular NF-κB activation, using functional analyses, in order to gain a deeper understanding of the pathomechanisms of TRAPS.

7. SUBJECTS AND METHODS

7.1. The Finnish families (I, II, III, IV)

In this thesis we have studied three Finnish families with dominantly inherited periodic fever. One family has been previously reported (McDermott et al., 1999), but was studied in the context of the functional analyses of the fourth article of the thesis (IV). Two families (Families 1 and 3) with hereditary periodic fever were revealed when patients with episodes of fever and inflammation of unknown origin were admitted to the Helsinki University Central Hospital. One family (Family 2) was brought to our knowledge when a family member with recurring inflammatory attacks contacted our clinic. In all three families, the periodic fever demonstrated a dominant mode of inheritance. Through the index patients, symptomatic and asymptomatic members of the same family were contacted and with the written and informed consent of each patient and healthy family member bloodsamples for scientific purposes were withdrawn. We have not systematically searched for potential patients for the study in hospital records.

In 1985, Välimäki and co-authors reported a Finnish family with periodically occurring attacks of fever and abdominal pain (Välimäki et al., 1985). The clinical picture mimicked that of familial Mediterranean fever (FMF), but the duration of the attacks in the Finnish patients was longer than the duration typically seen in FMF. A subsequent report by Karenko and co-workers established an autosomal dominant mode of inheritance in the same Finnish family with recurring fever (Karenko et al., 1992). The genetic background of the dominantly inherited periodic fever was established in 1999 by McDermott and co-workers, who localized the causative gene to chromosome 12p13, using linkage analysis (McDermott et al., 1999). An interesting candidate gene in this chromosomal area was the one coding for the tumour necrosis factor (TNF) receptor type 1 (TNFRSF1A). In seven families, one of which was Finnish, six different missense mutations of *TNFRSF1A* were discovered. In the Finnish family, a G -> A transition at nucleotide 350, causing an amino acid substitution of cysteine for tyrosine at residue 88 (C88Y) in exon 4 of the second extracellular domain of TNFRSF1A was detected. Originally, the C88Y family (Family 1) was studied as two separate families with periodic fever, but genealogic analysis revealed a common ancestry between the two. The patients are a father (patient 1) and his son (patient 4), as well as one sister of the father (patient 3), in one branch of the family, and a male patient (patient 2) and his, now deceased, father in the other branch of the family (Figure 7). All patients have suffered from periodic attacks of fever and abdominal pain since childhood or youth and the C88Y mutation was detected in each of the patients. Anamnestic information revealed recurring fever in the father and paternal grandmother of the patients in the first branch of the family (patients 1 and 3), as well as in the paternal grandmother in the other branch of the family (patient 2). Symptoms in the patients have been alleviated by indomethacin 75-150 mg daily or short courses of glucocorticoids. Patient 1, now aged 65, and his son, patient 4, 40 years of age, have had febrile attacks since the age of 24 and 15, respectively. The attacks are characterized by intense abdominal pain and appear to be provoked by physical strain or heavy meals (Välimäki et al., 1985; Karenko et al., 1992). Patient 3, the sister of patient 1, 56 years of age at present, has had recurring attacks of fever and abdominal pain since her teens, but with increasing age the severity of the attacks have decreased and the patient is nearly asymptomtic at present. Patient 2, now 58 years of age, developed attacks of high fever accompanied by abdominal and joint pain at the age of 9. At the age of 31, the patient developed proteinuria, and a kidney biopsy revealed amyloid A (AA) amyloidosis. Subsequently, the patient developed nephrotic syndrome and renal insufficiency. He received a renal transplant at the age of 53. The patient has an ongoing medication of methylprednisolone 6 mg every other day

and cyclosporine 125 mg daily, and has been free of febrile attacks for several years. This patient is the only Finnish TRAPS patient to have developed amyloidosis.

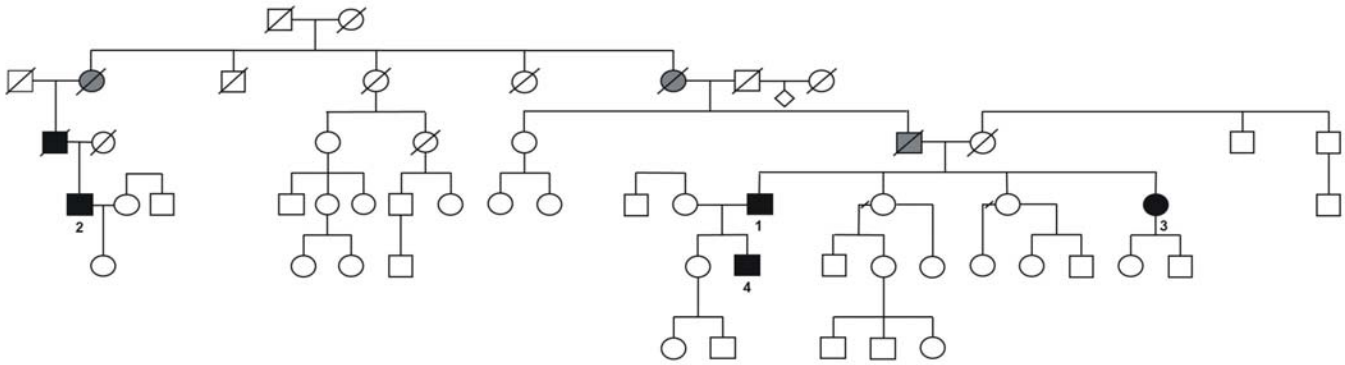

Figure 7. Pedigree of Family 1. The black symbols represent symptomatic patients in whom the C88Y mutation has been genetically confirmed, the grey symbols represent deceased family members known to have suffered from febrile and inflammatory episodes, but of whom DNA for genetic analysis was not available.

A second Finnish family, Family 2, with periodic fever occurring in three generations was subsequently studied and reported (I). Genetic analysis revealed a T -> A transition at nucleotide 421, resulting in the amino acid substitution of phenylalanine for isoleucine at residue 112 (F112I) in exon 4 in the third extracellular domain of *TNFRSF1A*. The mutation is located close to a conserved cysteine at residue 114, involved in a disulphide bond, and was found in all five symptomatic family members, as well as in an asymptomatic half-sister of the index patient (Figure 8). The index patient, patient 7, is a 45-year old woman, who has suffered from periodic attacks of high fever and abdominal pain since the age of 12. Her children, a daughter (patient 9) 20 years of age and son (patient 10) 18 years of age, also suffer from recurring fever and abdominal pain. The daughter has also experienced pleuritic chest pain in association with the febrile attacks. The index patient's father (patient 11), who is of Swiss ancestry and 69 years of age at present, has had similar attacks of fever and abdominal pain, as well as pleuritic chest pain, since childhood. The half-sister of the index patient (patient 8, now aged 31) developed similar febrile attacks involving intense abdominal pain at the age of 22. Her sister (patient 12, aged 30) was found to carry the F112I mutation, but has not manifested any symptoms of TRAPS. The symptoms have responded to nonsteroidal anti-inflammatory drugs and courses of corticosteroids.

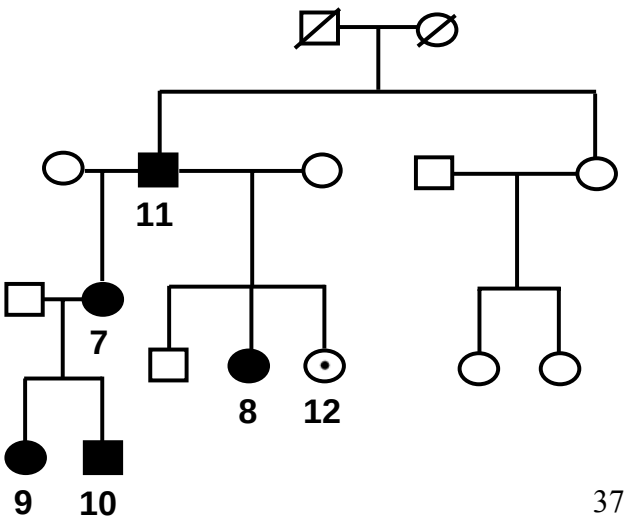

Figure 8. Pedigree of Family 2. Black symbols represent symptomatic patients with the F112I mutation, the symbol with the black dot represents an asymptomatic individual with the F112I mutation. The figure has been previously published in the first article of this thesis (I).

The third Finnish family (Family 3) investigated (II) comprises a mother and her daughter (figure 9), in whom sequencing revealed a T -> C transition at nucleotide 304, causing an amino acid substitution of cysteine for arginine at residue 73 (C73R) in exon 3 in the second extracellular domain of *TNFRSF1A*. Both patients have had recurring attacks of high fever, severe abdominal pain, as well as episcleritis, pleuritis, myalgia, and tendinitis. Other symptoms include periorbital cellulitis in the case of the mother and erythema localized to the ankles in the case of the daughter. The mother (patient 5), now aged 56, has had symptoms since the age of 10 and also the daughter (patient 6), now 31 years of age, has been symptomatic since childhood. With age, the frequency and the severity of the attacks have abated in the mother, whereas the daughter still continues to have TRAPS attacks several times a year. Short courses of corticosteroids have relieved the symptoms.

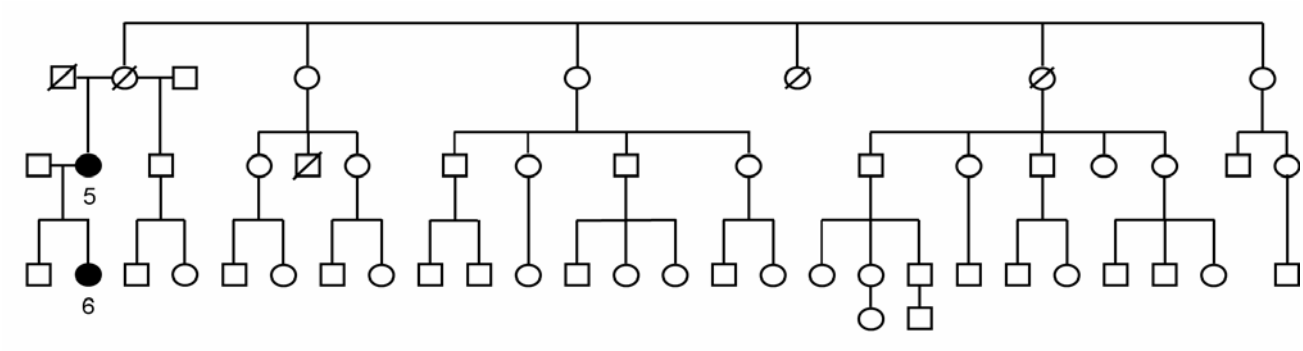

Figure 9. Pedigree of Family 3. Black symbols represent symptomatic patients in whom the C73R mutation has been genetically confirmed. The relatives on the maternal side of the family presented are all asymptomatic. Part of the figure has been previously published in the second article of this thesis (II).

7.2. Patients and control persons (I, II, IV)

In addition to the familial cases, serum samples of 113 patients with recurring febrile attacks of unknown etiology were sent to our clinic from other hospitals in Finland, Sweden and Norway for determination of serum concentrations of soluble (s)TNFRSF1A. Serum concentrations of sTNFRSF1A were likewise determined of 80 healthy control persons and asymptomatic family members of the three families studied. The asymptomatic persons served as negative controls. The clinical symptoms of patients with levels below the normal range, as defined by the manufacturer of the ELISA-kit (R&D Systems, Abingdon), have been carefully assessed. In the case of a strong suspicion of TRAPS, blood samples for the extraction of DNA and subsequent analysis for a possible genetic defect in *TNFRSF1A* have been collected through the referring physician.

7.3. Extraction of DNA (I, II, IV)

Genomic DNA was extracted from peripheral blood lymphocytes for mutation analysis. Samples of 20-30 ml whole blood, collected into tubes containing EDTA, were diluted with balanced salt solution (PBS) to produce a 1:2 solution, which was pipetted into two Falcon tubes. Lymphocytes were separated from the whole blood:PBS-solution, by adding 10 ml Ficoll-Paque Plus (Amersham Biosciences) into each Falcon tube, then centrifuging the tubes at 400 x g for 30-40 minutes. The interphase, containing lymphocytes, platelets and monocytes was recovered, washed and centrifuged twice with PBS to recover the lymphocytes. 5 ml of lymphocytes was pipetted into Falcon tubes. 45 ml of a buffer containing 320 mM Ultra pure sucrose, 1% Triton-X-100, 5 mM

$MgCl_2$ and 10 mM Tris-HCl pH 7.6, was added and the tubes were incubated for 10 minutes at 4 °C, then centrifuged for 10 minutes at 4 °C. The cell pellet was suspended in 4.5 ml of a solution, containing 25 mM EDTA pH 8.0 and 75 mM NaCl. 500 µl of 10% SDS and 50 µl of 10 mg/ml proteinase K were added and the solution was incubated over night at 37 °C in a shaker. After the over-night incubation, 5 ml Tris-EDTA (TE)-phenol (1:1) was added to the suspension. The solution was vortexed, then centrifuged for 10 minutes at 18˚C or 25˚C, at 3000 rpm. Using a Pasteur pipette, the upper phase was transferred to a clean Falcon tube and 5 ml of a 1:1 solution of TE-phenol: chloroform (96%)-isoamyl alcohol (4%) was added. Following vortexing, the solution was centrifuged for 10 minutes at 18˚C or 25˚C, at 3000 rpm. The upper phase was transferred to a clean Falcon tube using a Pasteur pipette and 5 ml chloroform (96%)-isoamyl alcohol (4%) was added, followed by vortexing and centrifuging for 10 minutes at 18˚C or 25˚C, at 3000 rpm. The upper phase was recovered as before and transferred to a clean Falcon tube. 500 µl of 3M sodium acetate (NaAc) pH 7.5 and 15 ml ethanol (C_2H_5OH) was added. The solution was centrifuged for 30 minutes at 4˚C, 10 000 rpm. The supernatant was removed and the pellet was washed with 70% ethanol, then centrifuged for 30 minutes at 4˚C, 10 000 rpm. The supernatant was removed and the pellet of DNA left to dry for 2-4 hours in a tube covered with plastic film. The DNA was resolved in 300 µl pure water and stored at 20˚C until analysed.

7.4. Mutation detection (I, II, IV)

Genomic DNA was amplified by polymerase chain reaction (PCR), using oligonucleotide primer pairs specific for exons 1-10 of *TNFRSF1A*. The primers are listed in the table below (Table 2).

Table 2. Primers for exons 1-10 of *TNFRSF1A*.

Primer	Sequence (in the 5’ to 3’ direction)
exon 1 F	ACT TGG GAC GTC CTG GAC AGA CC
exon 1 R	GGA AGG TGC CTC GCC CAC CAG
exon 2 F	CTG CTA CCC CTA AGC TTC CCA TCC C
exon 2 R	ACT GGA AGA AGC AGA GAA AGA AGC
exon 3 F	GCA TGG GGC TCC TTC CTT GTG TTC
exon 3 R	GCA CAT AGA CAG GCA CCC ACA CAC
exon 4 F	TCA GGA AGG GGA TGC AGG GAC AGG
exon 4 R	GAA AGG AAG TGC CAC CGC ATG GG
exon 5 F	TCA CTT CCT CTG TCC TGT GGG GTG
exon 5 R	TTC CCA CCA GTC ACC CGT CCC AAC C
exon 6 F	GTG GTT GTT TTT CTG TGT TCC TCC
exon 6 R	ATA GAT GGA TGG GTG GGA TGG ATG
exon 7 F	ATC CCA CCC ATC CAT CTA TCC CTG
exon 7 R	ACA TGT CCA TCG CAC CCA CCC ATG
exon 8 F	ATG TCA CCA CAA GTC CCC ACT GCC
exon 8 R	AGG ATG ATT CCA GGG GAT CTG AGC
exon 9 F	CTC TAA GTC CCA ACC CCC ACG TAG
exon 9 R	GCC TCT CGT GGT CCC CTC TGG GAG
exon 10 F	CTC CCA GAG GGG ACC
exon 10 R	GCT GCT AGC TCC TGC

The PCR conditions were optimized for each primer pair. The annealing temperatures ranged from 53 ˚C to 70 ˚C. The initial denaturation occurred at 95 ˚C for 1 minute, followed by 35 cycles of 95 ˚C for 30 seconds, 53 ˚C to 70 ˚C for 30 seconds, and 72˚C for 45 seconds, and a final 10-minute elongation step at 72˚C. The PCR products were separated by 1.5% agarose gel electrophoresis. Gel slices containing the amplicons were cut and DNA eluted from the agarose and cleaned using Amersham GFX PCR DNA and gel purification kit (Amersham Biosciences, Piscataway, NJ, USA). The purified amplicons or alternatively the PCR-products directly were sequenced, using the ABI Prism Big Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA, USA), in an ABI310 or ABI3100 automated sequencer (PE Applied Biosystems). Alignment and sequence analysis was performed using the NCBI BLAST program. The presence of each specific mutation was confirmed by sequencing in the reverse direction. PCR and sequencing for exon 1 was performed at the laboratory of Dr Michael F McDermott, at the Queen Mary's School of Medicine and Dentistry, London, UK. PCR for exons 2-10 was performed at the Skin- and Allergy hospital, the Department of Dermatology, Allergology and Venereology, Helsinki University Central Hospital or Biomedicum, Helsinki, and sequencing at the Institute of Medical Technology, University of Tampere, and Tampere University Hospital, or at the Biomedicum sequencing unit (BSU), University of Helsinki, using Applied Biosystems Bid Dye Terminator (v1.1) kit for PCR and Applied Biosystems ABI Prism 3130xl 16 capillary Genetic Analyzer for the flow cytometric analysis.

7.5. Determination of sTNFRS1A levels in serum (I, II, IV)

The levels of soluble TNFRSF1A were measured in samples of serum, obtained from patients during an asymptomatic period between attacks, using the Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Abingdon, UK). The absorbance-values were calculated according to the manufacturer's instructions. The levels of sTNFRSF1A were graphically extrapolated to picograms/millilitre in function of the absorbance. Serum samples from healthy control persons and asymptomatic family members were obtained and analyzed in the same manner.

7.6. Analysis of TNFRSF1A shedding (I, II)

TNFRSF1A shedding from the cell surface was investigated using flow cytometric analysis. Samples of heparinized peripheral blood from patients and healthy controls were left unstimulated or stimulated with phorbol myristate acetate (PMA, Sigma Chemical Company, St Louis, USA) at concentrations of 0.1 ng/ml to 100 ng/ml for 10 to 60 minutes at 37 ˚C in the CO_2 incubator. The optimal conditions proved to be a PMA concentration of 1 ng/ml and a time of stimulation of 20 minutes. The cells were washed and erythrocytes lysed with FACS lysing solution (Becton Dickinson, Mountain View, CA, USA). Following a subsequent washing, the cells were incubated either with monoclonal phycoerythrin (PE)-conjugated anti-CD120a antibody (Caltag Laboratories, Burlingame, CA, USA), specific for TNFRSF1A, or with monoclonal PE-conjugated anti-CD120b antibody (Caltag Laboratories), specific for TNFRSF1B, as well as with monoclonal fluorescein isothiocyanate (FITC)-conjugated anti-CD14 antibody (Becton Dickinson), for identification of monocytes, and FITC-conjugated anti-CD16 antibody (Becton Dickinson), for identification of granulocytes, for 20 minutes of time. After washing, the cells were analysed with a fluorescence-activated cell sorter (FACS) Calibur Flow Cytometer (Becton Dickinson, San Jose, USA). Monocytes and granulocytes were gated together to determine the CD120a (TNFRSF1A) and CD120b (TNFRSF1B) expression on these cells. CD14-positive cells were identified as monocytes

and CD16-positive cells as granulocytes. Lymphocytes were excluded from the analysis. PE-conjugated mouse IgG1 antibody (Caltag Laboratories) was used as the isotype control. The amount of monocytes and granulocytes expressing CD120a or CD120b was defined by subtracting the FACS histograms of monocytes and granulocytes stained with the corresponding antibodies, from the FACS histograms of monocytes and granulocytes stained with the mouse isotype control. The data processing was done using the CellQuest software and results were expressed as the mean fluorescence intensity (MFI).

7.7. First-strand synthesis (II)

First-strand synthesis was performed on total RNA for mutation screening in the TNFRSF1A gene, using primers for exons 1, 2 and 6 and following the Amersham Biosciences protocol. mRNA was purified from patient blood samples using the QuickPrep mRNA (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The RNA sample was placed in a microcentrifuge tube and RNAse free water was added to yield a volume of 8 µl. The RNA solution was heated to 65 °C for 10 minutes of time, after which the solution was chilled on ice. A reaction mix of 5 µl of bulk first-strand cDNA mix (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 1 µl of primer, 1 µl of DTT solution and 8 µl of total RNA, which had been heat-denaturated. The solution was carefully mixed and incubated at 37 °C for 1 h. The yielded cDNA was heated to 90 °C for 5 minutes to denature the RNA-cDNA complex and inactivate the reverse transcriptase, after which the solution was chilled on ice. A reaction mixture of 2 µl cDNA, 5 µl 10x PCR buffer, 1 µl 20 mM dNTP mix, 1 µl of upstream primer at a concentration of 10 pmol/ µl, 0.2 µg of downstream primer pd $(N)_6$, 40 µl of sterile water and 2.5 U Taq DNA polymerase (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was pipetted into a sterile microcentrifuge tube and covered with a layer of mineral oil. PCR was performed in a thermal cycler with an initial denaturing step at 95 °C for 1 minute, followed by an annealing step at 55 °C for 30 seconds and a polymerization step at 72 °C for 30 seconds for 30 cycles total. The PCR products were separated by 1.5% agarose gel electrophoresis and sequenced by automatic cycle sequencing, using the ABI Prism Big Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA, USA) in an ABI 13100 automated sequencer (PE Applied Biosystems). The sequences were analysed using the NCBI BLAST program.

7.8. *Ex vivo* stimulation and immunolabelling of blood samples (IV)

A 4-ml blood sample from patients or healthy control persons was divided directly after venesection into 35 aliquots of 90 µl in Falcon polystyrene tubes (Becton Dickinson, Lincoln Park, NJ) at 37 ^{o}C for further analysis: 1) to study the dose response of TNF, 2) to study the time course of NF-κB and p38 phosphorylation, and 3) to check the specificity of the findings by stimulating leukocytes with ligands other than TNF. All tubes were kept at 37 ˚C and were studied consecutively, so that all tubes had been analyzed within about 3 hours. The results of optimization experiments showed that incubation of 90-µl aliquots of citrate-anticoagulated blood samples at 37 ˚C up to six hours did not affect the TNF-induced phosphorylated NF- κB (pNF-κB) fluorescence intensity. The leukocytes were treated according to the BD Phosflow Protocol III for Human Whole Blood (http://www.bdbiosciences.com/pharmingen/products/display_product.php?keyID=94#3).
The first set of tubes, used to determine the dose response of TNF, was supplemented with 10-µl aliquots of FITC-conjugated mouse anti-CD14 monoclonal antibody (mAb) clone MφP9 (IgG_{2b}) (BD Biosciences, San Jose, CA, USA), vortexed and incubated for 10 min at 37 ^{o}C. TNF (R&D, Minneapolis, MN, USA) at final concentrations of 0, 0.01, 0.1, 10, 10 and 100 ng/ml was added, after which, the tubes were re-vortexed and further incubated for 5 min at 37 ^{o}C. Following

incubation, 1x BD PhosFlow Lyse/Fix Buffer (1.9 ml) pre-warmed to 37 ^{o}C was added. The tubes were subsequently incubated for 10 min at 37 ^{o}C, centrifuged (500 g for 8 min at room temperature) and washed once with Hank's balanced salt solution (Life Technologies, Paisley, UK). Permeabilization of the cells was performed by re-suspending the cell pellet in 1 ml of pre-cooled (-20 ^{o}C for one hour) BD Phosflow Perm Buffer III. The tubes were stored at -20 ^{o}C until stained with phospho-specific monoclonal antibodies (mAbs).

To determine the time course of pNF-κB and p38 phosphorylation, TNF at a final concentration of 10 ng/ml was added to certain tubes, or alternatively tubes were left without the stimulus, before further incubated at 37 ^{o}C for 2.5, 5, 10 and 20 min. Ten-µl aliquots of CD14-FITC mAb solution were added 15 min prior to fixation with 1.9-ml aliquots of 1x BD PhosFlow Lyse/Fix Buffer. The samples were processed further and finally stored in BD Phosflow Perm Buffer III at -20 ^{o}C as described above.

To check the specificity of the findings, using stimuli other than TNF, the third set of tubes was supplemented first with the aliquots of CD14-FITC mAb and 5 min later with 10 ng/ml and 100 ng/ml of *Esherichia coli* O111:B4 lipopolysacchardes (LPS) (Sigma, St. Louis, MO, USA) or 5 ng/ml and 50 ng/ml of IFN-γ (R&D), or left without the stimulus. The tubes were further incubated for 10 min at 37 ^{o}C. After incubation, red cells were eliminated and leukocytes permeabilized with BD Phosflow Perm Buffer III for one hour at -20 ^{o}C as above. The results of optimization of the experiments revealed that permeabilization of up to six hours did not decrease the pNF-κB fluorescence intensity.

The three sets of permeabilized cell samples were washed twice with BD Pharmingen Stain Buffer at room temperature and re-suspended in 100 µl of the buffer. 5-µl aliquots of Alexa 647-labelled and unlabelled phospho-specific mAbs to pNF-κB p65 (pS529) clone K10-895.12.50 (IgG_{2b}) or to pp38 (pT180/pY182) clone 36 (IgG1), were added to the TNF- and LPS-treated samples and respective control samples left without stimulus, and 5-µl aliquots of pSTAT1 (pY701) clone 4a (IgG_{2a}), was added to samples treated with IFN-γ. and non-stimulated control cells. The samples were incubated for 30 min at room temperature protected from light, washed once with 1 ml of BD Pharmingen Stain Buffer, after which, the cell pellets were re-suspended in 500 µl of the stain buffer. The samples were stored at 0 ^{o}C and analyzed by flow cytometry within 3 hours.

7.9. Flow cytometric analysis, using phospho-specific monoclonal antibodies (IV)

Flow cytometric analysis was performed on monocytes, lymphocytes and neutrophils to determine the intracellular levels of pNF-κB, pp38 and pSTAT1, using FACS Calibur flow cytometer and Cell Quest software (BD Biosciences, San Jose, CA, USA). The cells were fixed, permeabilized and labelled with phospho-specific mAbs or isotype controls as described above prior to analysis. The cells were identified and gated, monocytes according to CD14-FITC expression, lymphocytes and neutrophils by their light scatter properties. pNF-κB and pp38 histograms were produced for each cell type and as a function of a) the time of stimulation with TNFα and the concentration of TNFα. The fluorescence intensity was expressed as the median of relative fluorescence units (RFU).

7.10. Immunoblot analysis (IV)

Immunoblot analysis of whole blood samples was used to determine the intracellular maximal pNF-κB and pp38 levels following stimulation with TNFα. Blood samples from healthy volunteers were

stimulated as 5 ml aliquots for 0-60 minutes at 37˚C with TNF (R&D) at concentrations of 0-100 ng/ml. Erythrocytes were lysed, using 45 ml FACS Lysing solution (BD Biosciences;1:10 in aqua), incubating for 10 minutes at RT. The solution was vortexed once during the incubation. The cells were subsequently pelleted and lysed in a buffer (pH 7.6), constituted of 150 mM NaCl, 10 mM TRIS-HCl, 1 mM EDTA, 1 mM EGTA, 1% TritonX-100, 0.5% NP-40, 1xComplete™ protease inhibitor cocktail (Roche, Mannheim, Germany), 1 mM Pefabloc SC (Roche), 1 mM sodium fluoride and 1 mM sodium orthovanadate (Sigma, St. Louis, MO, USA). The cell suspension was incubated for 1 h at 0˚C, then vortexed. Colorimetric Bradford assay was used to determine protein concentrations and whole cell protein extracts were diluted at equal amounts (50 µl) in Laemmli sample buffer with 5% mercaptoethanol. Following a 5-minute incubation at 95˚C, the samples were resolved in 10% polyacrylamide gels in Tris-glycine-SDS buffer. The gels were transferred to nitrocellulose membranes, blocked in Odyssey blocking buffer (LI-COR, Linoln, NE, USA) and incubated overnight with unlabelled phospho-specific antibodies for pNF-κB p65 (pS529) clone K10-895.12.50 (IgG_{2a}) (1:2500), pp38 (pT180/pY182) clone 36 (IgG1) (1:2000v/v), p38α/SAPK2a clone 27 (IgG1) (1:2500 v/v) or with NF-κB p65 clone 20 (IgG1) (1:500 v/v) antibody (BD Biosciences, San Jose, CA, USA) and with rabbit anti-GADPH (1:5000 v/v) antibody (Santa Cruz, Biotechnology Inc, Santa Cruz, CA, USA) in Odyssey blocking buffer + 0.1% Tween at 4˚C. Following incubation, the membranes were washed three times in Dulbecco's phosphate buffered saline (PBS) (Life Technologies, Paisley, UK) + 0.1% Tween and incubated for 1 hour with goat anti-mouse IgG IRDye 800 (1:15000 v/v) (LI-COR, Lincoln, NE, USA) and with goat anti-rabbit IgG Alexa 680 dye (1:15000 v/v) (Molecular Probes, Eugene, OR, USA) in Odyssey blocking buffer + 0.1% Tween. Odyssey infrared imager was used to visualize the protein bands in the membranes. Quantitative densitometric analysis was performed, using the Odyssey Imaging System Application Software (LI-COR, Lincoln, NE). The optimal incubation conditions, at which maximal pNF-κB/pp65 and pp38 levels were observed, proved to be an incubation time of 5 minutes and a TNF concentration of 0.1 ng/ml for p65 and 10 ng/ml for p38.

7.11. Electrophoretic Mobility Shift Assay (EMSA) (IV)

Protein concentrations in nuclear and cytoplasmic protein extracts, prepared according to the protocol presented by Schreiber et al. (Schreiber et al., 1989) were determined to evaluate NF-κB activation following stimulation with TNF. Peripheral blood mononuclear cells, purified on Ficoll, were washed with 10 ml TBS, then pelleted by centrifugation at 1500 x g for 5 minutes, resuspended in 1 ml TBS, transferred to an Eppendorf tube and pelleted again by centrifugation in a microfuge. After removal of the TBS, the pellet was resuspended in 400 µl of cold dilution buffer, constituted of 10 mM HEPES pH 7.9, 10 mM KCL, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF, and incubated on ice for 15 minutes. 25 µl of a 10% solution of Nonidet NP-40 (Fluka) was added and the tube vortexed, then centrifuged for 30 seconds in a microfuge. The cytoplasm- and RNA-containing supernatant was transferred to a tube containing 400 µl of a buffer (B) containing 10 mM Tris pH 7.5, 7 M urea, 1% SDS, 0.3 M NaAc, and 20 mM EDTA, as well as 600 µl of phenol/chloroform (1:1), then mixed. The nuclear pellet was resuspended in 50 µl of a cold buffer, containing 20 mM HEPES pH 7.9, 0.4M NaCl, 1mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, mixed for 15 minutes at 4˚C and centrifuged for 5 minutes at 4˚C in a microfuge. The supernatant, containing the nuclear extracts, was collected and an aliquot of 10 µg was incubated in 20 µl binding buffer for 20 minutes at room temperature together with 0.5 µg poly [d(I-C)], 0.05% NP-40, 2,5 mM DTT, 0.23% Tween-20 and 0.8 µg IRD YE 700 end labelled double stranded oligonucleotide probe containing the NF-κB binding motif (LI-COR, Lincoln, NE, USA). The DNA-protein complexes were resolved by electrophoresis on a 5% non-denaturating polyacrylamide gel in a TBE-buffer containing 22.mM Tris, 22.5 mM boric acid, and 0.5 mM

EDTA, pH 8.3 at 100 V and 4˚C for 1.5-2 hours. The bandshift experiment was imaged with Odyssey infrared imager.

7.12. Data analysis (IV)

NF-κB and p38 phosphorylation levels in response to stimulation with TNFα in different leukocytes were interpreted as the fluorescence intensity, expressed as the median of relative fluorescence units (RFU). The areas under curve (AUC) for the p65 NF-κB and p38 phosphorylation levels in response to different concentrations of TNFα (AUC^{dose}, RFU x ng/ml) and to different time periods of stimulation (AUC^{time}, RFU x min) were calculated using the trapezoidal rule. To evaluate the signalling capacity of the patients and the control persons, the Mann-Whitney U non-parametric test was used to calculate p-values based on the AUCs. A p-value of 0.05 was considered to be statistically significant. Analysis of variance with repeated measures was used to compare the dose response and time course curves of the patients and the control persons. The difference between the shapes of the curves represents the within-subjects effects and the difference between RFU levels of the curves represents the between-subjects effects. Fold increase values were calculated for each cell type and all patients and controls, by dividing the RFU-value of stimulated cells by that of unstimulated cells. The coefficient of variation calculated for pNF-κB and pp38 was 5% within experiments and 10% between experiments.

7.13. Ethical considerations (I-IV)

This study was approved by the Ethical Review Board of the Department of Medicine at the Joint Authority for the Hospital District of Helsinki and Uusimaa, Finland. A written informed consent was received from all patients and healthy family members participating in the study.

Although TRAPS is not a continuous or progressive disease, the recurring symptoms of fever and inflammation may be severe and may ultimately result in systemic amyloidosis, which is a potentially lethal complication of the disorder. TRAPS is defined by the presence of a genetic defect in *TNFRSF1A* (Hull et al., 2002a) and as there is no established method of screening for TRAPS at present, the verification of a mutation in the causative gene to support the clinical suspicion is an important aspect of diagnostics of the autoinflammatory disorders (Stojanov and Kastner, 2005). Furthermore, as the age of onset of the disorder varies within a family and may occur past the age of 60 (Dodé et al., 2002a), it was considered justified to perform genetic analysis also on asymptomatic family members, according to the wishes of the families concerned.

8. RESULTS

8.1. Two novel *TNFRSF1A* mutations, F112I and C73R, in Finnish patients (I, II)

Two families with periodically occurring fever and inflammation, inherited in an autosomally dominant manner, were investigated for mutations in *TNFRSF1A* to verify the clinical suspicion of TRAPS. At the time Family 2 was studied, mutations in exons 2-4 in the first and second extracellular domains had been reported (McDermott et al., 1999; Galon et al., 2000; Dodé et al., 2000; Aganna et al., 2001; Simon et al., 2001). None of the earlier reported mutations were

observed in our patients, wherefore all *TNFRSF1A* exons were screened for mutations. Sequencing of genomic DNA revealed a novel missense mutation in exon 4 of the third extracellular domain of *TNFRSF1A* in this family (I). Another family with periodic fever, Family 3, was similarly investigated and a novel missense mutation in exon 3 of the second extracellular domain of *TNFRSF1A* was revealed (II). Previous studies had reported 33 true mutations in exons 2-4 in the first, second and third extracellular domains of *TNFRSF1A.*

The mutation in Family 2, a T to A transition in position 421, results in an amino acid substitution of phenylalanine to isoleucine (F112I). The mutation was detected in three generations and in six family members, five of whom had experienced periodic attacks of fever and abdominal pain and one patient having suffered also from pleurisy. The sixth family member (patient 12) in whom the F112I mutation was detected had remained free of inflammatory symptoms at the age of 23 years. The mutation was confirmed by DNA sequencing in the reverse direction and the 155 bp mutant allele was visualised as segregated from the healthy allele by microsatellite genotyping. The F112I or any other *TNFRSF1A* mutation was not detected in the 25 randomly chosen, healthy control persons screened. In association with the functional analyses, another member of Family 2 was screened for the *TNFRSF1A* mutation (patient 10). The patient had suffered from fever and abdominal pain since the age of 12, the symptoms ressembling those of his sister (patient 9), his mother (patient 7) and his grandfather (patient 11). Genomic sequencing revealed the F112I mutation also in this patient.

The mutation in Family 3, a T to C transition at position 304, causing an amino acid substitution of cysteine to arginine at residue 73 (C73R), was detected in two patients, a mother (patient 5) and her daughter (patient 6). Both patients had suffered from attacks of abdominal pain accompanied by fever, or in the case of the daughter, occasionally also in the absence of fever. Other symptoms included myalgia, periorbital swelling and episcleritis, as well as, arthritis in the mother and erythema, tendonitis and pleurisy in the daughter. The mutation was confirmed by DNA sequencing in the reverse direction and by first-strand synthesis of total RNA and cDNA sequencing. The mutation was not found in the patients' asymptomatic son/brother, or in any of the 50 healhty control persons screened.

The F112I mutation was the first mutation to be reported in the third extracellular domain of *TNFRSF1A.* The 112 residue lies close to a cysteine at residue 114, involved in the formation of a disulfide bond. The C73R mutation affects the second extracellular domain of the receptor and directly affects the disulfide bond between C73 and C88 (McDermott et al., 1999). Cysteine *TNFRSF1A* mutations have been associated with a more severe phenotype than non-cysteine mutations (Aksentijevich et al., 2001). The two C73R patients have a clinical picture with more diverse symptoms, than do the TRAPS patients with the F112I mutation or the previously reported Finnish patients (McDermott et al., 1999) with another cysteine-mutation, C88Y (Table 1), but the attacks do not markedly differ in severity between the three Finnish families.

Table 3. Clinical phenotypes associated with three different *TNFRSF1A* mutations in Finnish families. Patients 1-10 participated in the functional analyses of the fourth article of this thesis (IV), from which the table is adapted.

Patient	Age at the onset of the symptoms/ at present (years)	Gender	*TNFRSF1A* exon/ extracellular domain/ mutation	Main clinical features	Soluble TNFRSF1A level(pg/ml) normal:746-1966 pg/ml
1	24/65	male	exon 4/ 2/ C88Y	Fever, abdominal pain; attacks provoked by physical strain or heavy meals	474
2	9/58	male	exon 4/ 2/ C88Y	Fever, abdominal and joint pain, amyloidosis	2160
3	teens/56	female	exon 4/ 2/ C88Y	Fever, abdominal pain	465
4	15/40	male	exon 4/ 2/ C88Y	Fever, abdominal pain; attacks provoked by physical strain or heavy meals	504
5	10/56	female	exon 3/ 2/ C73R	Fever, abdominal pain, arthritis, myalgia, periorbital swelling, episcleritis	460
6	childhood/31	female	exon 3/ 2/ C73R	Abdominal pain, with or without fever, pleurisy, myalgia, tendonitis, erythema, periorbital swelling, episcleritis	485
7	12/45	female	exon 4/ 3/ F112I	Fever, abdominal pain	573
8	22/31	female	exon 4/ 3/ F112I	Fever, abdominal pain	605
9	11/20	female	exon 4/ 3/ F112I	Fever, abdominal pain, pleurisy	527
10	12/18	male	exon 4/ 3/ F112I	Fever, abdominal pain	1077
11	15/70	male	exon 4/ 3/ F112I	Fever, abdominal pain, pleurisy	532
12	-/30	female	exon 4/ 3/ F112I	No symptoms developed to date	465

8.2. Levels of soluble TNFRSF1A in serum (I, II, IV)

Serum concentrations of sTNFRSF1A have been determined in patients with recurring febrile attacks of unknown etiology, as well as in healthy control persons and asymptomatic family members, using the Quantikine ELISA-kit, as described in the Subjects and Methods section. Sporadic cases, as well as familial cases have been studied. The normal sTNFRSF1A range according to the manufacturer's instructions is 749-1966 pg/ml. The sTNFRSF1A concentration determined of the asymptomatic family members (n=44) ranged from 315-2651 pg/ml, low levels being observed in seven C88Y family members (315-690 pg/ml, median 675 pg/ml) and in seven C73R family members (470-700 pg/ml, median 620 pg/ml) (Figure 10). The C73R family members were all from the maternal side of the family, as no information of the paternal side of the family was available. The range of healthy controls (n=11) was 577-2246 pg/ml, low levels (below 749 pg/ml) being observed in four controls (466-702 pg/ml, median 602 pg/ml) in a single assay (Figure 10). In repeated assays, these four controls had normal sTNFRSF1A concentrations, as defined by the manufacturer.

The patients with *TNFRSF1A* mutations, with the exception of two patients, presented with sTNFRSF1A levels below the normal range established by the manufacturer, and all, but one patient, had sTNFRSF1A concentrations below the values of the healthy control persons studied at the same time. The sTNFRSF1A range for all patients was 460-2160 pg/ml (median 527 pg/ml), and for the nine patients with sTNFRSF1A levels below the manufacturer's normal range, 460-605 pg/ml (median 504 pg/ml). Of the two patients who did not have low levels of sTNFRSF1A as determined by the manufacturer's reference values, one patient, with the F112I mutation (patient 10), had a sTNFRSF1A concentration of 1077 pg/ml and another patient, with the C88Y mutation (patient 2), had a sTNFRSF1A concentration of 2160 pg/ml. Although the F112I patient had a sTNFRSF1A value within the normal range, according to the manufacturer's instructions, the sTNFRSF1A concentration was below the range of the values of the healthy controls studied in the same experiment (1112-2246 pg/ml). The C88Y patient was thus the only person with a sTNFRSF1A concentration, which was clearly elevated both according to the normal range defined by the manufacturer, as well as in comparison to the healthy controls studied at the same time.

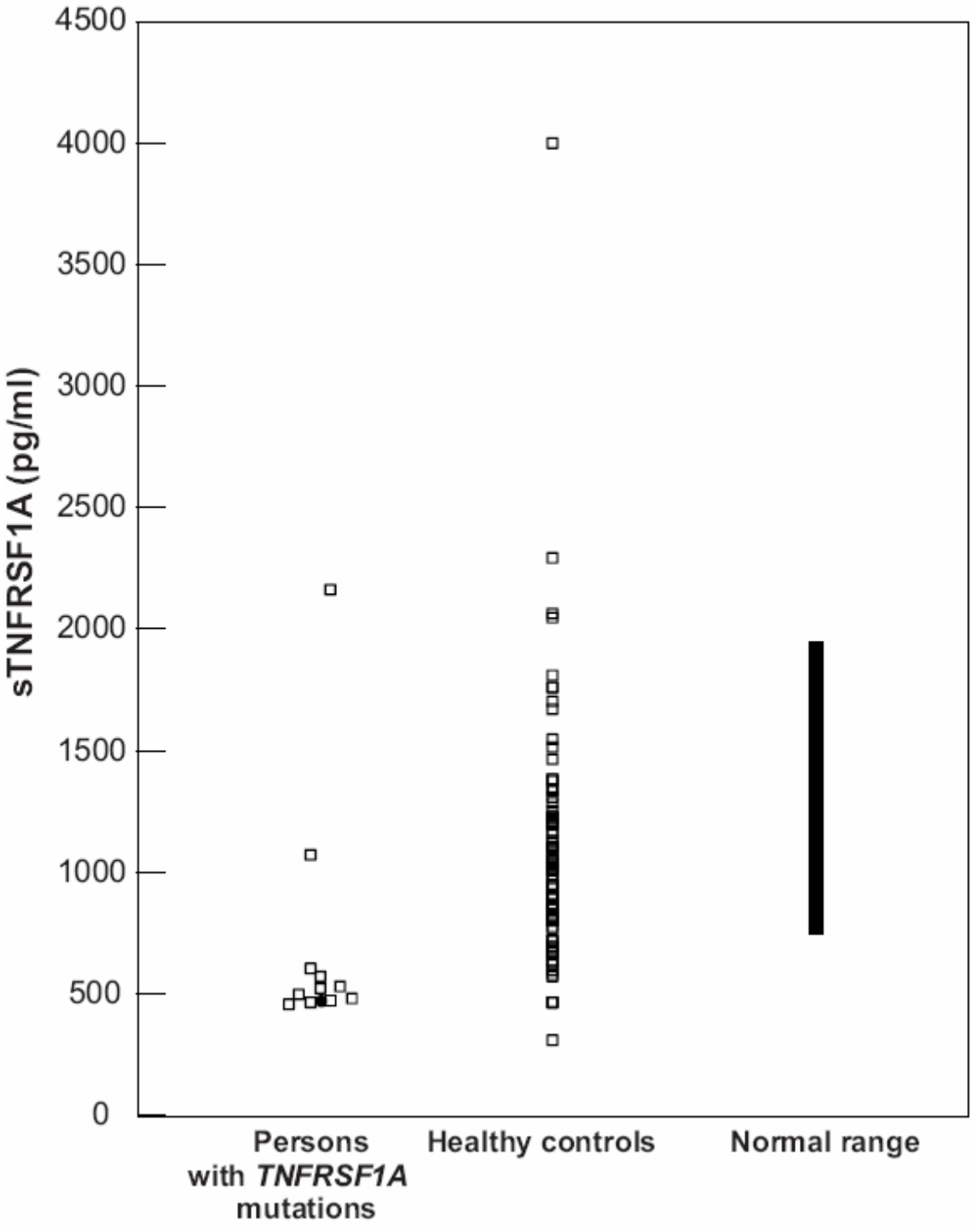

Figure 10. sTNFRSF1A concentrations in 11 symptomatic TRAPS patients from the three Finnish TRAPS families with different *TNFRSF1A* mutations and in one asymptomatic carrier of the F112I *TNFRSF1A* mutation (represented with a black square), as well as in healthy controls and asymptomatic family members. The normal range, 749-1966 pg/ml, is the value established by the manufacturer of the ELISA-kit (R&D Systems, Abingdon, UK).

8.3. Shedding of TNFRSF1A from the cell surface (I, II)

In the Finnish TRAPS families, TNFRSF1A surface expression and shedding was studied, using flow cytometric analysis and anti-CD120a (anti-TNFRSF1A) and anti-CD120b (TNFRSF1B) monoclonal antibodies, of monocytes and granulocytes before and after stimulation with PMA (for details, see the Subjects and Methods section).

In three patients with the F112I mutation studied, no decrease in the CD120a (TNFRSF1A)-expressing cells, following PMA-stimulation was observed, but on the contrary an increase of 1 per cent, 3 per cent and 15 per cent, suggesting that no marked TNFRSF1A-shedding occurred. In one F112I patient, cell-surface CD120a expression decreased by 16 per cent, following stimulation with PMA, indicating partial receptor shedding. In the healthy control persons studied there was a decrease in the CD120a cell surface expression of 4-22 per cent (median 16 per cent, mean 14.5 per cent), indicating a significant TNFRSF1A shedding in three out of four control persons. The CD120b (TNFRSF1B) shedding in patients and healthy controls did not differ from each other.

In the C73R family, FACS analysis of both patients and of two healthy control persons revealed a similar cell surface expression of CD120a and CD120b before stimulation with PMA. The initial values for CD120a cell surface expression were 25 mean fluorescence intensity (MFI) units and 27 MFI for the patients and 26 MFI and 20 MFI for the controls. The respective values for CD120b were 45 MFI and 53 MFI for the patients and 46 MFI and 37 MFI for the controls. Following PMA-stimulation, there was a comparable change in CD120a expression on the cell surface of both patients and controls, CD120a values being 28 MFI and 29 MFI for the patients and 26 MFI and 18 MFI for the controls, and CD120b values being 44 MFI and 51 MFI for the patients and 46 MFI and 36 MFI for the controls.

8.4. Clinical characteristics of TRAPS and their implications in differential diagnostics (III)

We present a report of a Finnish TRAPS patient, illuminating the differential diagnostic problems associated with rare hereditary diseases and presenting a hypothesis that an initial attack of TRAPS may have been the underlying cause of severe complications (III). The patient, a member of the previously reported family with the F112I *TNFRSF1A* mutation (I), has suffered from attacks of high fever, abdominal pain and pleurisy since the age of 11. Normally, the attacks recur 1-2 times a year and last 1-3 weeks at a time, the pain localizing to the right side of her lower abdomen. The inflammatory attacks, and in particular the abdominal pain, have responded well to nonsteroidal anti-inflammatory drugs, although short courses of prednisone, at an initial dose of 30 mg/day, tapered down by 5-10 mg/week over the following 4-6 weeks, have occasionally been required in addition to NSAIDs.

At the age of 16, on January 1st, 2003, the patient was admitted to hospital because of high fever and abdominal pain. Contrary to previous inflammatory attacks in association with TRAPS, an additional symptom upon admission was nausea, and the abdominal pain localized more diffusely to the lower abdomen than during the previous attacks, when the pain had been predominantly on the right hand side. Clinical findings included muscle guarding, indicative of peritoneal irritation, leucocytosis of 20.7 x 10^{-9} L^{-1} (normal 4.5-13 x 10^{-9} L^{-1}) and a rise in CRP levels of 55 mg L^{-1} (normal <10 mg L^{-1}). X-rays of the abdomen revealed dilated bowels and fluid retention in the colon. The diagnosis of TRAPS initially led to believe that the symptoms were manifestations of the autoinflammatory disease but a decline in the patient's clinical condition, with a continuing rise

in CRP levels to 358 mg L^{-1}, raised the possibility of an enteric bacterial infection, upon which intravenous cefuroxime and metronidazole treatment was initiated on the third day of admission. The option of an underlying TRAPS attack, possibly in addition to a bacterial infection, was not discarded and intravenous corticosteroid was commenced on the fifth day of hospitalization. The combination of antibiotics and corticosteroids initially resulted in a decrease in CRP levels, which began to rise again on day 9 after admission, reaching a level of 101 mg L^{-1}. A computer tomography (CT) of the abdomen revealed no signs of perforation and no abnormal findings in the internal organs. An echocardiography showed no signs of pericarditis or valvular vegetations. The patient's clinical condition continued to decline, despite ongoing medication, and the patient was transferred to the Helsinki University Central Hospital for further examinations.

At the university central hospital a new CT of the abdomen revealed signs of a collection of fluid in the lower abdomen, suggesting an intraperitoneal abscess. Emergency laparotomy was performed four days after admission, the operation revealing a large abscess attached to the peritoneum and involving part of the terminal ileum, which had undergone necrosis, in the lower abdomen. The abscess was drained, the necrotic bowel segment was resected and, following decompression of the small intestine, an ileostomy was created. After the laparotomy, the patient recovered and CRP levels normalized. The patient was discharged 18 days post-operatively. Histology of the resected bowel showed necrosis in the intestinal wall, but no signs of inflammation, thrombosis or amyloidosis.

8.5. TNF-induced phosphorylation of transcription factors NF-κB and p38 (IV)

TNFα-induced intracellular signalling mechanisms and a possible abnormal signalling in association with *TNFRSF1A* mutations were evaluated by determining the levels of phosphorylated p65 NF-κB and p38 of ten Finnish TRAPS patients with the C88Y, C73R and F112I mutations, as well as of ten healthy control persons, following stimulation with TNF and using phosphospecific monoclonal antibodies and flow cytometric analysis (see the Subjects and Methods section for details).

The dose response and time course of TNFα-induced NF-κB and p38 phosphorylation curves appeared to have a similar shape for monocytes, lymphocytes and neutrophils of both TRAPS patients and controls, a finding supported by the analysis of variance with repeated measures. However, the phosphorylated NF-κB (pNF-κB) and p38 (pp38) values tended to be lower in the patients than in the respective controls (the between-subjects effect), with the exception of the monocyte pp38 dose response curve, where RFU values for the patients and healthy subjects was comparable (Figure 11).

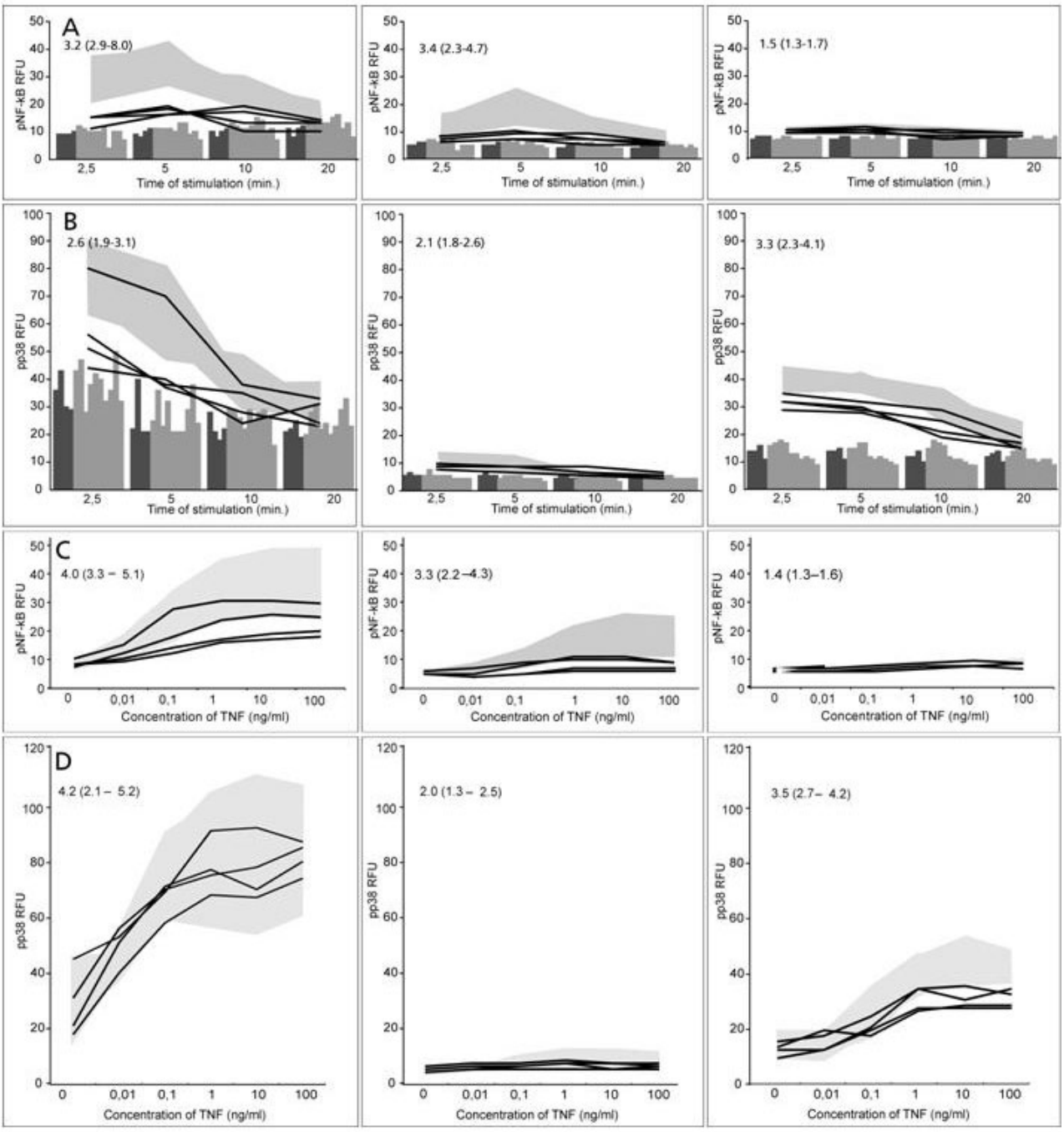

Figure 11. The TNF-induced NF-κB and p38 phosphorylation levels in whole blood samples from 10 healthy subjects and four patients with the C88Y mutation. Panels A and B show the time course and panels C and D the dose response curves of the NF- κB and p38 phosphorylation. The left column presents monocytes; the middle column, lymphocytes; the right column, neutrophils. The solid lines represent patients and the shaded area (upper) the range of ten controls. RFU = relative fluorescence units. The median of fold-increase (the TNF-stimulated value, divided by the unstimulated value) of ten healthy subjects are shown in the upper left hand corner of each figure. In panels A and B the shaded areas (bottom) denote unstimulated cells from patients (black) and controls (grey). pNF-κB= phosphorylated NF- κB; pp38= phosphorylated p38. The figure is part of the fourth article of this thesis (IV).

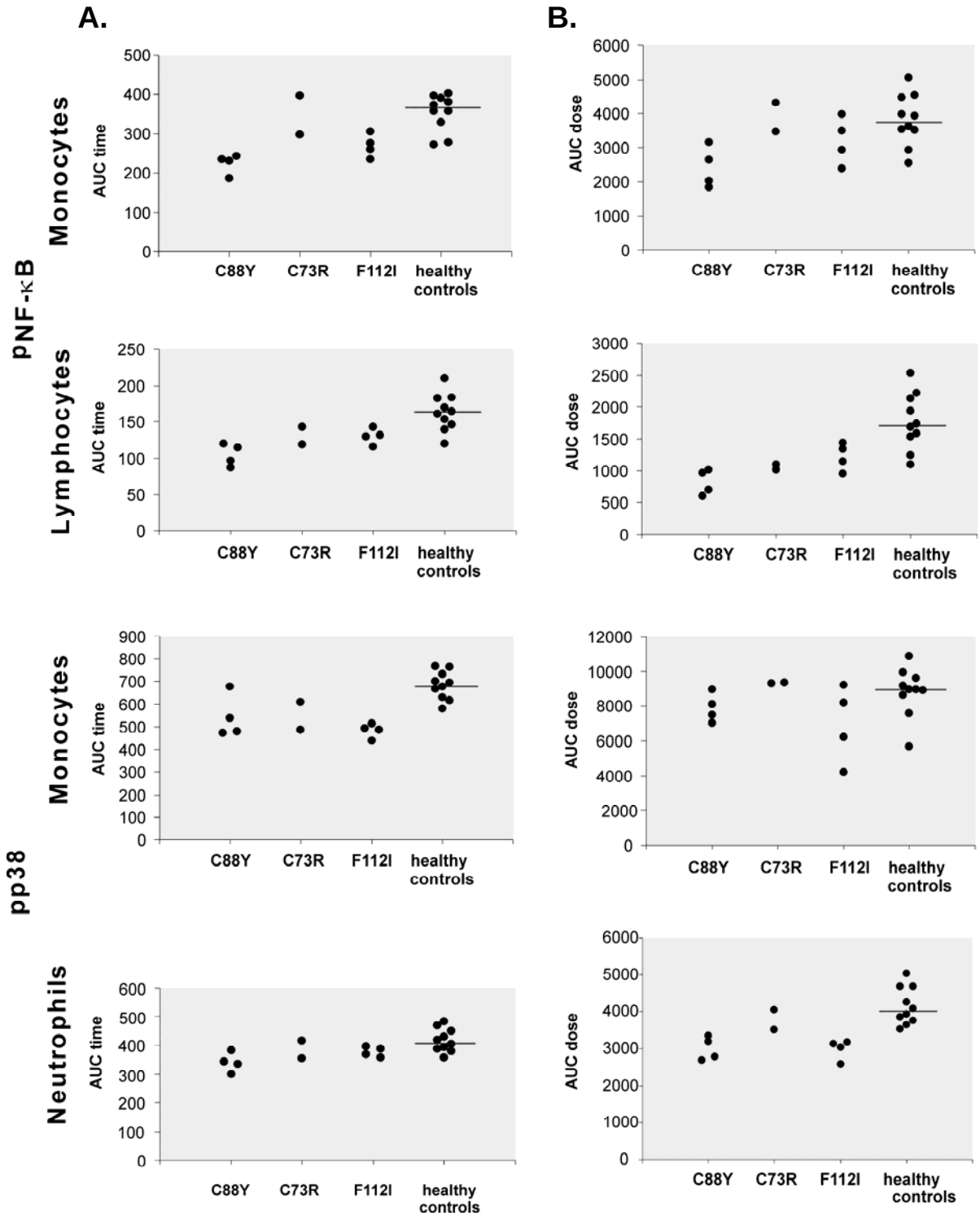

Figure 12. The areas under curve (AUC) for pNF-κB and pp38 values. The TNF-response of the TRAPS patients with the C88Y, F112I and C73R, and of ten healthy control subjects were compared by calculating the AUC values for pNF-κB and pp38 levels as a function of the time of stimulation with TNF (AUC^{time}) (A) and the concentration of TNF (AUC^{dose}) (B). The figure is part of the fourth article of this thesis (IV).

In monocytes, pNF-κB RFU fold increase values were 3.3-5.1 for the dose response and 2.9-8.0 for the time course activation. The monocyte pp38 RFU fold increase values were 2.1-5.2 for the dose response and 1.9-3.1 for the time-course activation. Based on the fold increase values, all four monocyte subscores would appear reliable for comparing phosphorylation levels. In lymphocytes,

pNF-κB RFU fold increase values for dose response was 2.2-4.3 and 2.3-4.7 for time-course activation, indicating a reliable response to TNFα. Lymphocyte pp38 RFU fold increase values were 1.3-2.5 for dose response and 1.8-2.6 for time-course activation. Based on the low fold increase values for the dose response and on the low RFU values, the TNFα-induced p38 phosphorylation in lymphocytes is poor and therefore not suited for comparing phosphorylation levels between patients and healthy control persons. In neutrophils, pNF-κB dose response RFU fold increase values were 1.3-1.6 and the time-course activation RFU fold increase 1.3-1.7, revealing a poor response to TNFα. pp38 RFU fold increase values in neutrophils were 2.7-4.2 for the dose response and 2.3-4.1 for the time-course activation. AUC-values were calculated for the four monocyte subscores, the two pNF-κB lymphocyte subscores and the two pp38 neutrophil subscores, showing a significant response to stimulation with TNFα, to evaluate the phosphorylation profiles of TRAPS patients in comparison to healthy controls. The neutrophil pNF-κB and lymphocyte pp38 subscores were not included in further analyses due to the poor TNFα-response.

The AUCs for monocyte pNF-κB and pp38 values, for lymphocyte pNF-κB values and neutrophil pp38 values were calculated for both the dose response and time-course activation curves (Figure 12).

All four C88Y patients had lower monocyte pNF-κB AUC^{time}-values, lower lymphocyte pNF-κB AUC^{dose}-values and lower neutrophil pp38 AUC^{dose}-values than the lower range of the control values, indicating low NF-κB and p38 phosphorylation levels following stimulation with TNF (Figure 12, Table 4).

In the F112I family, phosphorylation levels were less consistent than in the C88Y family. In all four F112I patients monocyte pp38 AUC^{time}- and neutrophil pp38 AUC^{dose}-values were below the range of the control values. One patient had AUC-values below the lowest value of the respective controls in 7/8 subscores, AUC-values being at the range of the controls only for the dose response in monocytes, and another patient had AUC-values below the range of the controls in ¾ of the p38 subscores and in ¼ of the NF-κB subscores (Figure 12, Table 4). The different phosphorylation levels in four related patients, with the same mutation and a similar clinical phenotype, would indicate that this particular *TNFRSF1A* mutation does not correlate with the TNF-induced phosphorylation profile.

Phosphorylation levels of the two C73R patients, a mother and her daughter, showed a marked divergence. In the mother (patient 5), none of the assays gave AUC-values below the lowest values of the respective control group, whereas in the daughter (patient 6), AUC-values below the range of the controls were observed for pNF-κB in lymphocytes, for pp38 in monocytes as a function of the time of stimulation and for pp38 in neutrophils (Figure 12, Table 4). The clinical phenotype, displaying more diverse symptoms than in the other families, was similar in both patients, although the mother, having normal NF-κB and p38 phosphorylation levels, suffers from inflammatory attacks less frequently with increasing age.

In comparing phosphorylation levels in patients and in controls, the calculated p-values (Mann-Whitney U) were statistically significant for monocyte, lymphocyte and neutrophil pNF-κB and pp38 AUC-values, with the exception of monocyte pp38 AUC^{dose} ($p=0.199$) (Table 4).

Table 4. Comparison of the intracellular responses to TNFα in TRAPS patients and in healthy control persons based on the AUC values for NF-κB and p38.

	C88Y		C73R		F112I		all patients		controls		MWU p-value
	range	median	range	median	range	median	range	median	range	median	
NF-κB mo AUC^{time}	186-241	232	298-396	347	234-305	268	186-396	250	271-404	365	0.005
NF-κB mo AUC^{dose}	1837-3153	2336	3479-4324	3902	2386-3967	3209	1837-4324	3041	2540-5071	3784	0.034
NF-κB ly AUC^{time}	86-120	106	119-142	130	116-142	131	86-142	120	120-210	163	0.001
NF-κB ly AUC^{dose}	599-1008	826	1003-1098	1050	943-1442	1240	599-1442	1006	1093-2528	1858	0.001
p38 mo AUC^{time}	471-678	508	488-610	549	439-512	490	439-678	490	580-768	682	0.001
p38 mo AUC^{dose}	7014-8956	7790	9311-9346	9328	4190-9233	7204	4190-9346	8139	5681-10875	8955	0.199
p38 pmn AUC^{time}	299-381	340	355-415	385	358-395	378	299-415	363	359-482	412	0.007
p38 pmn AUC^{dose}	2695-3554	2989	3496-4045	37770	2594-3173	3086	2594-4045	3156	3520-5028	4008	0.001

mo= monocytes; ly= lymphocytes; pmn= peripheral blood mononuclear cells; AUC^{time}= area under time course curve; AUC^{dose}= area under dose response curve; MWU= Mann-Whitney U non-parametric test

9. DISCUSSION

In this thesis, patients with recurring febrile and inflammatory attacks, as well as a positive family history of periodic fever, have been studied, revealing two new Finnish families with novel *TNFRSF1A* mutations, causing the autoinflammatory tumour necrosis factor receptor-associated periodic syndrome (TRAPS) (I, II). In addition to mutation analysis by genomic sequencing, serum concentrations of soluble TNFRSF1A by enzyme-linked immunosorbent assays, as well as cell surface expression and metalloproteinase-induced shedding of TNFRSF1A, using flow cytometric analysis, have been performed to enlighten the correlation between genotype and phenotype, and evaluate the reliability of these analyses in differential diagnostics. In the third article (III), we report a differential diagnostic problem in one TRAPS patient, in whom the cause of the severe abdominal pain and acute phase response proved to be an intraperitoneal abscess and present the hypothesis that an underlying autoinflammatory disorder may predispose to severe clinical

conditions. The fourth article (IV) is a functional study of tumour necrosis factor-induced intracellular signalling in TRAPS. We studied the two Finnish families with the novel TNFRSF1A mutations, as well as a third, previously reported Finnish family with TRAPS (McDermott et al., 1999), using phosphospecific monoclonal antibodies and flow cytometric analysis adapted for fresh whole blood, to evaluate the phosphorylation levels of proinflammatory NF-κB and p38.

9.1. The genotype and the phenotype of the novel F112I and C73R, and one previously reported C88Y, TNFRSF1A mutations (I, II, III, IV)

Following the discovery of *TNFRSF1A* as the causative gene in TRAPS and of the initial six *TNFRSF1A* missense mutations in seven different families, novel mutations and detailed descriptions of the phenotype associated with each mutation have been reported in an increasing number. As all reported *TNFRSF1A* mutations affect the cysteine-rich extracellular domains of the receptor (INFEVERS database. URL: fmf.igh.cnrs.fr/infevers), a change in the three-dimensional structure of the receptor, leading to its dysfunction has been presented as the central pathomechanism behind TRAPS (Galon et al., 2000; Rebelo et al., 2006). In particular mutations affecting cysteine residues would be expected to alter the conformation of the receptor, as the formation of disulphide bonds is hindered, and thus promote a more severe phenotype than non-cysteine mutations (Aksentijevich et al., 2001). Mutations affecting proline residues or residues involved in hydrogen bonds are also expected to have a pronounced effect on the secondary structure of the receptor (Rebelo et al., 2006).

In the context of this thesis, we have reported two Finnish families with novel *TNFRSF1A* mutations (I, II). The dominant mode of inheritance of the disorder and a clinical phenotype suggestive of TRAPS in both families gave cause to suspect a genetic defect in the gene coding for the cytokine receptor. The first study (I) reported a F112I mutation in exon four of *TNFRSF1A* in a family with periodic inflammatory attacks occurring in three generations. This was the first report of a mutation in the third extracellular domain of the receptor, this region (CRD3) being involved in the binding of TNF trimers. Previous studies had reported missense mutations and one splice junction mutation in exons 2-4 in the first and second extracellular domains of the gene (McDermott et al., 1999; Galon et al., 2000; Dodé et al., 2000; Aganna et al., 2001; Simon et al., 2001).

The F112I mutation is located in the proximity of a conserved cysteine residue and in a region responsible for binding lymphotoxins (Banner et al., 1993). The phenylalanine in position 112 would also appear to be closely involved in the formation of disulphide bonds at cysteine residue C114, as the amino acids F112, O113 and C114 are conserved in all mammalian TNFRSF1A proteins and a mutation at F112 could potentially hamper bond formation. The F112I mutation was confirmed in a sixth symptomatic family member (patient 10) in association with the fourth (IV) article. 5/6 family members with the F112I mutation had experienced periodically recurring high fever and abdominal pain since childhood or youth (Table 1). Additional manifestations of TRAPS included pleurisy in two of the patients and articular pain of the hip joint in one patient. One family member (patient 12), now aged 30, in whom the F112I mutation was confirmed has remained asymptomatic. The age of onset of the symptoms in TRAPS has been reported to vary within a given family, ranging from 2-63 years (Dodé et al., 2002a), wherefore it is possible, that the autoinflammatory disorder has not yet manifested itself. Other reports of patients bearing a *TNFRSF1A* mutation without clinical signs of TRAPS (Galon et al., 2000; Kusuhara et al., 2004; Cigni et al., 2006) would indicate an incomplete penetrance of the disorder, which may also be the case for the F112I mutation.

The second novel *TNFRSF1A* mutation reported (II) was the C73R mutation in exon 3 in the second extracellular domain (CRD2) of the receptor, CRD2 and CRD3 being the sites of interaction with TNFα. The mutation was detected in a mother and her daughter, both of whom had suffered from recurring inflammatory attacks since childhood. This mutation was the third mutation to be reported in a Finnish family. A Swedish family with autosomal dominant periodic fever has been reported in 1964 (Nilsson and Floderus, 1964) and in 1968 (Bergman and Warmenius, 1968), but in addition to the three Finnish TRAPS families, a Danish family with a novel *TNFRSF1A* mutation (Weyhreter et al., 2003), are the only patients with genetically confirmed TRAPS in the Nordic countries.

The cysteine at residue 73 is involved in the second disulphide bond of the second cysteine-rich extracellular domain of TNFRSF1A, the other cysteine of the bond being at position 88 (Galon et al., 2000). By causing a substitution of cysteine for arginine, the C73R mutation is likely to affect disulphide bonding and possibly cause a more severe clinical phenotype than might a non-cysteine mutation (Aksentijevich et al., 2001; Rebelo et al., 2006). With increasing age, the frequency and severity of the TRAPS attacks usually decrease, as in the case of the Finnish TRAPS patients. In the C73R family, the mother (patient 5), now aged 56, is nearly asymptomatic at present, whereas the daughter (patient 6), aged 31, continues to suffer from inflammatory attacks, with or without fever, twice a year.

In the third Finnish family, which was reported in association with the discovery of the genetic background to TRAPS (McDermott et al., 1999), the C88Y mutation was discovered in five family members, four of whom are alive at present. The C88Y patients also participated in the functional studies associated with the fourth (IV) article of this thesis. The C88Y mutation is likely to affect the second disulphide bond of the second extracellular domain of TNFRSF1A. The other participant of the bond is the cysteine at residue 73 (C73), the mutation of which causes TRAPS in the above mentioned Finnish family (II).

All four C88Y patients have suffered from episodes of fever and abdominal pain, the inflammatory episodes occasionally being launched by heavy meals or physical strain in two of the patients, a father (patient 1) and his son (patient 4) (Table 1). One patient (patient 2) also experienced joint pain during the attacks and is the only Finnish TRAPS patient to develop amyloidosis, with nephrotic syndrome and renal insufficiency as a consequence, leading to renal transplantation. Due to the renal transplant, the patient has a continuous medication of cyclosporine and methylprednisolone. Within the context of an international study involving AA amyloidosis in TRAPS, genomic sequencing of the FMF gene, *MEFV,* revealed the E148Q polymorphism in this patient (Aganna et al. 2004). In our laboratory we have not been able to detect this polymorphism, despite genomic sequencing, but it is possible, that PCR has failed to reproduce this affected allele. Nonetheless, the presence of a *MEFV* polymorphism in addition to the *TNFRSF1A* mutation, may explain why this patient is the only one of the C88Y or any other Finnish TRAPS family to develop amyloidosis. Although the E148Q variant is considered a low-penetrance polymorphism, which also is quite frequent in the healthy population (Aksentijevich et al., 1999; Booth et al., 2001), it is possible that the *TNFRSF1A* mutation and the *MEFV* variant together potentiate the effects of inflammation (Aganna et al., 2004). Following the transplantation, the patient, now aged 58, has not experienced any inflammatory attacks and also a female C88Y patient, aged 56 at present, is now asymptomatic. The TRAPS attacks in the Finnish patients appear to decrease in intensity and frequency with increasing age, which may explain the absence of symptoms in these two patients, but it is also possible, that the immunosuppressive medication of patient 2 may prevent the febrile episodes.

As the C73R and the C88Y mutations affect the same disulphide bond, the consequences on the TNFRSF1A conformation, as well as, the phenotypes of the patients would be expected to be similar. The clinical phenotype in association with the C73R mutation caused symptoms of a broader spectrum than the phenotype in association with the F112I or with the earlier reported C88Y mutation (Figure 1), but the frequency of the attacks, two to three times a year, was similar in all three mutations. It would also seem that the phenotypes associated with the same *TNFRSF1A* mutation may vary from one individual to another, as is the case for the mevalonate kinase deficiencies, where certain *MVK* mutations appear to be associated with either HIDS or MA (Mandey et al., 2006). The small number of patients, in particular in Family 3, makes it difficult to draw reliable conclusions concerning the variations of the clinical phenotype within a given family. Nonetheless, based on the three Finnish families studied, there appears to be no clear correlation between the genotype and the phenotype of TRAPS. Furthermore, the severity of the phenotype appears not to be automatically designated by the presence of a cysteine mutation, but appears to be regulated by additional mechanisms to the disruption of disulphide bond formation.

9.2. The role of low levels of sTNFRSF1A in serum and reduced shedding in association with mutations of TNFRSF1A (I, II, IV)

Based on the initial studies concerning TRAPS it was hypothesized that impaired cleavage of mutated TNFRSF1A from the cell surface, as a result of the conformational changes entrailed by the mutations of the gene, was the main mechanism causing the proinflammatory phenotype of TRAPS (McDermott et al., 1999; Galon et al., 2000). The finding of low concentrations of sTNFRSF1A in the serum of TRAPS patients, as well as reduced metalloproteinase activation-induced shedding of TNFRSF1A from the cell surface in the initially reported mutations, lended support to this theory (McDermott et al., 1999; Galon et al., 2000; Aksentijevich et al., 2001; Aganna et al., 2003; Xanthoulea et al., 2004). Subsequently, PMA-induced receptor shedding and sTNFRSF1A concentrations in serum, in between febrile attacks, have been used as differential diagnostic markers in patients with periodic fever and in whom TRAPS is suspected, although normal shedding has been demonstrated in association with some *TNFRSF1A* mutations and differences in shedding appear to be cell-type dependent (Huggins et al., 2004).

Our results of TNFRSF1A shedding following metalloproteinase activation in four F112I (I) and two C73R (II) patients, revealed no or reduced shedding in the F112I family, whereas no clear defect in the shedding of TNFRSF1A from the cell surface could be established in the C73R patients or in the two healthy controls examined concurrently. In three F112I patients, there was a nearly complete defect in TNFRSF1A shedding following stimulation with PMA, whereas TNFRSF1A shedding was normal in the healthy control persons studied simultaneously. Only in one young family member with the F112I mutation (patient 9) some receptor shedding did occur. There is no certain evidence on when a possible shedding defect develops in TRAPS, but the patient had only recently developed symptoms of serosal inflammation and fever, in agreement with the finding that symptomatic TRAPS is not present at birth, but appears during childhood or youth (Kimberley et al., 2007). It is possible to speculate that a shedding defect had not yet developed in this person, although, to my knowledge, there are no studies available at present to support this hypothesis. It would therefore be interesting to be able to study TNFRSF1A shedding in families with a *TNFRSF1A* mutation before and after the appearance of clinical symptoms, and on the other hand, in patients, whose symptoms have diminished with increasing age.

The F112I mutation affects the third and the C73R mutation the second extracellular domain of TNFRSF1A, whereas the site of cleavage of the TNFRSF1A ectodomain is located close to the transmembrane region at Asn172/201-Val173/202 (Gullberg et al., 1992). Nonetheless, reduced shedding was observed in association with the F112I mutation. TNFRSF1A has been demonstrated to be released into soluble form also from exocytosis vesicles (Hawari et al., 2004), a process, which does not require metalloproteinase-induced cleavage of the receptor. As cell surface expression of TNFRSF1A before and after stimulation with PMA was used to evaluate receptor shedding, the finding cannot be explained by possible impaired release of exosomal TNFRSF1A (eTNFRSF1A). With the exception of an I199N mutation, which directly affects the ectodomain cleavage site, through a new hydrogen bond (Kriegel et al., 2003), also the other mutations in association with which a shedding defect has been observed, affect the extracellular domains at regions other than the cleavage site (McDermott et al., 1999; Aksentijevich et al., 2001; Aganna et al., 2003; Xanthoulea et al., 2004). It is therefore likely that these mutations alter other regulatory mechanisms involved in receptor cleavage, such as ARTS1 (aminopeptidase regulator of TNFR1 shedding), a membrane-associated protein, which specifically binds to the extracellular domain of TNFRSF1A, promoting metalloprotease-induced shedding of the receptor (Cui et al., 2002).

As TNFRSF1A shedding may or may not be impaired in TRAPS, shedding analysis is not suitable for differential diagnostic use. Nonetheless, determining receptor cleavage from the cell surface is valuable in evaluating possible correlations between genotype and phenotype, and in furthering the knowledge of possible additional regulatory mechanisms involved in receptor shedding.

TRAPS has also been associated with reduced levels of serum sTNFRSF1A in patients in between febrile attacks (McDermott et al., 1999; Aganna et al., 2003), which appeared to be in agreement with the shedding hypothesis. In the F112I family, serum concentrations of sTNFRSF1A were below normal in 5/6 patients studied, and in the range of normal, but below the values of the healthy controls studied concurrently, in one patient. Thus, there would appear to be a reduction in sTNFRSF1A levels also in the sixth patient, although to a lesser extent than in the other family members with the F112I mutation.

Low levels of sTNFRSF1A in between febrile attacks were detected in both C73R patients, whereas nearly all healthy relatives on the maternal side of the family had normal sTNFRSF1A concentrations. All of the maternal relatives were asymptomatic, whereas no information was available of the paternal side of the family. This would indicate that low sTNFRSF1A concentrations are associated with TRAPS in the case of the C73R mutation. Serum concentrations of sTNFRSF1A were determined in patients and healthy relatives also in the C88Y family. The blood samples from the patients were drawn during an asymptomatic period in between inflammatory attacks. sTNFRSF1A levels were below normal in three out of four C88Y patients and elevated in the patient with the renal transplant (patient 2). The patient with the renal transplant was on cyclosporine and methylprednisolone also at the time of analysis and had normal serum creatinine levels. In seven healthy family members low sTNFRSF1A concentrations were observed, but these persons have not presented with inflammatory symptoms suggesting TRAPS.

In our findings, sTNFRSF1A concentrations of all but one patient were below the range of the values of the healthy controls studied simultaneously. The only patient with elevated sTNFRSF1A levels is the C88Y patient (patient 2), who developed amyloidosis, necessitating a renal transplantation in January of 2001 and subsequent immunosuppressive medication. Interestingly, sTNFRSF1A concentrations measured both before and after the renal transplantation are elevated. In the case of the Finnish patients, therefore, it would appear that a low sTNFRSF1A concentration in between attacks often is indicative of TRAPS. The majority of the asymptomatic relatives of the

patients and the healthy control subjects had sTNFRSF1A concentrations within the range of normal, as determined by the manufacturer of the ELISA Quantikine kit, although low sTNFRSF1A levels were observed also in some healthy relatives and controls. We have also observed reduced sTNFRSF1A levels in some patients with recurring fever of unknown etiology, although we have not been able to demonstrate a *TNFRSF1A* mutation in them (unpublished data).

Evaluating serum concentrations of sTNFRSF1A in patients with periodic fever and inflammation would appear helpful in screening for TRAPS, at least in the Finnish patients studied, although low sTNFRSF1A levels are not always indicative of TRAPS, and normal or elevated sTNFRSF1A levels do not necessarily exclude the autoinflammatory disorder. The clinical phenotype and possibly a positive family history of periodic fever are all the more important in deciding whether or not to pursue to mutation analysis.

9.3. Differential diagnostic problems and possible complications associated with TRAPS (III)

The discovery of the genetic backgrounds of the different autoinflammatory disorders has enabled verification of the diagnosis through mutation analysis, although a mutation in the target gene has not always been found in patients with periodic fever (Aksentijevich et al., 2002; Stojanov and Kastner, 2005). Prior to the diagnosis of TRAPS, the Finnish patients had undergone extensive diagnostic and therapeutic procedures brought about by the febrile episodes. The febrile attacks and abdominal pain had led to appendicectomy in six of the patients and in all cases the removed appendices were intact. Differential diagnostic options proposed included tuberculous peritonitis, Crohn's disease and salphingo-ophoritis. A positive family history of periodic fever indicating a hereditary disorder and comparison to previous inflammatory episodes have been guidelines in differential diagnostics and treatment of the patients during the acute phase. The genetic verification of TRAPS has enabled early treatment of the symptoms and has psychologically been a great relief to all patients.

The diagnosis of TRAPS or other rare genetic disorders may on the other hand be a misleading factor in the case of an acute illness. The case of the Finnish TRAPS patient described by us (III) emphasizes the difficulty in discerning the acute symptoms involved with a serious disease from those of the diagnosed disorder. In the female patient, the symptoms of fever and abdominal pain were initially indistinguishable from the symptoms associated with TRAPS attacks. The localization of the pain to the lower right of the abdomen and the nausea were the only clinical features different from previous episodes. The increasing severity of the symptoms and the intensifying of the acute phase reactants, despite treatment for both a possible bacterial infection and an underlying TRAPS attack, were suggestive of an etiology other than TRAPS behind the symptoms. Only after a renewed CT of the abdomen an intraperitoneal abscess was revealed as the underlying cause of the severe clinical condition.

As the TRAPS patient was a young woman, without predisposition to infections, it is surprising that an abscess had developed. In association with systemic inflammation, pro-inflammatory cytokines and nitric oxide have been shown to cause dysfunction of intestinal epithelial cells and disruption of intercellular tight junctions (Fink, 2003). TNFα-mediated NF-κB activation has been demonstrated to disrupt tight junctions (Poritz et al., 2004) and increase tight junction permeability (Ma et al., 2004) in the intestinal epithelium, whereas another study using *in vivo* rabbit intestinal segments revealed enhanced intestinal permeability as a result of a rise in intraluminal pressure (Swabb et al., 1982). Systemic inflammation is also a characteristic of TRAPS, caused by abnormally functioning

TNFRSF1A. It is therefore possible, that an initial TRAPS attack in the patient could have caused intestinal paralysis and increased intraluminal pressure, leading to increased epithelial cell permeability and subsequent bacterial extravasation. The additional effect of TNFα and other proinflammatory cytokines may have accelerated the process, resulting in the development of an intra-abdominal abscess.

This case report presents the challenges of differential diagnostics in patients with rare genetic disorders and stresses the importance of critical interpretation of the clinical findings and of the general condition of the patient in such disorders. In the case of this patient, the diagnosis of TRAPS served as a misleading factor, which possibly prevented the abscess from being revealed sooner, although an initial TRAPS attack may have predisposed to the formation of the abscess. It is important to note that not all symptoms may be attributed to the underlying autoinflammatory disorder, especially if the symptoms are abnormally severe or differ significantly from previous disease attacks. On the other hand, it would appear that a serious clinical condition of this nature may be the result of an underlying autoinflammatory disorder, further emphasizing the necessity for early intervention and treatment at the appearance of inflammatory symptoms.

9.4. NF-κB signalling in TRAPS (IV)

In the fourth (IV) article of this thesis, we studied intracellular signalling in ten TRAPS patients, with the C73R, C88Y and F112I mutations, by determining levels of phosphorylation of NF-κB and p38 in response to TNFα (see Figure 2 for the TNFα-induced intracellular signalling cascades). Transfection experiments have previously shown both an increase (Yousaf et al., 2005) and a decrease in NF-κB activity (Siebert et al., 2005b; Lobito et al., 2006) in association with *TNFRSF1A* mutations. A study, using cultured fibroblast from a patient with the C43S mutation, also demonstrated reduced NF-κB activity, as well as decreased apoptosis, in mutated cells (Siebert et al., 2005a). To minimize ex vivo activation of the cells studied, we adapted a method of flow cytometric analysis tailored for fresh whole blood, drawn directly from the patients. The blood samples were labelled with phosphospecific antibodies, recognizing the phosphorylated forms of p65 NF-κB and p38. Based on the pNF-κB and pp38 levels, AUC-values were calculated and the Mann-Whitney U non-parametric test and the analysis of variance with repeated measures were used to evaluate the phosphorylation profile in comparison to the ten healthy subjects studied concurrently.

The study revealed low NF-κB and p38 phosphorylation levels in the C88Y and the F112I patients and in one of two C73R patients, in all three types of leukocytes studied (monocytes, lymphocytes and neutrophils), in comparison to the healthy persons studied. The finding that not all patients showed aberrant transcription factor phosphorylation, would indicate that the development of TRAPS does not require a low signalling profile, and that a low signalling phenotype is not a characteristic of a particular type of TNFRSF1A mutation.

The clinical picture in association with the three different mutations was quite similar, although the symptoms in the C73R family were of a broader spectrum than the symptoms in the C88Y and F112I families. In the C88Y family, in which all four patients had low phosphorylation profiles, the symptoms were not particularly severe, but one C88Y patient did develop amyloidosis, being the only Finnish TRAPS patient in which amyloidosis has been diagnosed. Despite the fact that none of the other C88Y patients have presented any signs of amyloidosis, the histologic confirmation of AA amyloidosis in this particular patient, indicates it to be a consequence of the autoinflammatory disorder. The C73R and F112I patients with NF-κB and p38 phosphorylation patterns in the range

of the controls both suffer from febrile episodes and symptoms similar to those of the other patients with the respective mutations. Based on these results, there would appear to be no correlation between the phosphorylation profile and the clinical phenotype of TRAPS in between mutations or in patients with the same mutation.

The low NF-κB phosphorylation levels observed in the majority of our TRAPS patients is in agreement with the in vitro experiments of cultured fibroblasts from a C43S TRAPS patient (Siebert et al., 2005a), the transfection experiments of B-cell lymphoma cells with the C30R, C43S, T50M and C52F *TNFRSF1A* mutations (Siebert et al., 2005b) and of HT1080 cells with the H22Y, C33G, T50M and C52F mutations (Lobito et al., 2006), these studies involving mutations other than the ones studied here. Reduced surface expression of TNFRSF1A (Siebert et al., 2005b; Lobito et al., 2006) or intracellular retention of the mutated and misfolded receptor (Lobito et al., 2006) may explain the low NF-κB phosphorylation levels, but in our patients FACS analysis revealed normal TNFRSF1A surface expression (I, II).

Deficient TNFα-induced apoptosis has also been observed in association with low NF-κB phosphorylation levels (Siebert et al., 2005a; Kimberley et al., 2007). Under normal conditions, TNF induces NF-κB activating pathways preferentially to caspase-mediated pro-apoptotic pathways (Muppidi et al., 2004), which are triggered as a result of reduced NF-κB activity and subsequent attenuation of the NF-κB-induced antiapoptotic regulatory mechanisms (Micheau et al., 2003; Deng et al., 2003; Lin et al., 2003; Varfolomeev et al., 2004). A recent study demonstrates that activation of the proapoptotic pathways necessitates the internalization of the receptor into the cytoplasm (Schneider-Brachert et al., 2004). A reduction in NF-κB activity would be expected to cause increased TNF-induced apoptosis, but it is possible, that the altered structure of mutant TNFRSF1A may hamper its endocytosis and thus lead to defective apoptosis (Schneider-Brachert et al., 2004; D'Osualdo et al., 2006).

On the other hand, 293T cells transfected with the T50K *TNFRSF1A* mutation, show self-association of the mutant receptor, as well as increased NF-κB activation in the absence of TNF (Yousuf et al., 2005). A mutation affecting the preligand domain of TNFRSF1A could lead to self-association of the receptor and constitutive activation. Misfolding and intracellular retention of the receptor, leading to self-aggregation and activation of intracellular proinflammatory signalling cascades may also explain the results (Siebert et al., 2005a, Lobito et al., 2006). In our study, self-association and subsequent ligand-independent signalling could not explain the aberrant NF-κB and p38 signalling profiles, as the response was specific to stimulation with TNF. LPS-induced NF-κB and p38 and IFN-γ-induced pSTAT1 phosphorylation levels were similar in patients and healthy controls alike.

Low NF-κB and p38 signalling profiles in leukocytes appear to be frequently associated with TRAPS. Although the number of patients studied is small and the results need to be interpreted with caution, 83 per cent of all Finnish patients alive with diagnosed TRAPS were included in the study and of these patients, low levels of NF-κB and p38 phosphorylation was observed in nine out of ten patients. As TRAPS is characterized by a hyperinflammatory phenotype and the systemic autoinflammatory disorders are considered to be the result of an overactivation of the innate immune system (Simon and van der Meer, 2007), the findings of the low phosphorylation profiles associated with the *TNFRSF1A* mutations studied here need to be analyzed carefully, in particular, as intracellular retention with ligand-independent signalling or reduced TNFRSF1A surface expression does not explain our results. Other hyperinflammatory disorders, such as Crohn's disease, which may be associated with mutations in the LRR motif of *NOD2/CARD15* and in association with which a reduction in NF-κB activation (Ogura et al., 2001) and NF-κB-induced

cytokine expression (van Heel et al., 2005; Korzenik et al., 2007) has been observed. A recent study using mice with IKKβ depleted macrophages and neutrophils demonstrated that prolonged inhibition of IKKβ, hindering NF-κB activation, resulted in an increase in plasma IL-1β concentrations, as a result of increased pro-IL-1β cleavage and caspase-1 activation, but also through an elevation in the caspase-1 independent IL-1β secretion (Greten et al., 2007). Pharmacological inhibition of IKKβ in myeloid cells, followed by stimulation with endotoxins, also caused an elevation of IL-1β-levels in the plasma, thereby reinforcing the results obtained by the knock-out mice model. The findings in this study demonstrate a compensatory posttranscriptional regulatory role of NF-κB on inflammatory pathways through the inhibition of pro-IL-1β processing, in addition to the established regulatory effect on pro-inflammatory gene transcription. The capacity of NF-κB to posttranscriptionally regulate proinflammatory signalling cascades would explain why sustained inhibition of NF-κB would result in increased IL-1β secretion, despite inhibition of the NF-κB-mediated IL-1β transcription. The hyperinflammatory phenotype of TRAPS may, thus, be the result of the compensatory posttranslational regulatory mechanisms, activated by low levels of cytokine-induced NF-κB phosphorylation, as was observed in association with the C88Y, F112I and partly with the C73R mutations studied here.

10. CONCLUSIONS

TRAPS is defined by mutations in *TNFRSF1A*, believed to cause conformational changes of the receptor (McDermott et al., 1999). These structural changes may result in the impaired metalloproteinase-induced cleavage of TNFRSF1A, leading to the continuous binding of TNFα, and thus a sustained activation of the intracellular protein cascades and transcription of proinflammatory cytokines (McDermott et al., 1999). The structural changes in TNFRSF1A may also cause retention of the misfolded receptor in the ER, resulting in reduced surface expression (Lobito et al., 2006) and subsequent incomplete binding and neutralisation of TNFα (Van Zee et al., 1992) or self-association of the receptor in the cytoplasm, causing ligand-independent activation of intracellular protein cascades (Todd et al., 2004; Lobito et al., 2006; Kimberley et al., 2007). Reduced TNFα-induced apoptosis (Siebert et al., 2005a; D'Osualdo et al., 2006; Kimberley et al., 2007) or aberrant intracellular signalling as a result of mutations in *TNFRSF1A* (Siebert et al., 2005a; Siebert et al., 2005b; Lobito et al., 2006; Yousaf et al., 2005) may further explain the proinflammatory response in association with TRAPS.

The two novel *TNFRSF1A* mutations reported in this thesis and the phenotype associated with each mutation have added to the knowledge of TRAPS. The F112I mutation was the first mutation to be reported in the third cysteine-rich extracellular domain of TNFRSF1A (I), thereby bringing a new aspect to possible pathomechanisms involved in the autoinflammatory disorder. The C73R mutation (II) is the third mutation reported in Finnish patients, elucidating the fact that TRAPS is not associated with a restricted number of mutations at certain residues, but that *TNFRSF1A* is vulnerable for nucleotide changes at numerous sites in the region coding for the extracellular domains of the receptor, in particular in exons 2-4. Even in a population as small as the Finnish, all three TRAPS families have different *TNFRSF1A* mutations, suggesting the absence of a founder effect in this dominant autoinflammatory disorder. The identification of these mutations in the Finnish families has facilitated the accurate molecular diagnosis of TRAPS and thus the proper treatment of the patients concerned. Based on these findings, consultations about Finnish, as well as Scandinavian patients have been directed to our clinics. We have applied the knowledge on *TNFRSF1A* mutations in analysing the samples of patients with clinical symptoms suggesting TRAPS, in particular if these are accompanied by low levels of sTNFRSF1A and a dominant mode

of inheritance, in the familial cases. The genetic analysis continues still, but to date, we have not been able to detect any *TNFRSF1A* mutations in these patients.

The third article (III) reported a differential diagnostic dilemma in association with TRAPS and presents the possibility of TRAPS as the underlying cause of complications, in this case an intra-abdominal abscess. The hypothesis that TRAPS may predispose to such a severe clinical condition brings a new dimension to the autoinflammatory disorders, as complications other than amyloidosis have not previously been associated with TRAPS. The second aim of the report was to elucidate for the clinician typical findings in TRAPS, as opposed to aberrant findings indicating pathologic conditions other than the underlying autoinflammatory disease. In conclusion, the importance of critical evaluation of the patients' condition is enhanced in the case of rare genetic diseases.

Functional studies of the Finnish TRAPS patients revealed low TNF-induced NF-κB and p38 phosphorylation levels, indicating aberrant signalling in leukocytes from nine out of ten patients (IV). To our knowledge, this is the first study to determine intracellular signalling using fresh whole blood from TRAPS patients. Our findings of low NF-κB and p38 phosphorylation profiles in association with *TNFRSF1A* mutations suggest that unverified regulatory mechanisms, causing the hyperinflammatory phenotype of TRAPS, are activated by the defectively functioning innate immune system

The aim of this thesis to 1) establish the genetic cause of hereditary periodic fever in Finnish patients and to delineate the clinical course of the disease was addressed in the first two articles of this thesis (I, II), where the clinical symptoms of patients in two new TRAPS families were described and two new TNFRSF1A mutations identified, as well as in the third article (III), presenting the clinical phenotype of TRAPS in light of a differential diagnostic dilemma and the hypothesis of TRAPS as the predisposing factor to severe complications. The two following aims of the thesis to 2) evaluate the clinical value of measuring sTNFRSF1A in TRAPS patients and 3) investigate metalloproteinase.induced TNFRSF1A shedding in leukocytes from TRAPS-patients, were investigated and discussed in articles I and II, presenting the novel *TNFRSF1A* mutations. The fourth aim of this thesis to 4) study intracellular NF-κB activation, in an attempt to gain a greater understanding of the underlying pathomechanisms of TRAPS, was met in part, as the functional studies presented in article IV did reveal an abberant NF-κB phosphorylation in association with *TNFRSF1A* mutations, although the exact implications on the pathomechanism of this defect still remains unclear.

The findings in the studies of this thesis have contributed to the knowledge of TRAPS, by shedding some light on genotype and phenotype associations, as well as on possible pathomechanisms involved. Our findings of an aberrant intracellular phosphorylation profile in association with TRAPS do not completely explain the pro-inflammatory phenotype typical of the autoinflammatory disorders and further investigations are necessary before the pathomechanisms of TRAPS can be established. Future molecular studies need therefore to be focused on the intracellular signalling associated with different *TNFRSF1A* mutations, as it is possible that the pathomechanisms involved may vary from one mutation to another. The increasing amount of information on the autoinflammatory disorders does, however, allow for the identification of patients affected, for getting a clear picture of the symptoms and natural history of the disease and for attaining the experience necessary for the development of more efficient forms of treatment. Investigations of the pathomechanisms behind the autoinflammatory disorders may also give a deeper understanding of the mechanisms associated with other chronic inflammatory diseases and help identify molecular targets for possible drug discovery.

11. ACKNOWLEDGEMENTS

This study was performed as a joint project of the Department of Dermatology, Allergology and Venereology and the Department of Medicine, University of Helsinki, and carried out at the Department of Dermatology, Helsinki University Central Hospital and Biomedicum during the years 2000-2008.

I would like to express my sincere gratitude to my supervisors, Professor Annamari Ranki, Head of the Department of Dermatology, Allergology and Venereology, and docent Tom Pettersson of the Department of Medicine, University of Helsinki, for their invaluable guidance throughout this study.
Professor Annamari Ranki is warmly acknowledged for providing excellent research facilities at the Department of Dermatology, for sharing her expertise in the field of scientific research with me and being a constant source of inspiration during this study.
Docent Tom Pettersson is greatly acknowledged for providing the means to carry out this project and for sharing his vast knowledge of inflammatory disorders, medicine in general and scientific writing.
I am greatly thankful to my supervisors for their enthusiasm and encouragement to complete this project, as well as for their ever understanding attitude, which has enabled family life and clinical work alongside the scientific work.

My sincere thanks are extended to docent Pekka Lahdenne and Professor Helena Kääriäinen for reviewing this thesis under a pressing deadline and for providing me with constructive and helpful advice.

My deepest gratitude is owed to all my co-authors. I want to thank, in particular, MD, PhD Leena Karenko for her guidance concerning methodological dilemmas and enlightening discussions on the autoinflammatory disorders, Professor Pärt Peterson for his invaluable advice on genetic analysis and docent Heikki Repo for patiently and readily taking the time to instruct me in questions ranging from data analysis to flow cytometry. I am truly privileged to have had the opportunity to work with and parttake of the expertise of the above mentioned. I also want to express my gratitude to PhD Vesna Blazevic, MD, PhD Arto Kivisaari, PhD Harri Mustonen, Professor Pauli Puolakkainen, as well as to MD Hanna Nevala and MD, PhD Maria Pesonen for teaching me the basic methods of genetic analysis and Professor Seppo Sarna for introducing me to the world of statistics.

The members of the research groups and the laboratory staff at the Skin- and Allergy Hospital and Biomedicum are warmly thanked for providing me with an enjoyable and supportive atmosphere to work in: Alexa, Alli, Jenita, Paula, Pilvi, Marja, Sari, and in particular Emilia, Harri, Katja, Marjut, Tiina and Veli-Matti for fruitful discussions and help when encountering problems in the realms of biochemistry, genetics and word processing. My most sincere gratitude is extended to Kaija Järvinen for teaching me the basics of laboratory work and for the adept technical assistance during all these years in the laboratory. I also want to express my warmest thanks to Sonja and Tiina, for all the thoughts and advice on finalizing a thesis, as well as to Sakari and Jonna for their much appreciated assistance.

Raija Sassi, Kaija Kosonen and Helena Minkkinen are cordially acknowledged for their outstanding work and help with manuscript and poster pictures, and Marianne Karlsberg is warmly acknowledged for her help with practical arrangements in finalizing this thesis.

My sincere gratitude is due to all patients and their families for participating in this study.

I wish to acknowledge my friends from childhood and youth and I especially want to thank Andrea, Caro, Jessica, Kata, Maria, Mascha, Mikaela and Nana, as well as “Sibbo-gänget”: André, Antte & Hanna, Antti, Hannu, Micke and Segge, for putting a smile on my face every now and then, as well as for reminding me that occasionally life is, in fact, all fun and games and for being there when it is not.

My most devoted gratitude is extended to my family and relatives. I want to thank my parents Hilkka and Bjarne Stjernberg for teaching me that aspirations are worth pursuing. Most of all I thank my parents and my brothers, Anders and Mats, for always having faith in me and my endeavours and for giving me and my family their unyielding support and help in whatever the matter. I also want to express my gratitude to my parents-in-law, Aila and Jarmo Salmela for having accepted me, without reservations, into the family and for always readily extending their help and assistance, despite the geographical distance separating us. My sisters-in-law, Anu and Hanna, are warmly thanked for their friendship and for being such outstanding god-mothers to our children.

Above all, I want to thank my beloved husband Mikko and our beautiful children Amos and Aino, without whom all efforts would appear futile and any achievements would be without meaning. Thank you, Mikko, for being my dearest friend, for tirelessly providing for our family and for your support and encouragement when I have despaired. My warmest affections are adressed to Amos, whose astute observations and insights never cease to amaze me and to Aino, whose sunny appearance and good humour is out of this world and makes it worth getting up in the morning.

Financial support from the Finska Läkaresällskapet, the Wilhelm and Else Stockmann Foundation, the Maud Kuistila Foundation, the Oskar Öflund Foundation, the Dorothea Olivia, Karl Walter and Jarl Walter Perkléns Memory Foundation, the Research Fundation of Orion Corporation, the Finnish Medical Society Duodecim foundations, and the Helsinki University Central Hospital Research Funds are greatefully acknowledged.

Lahti, July 2008.

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