NOVEL GENES AND MECHANISMS IN MONOGENIC AUTOINFLAMMATORY DISORDERS

A thesis submitted in total fulfilment of the degree of Doctor of Philosophy

by

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ABSTRACT

Monogenic autoinflammatory disorders are a heterogeneous group of rare conditions characterised by innate immune dysregulation. Patients often present early in life with recurrent fevers and features of systemic inflammation without high titres of autoantibodies or self-reactive T cells. Since the introduction of whole exome sequencing in the diagnostic evaluation of patients with suspected monogenic autoinflammatory disorders, the number of genetically defined conditions has greatly increased as has the phenotypic diversity.

This study aimed to optimise methods of validating the pathogenicity of previously undescribed variants *in vitro* and to establish a national registry for patients with suspected or confirmed monogenic autoinflammatory disorders.

Two variants in inflammasome forming proteins were evaluated. An inflammasome is a large multiprotein complex that forms in response to danger or pathogens. It serves as a platform for caspase-1 activation, resulting in cleavage of pro-IL-1β and pro-IL-18 to their active forms, as well as inflammatory cell death, pyroptosis. The first variant investigated was found through Sanger sequencing of *MEFV* in a family with a dominantly inherited suppurative dermatological condition. The novel variant p.Glu244Lys pyrin segregated with disease and was associated with increased inflammasome activation *in vitro*. This residue was shown using immunoprecipitation to be important for the binding of the regulatory proteins 14-3-3 and the substitution to lysine resulted in the auto-activation of pyrin.

The second novel variant was found in two unrelated children with autoinflammation and macrophage activation syndrome. Although different genetic sequencing techniques were used, both children were found to harbour heterozygous p.Trp655Cys NLRC4. *In vitro*

modelling revealed that this variant caused a Caspase-1-dependent increase in IL-1β and IL-18 release with priming alone. Through the evaluation of the potential mechanisms of auto-activation, a previously unknown leucine rich repeat interface was revealed to exist between two NLRC4 monomers in the oligomeric state. Furthermore, an additional distinct interface was shown to exist between p.Trp655Cys NLRC4 and residues of the adjacent leucine rich repeat domain.

Finally, the Australian Autoinflammatory Diseases Registry was established with fifteen tertiary hospitals across six Australian states currently involved. Thirty-seven patients with suspected autoinflammatory disorders have been recruited to date along with seventy-seven family members. The first twenty participants in whom no pathogenic mutation had been detected using National Association of Testing Authorities approved diagnostic testing underwent whole exome sequencing alongside their biological parents to determine variants that may be causing disease. The results of this analysis are presented here, including the identification of a novel variant in *SHARPIN*, encoding a component of the linear ubiquitin chain assembly complex involved in both the NF-κB and NLRP3 inflammasome pathways. This is the subject of ongoing investigation.

The work described in this thesis has led to the first ethically approved national Australian registry for patients with monogenic autoinflammatory disorders. Furthermore, the *in vitro* validations of several AIDs within this thesis provide exquisite examples of some of the techniques that can be utilised in the future evaluation of variants of interest generated through the recruitment and sequencing of patients through the Australian Autoinflammatory Diseases Registry.

DECLARATION

This is to certify that:

(i) The thesis is comprised of my original work towards the Doctor of Philosophy except were indicated in the preface.

(ii) Due acknowledgement has been made for the work of other authors.

(iii) The thesis is fewer than 100,000 words, exclusive of tables, maps, bibliographies and appendices.

Fiona E. Moghaddas

PREFACE

My overall contribution to the work presented in this thesis is 85%.

The following authors contributed 25% of the data within **Chapter 3**: Clinical and genetic information for Figures 1 and Supplementary Figure 1 and 2 was provided by RL, VG, AMV and JIA. Serum cytokine and primary cell ASC speck analysis for Figure 2 was provided by HMB, JJMG, PMdC, SC and PP.

The work presented in **Chapter 3** has been published: **Moghaddas F,** Llamas R, De Nardo D, Martinez-Banaclocha H, Martinez-Garcia JJ, Mesa-Del-Castillo P, Baker PJ, Gargallo V, Mensa-Vilaro A, Canna S, Wicks IP, Pelegrin P, Arostegui JI, and Masters SL. A novel Pyrin-Associated Autoinflammation with Neutrophilic Dermatosis mutation further defines 14-3-3 binding of pyrin and distinction to Familial Mediterranean Fever. *Annals of the rheumatic diseases* 2017.

The following authors contributed 25% of the data within **Chapter 4**: Clinical data, genetic analysis and serum cytokine analysis for Figure 1 and Supplementary Figure 1 was provided by HS, SB, SRH, RB, SW, KL, SC and CH. Clinical data, genetic analysis and serum cytokine analysis for Figure 2 was provided by PZ, YZ, YZ, BL, XC, LZ, SC and HZ.

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presentations needed to include ‘more science’, to the final meeting when we were discussing the manuscripts to be included in my thesis.

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ABBREVIATIONS

Abbreviation	Description
AADRY	Australian Autoinflammatory Diseases Registry
ACMG	American College of Medical Genetics and Genomics
AGHA	Australian Genomics Health Alliance
AGS	Aicardi–Goutières syndrome
AID	monogenic autoinflammatory disorder
AIFEC	autoinflammation with infantile enterocolitis
AIM2	absent in melanoma 2
ALPS	autoimmune lymphoproliferation
ANA	antinuclear antibody
APLAID	autoinflammation and PLCG2-associated antibody deficiency and immune dysregulation
ASC	apoptosis-associated speck-like protein containing a CARD
Asp	aspartic acid
BIRC	baculoviral IAP repeat-containing
BMDM	bone marrow derived macrophages
BS	Blau syndrome
BSA	bovine serum albumin
CADD	combined annotation dependent depletion
CANDLE	chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature
CAPS	cryopyrin associated periodic syndrome

CARD	caspase activation and recruitment domain
Cas	CRISPR associated
CD	cluster of differentiation
CDK	cyclin-dependent kinase
CDN	cyclic dinucleotides
cGAMP	cyclic guanosine monophosphate–adenosine monophosphate
cGAS	cyclic GMP-AMP synthase
cIAP	cellular inhibitor of apoptosis protein
CLR	C-type lectin receptor
CNS	central nervous system
CPI	Centre for Personalised Immunology
CPPD	calcium pyrophosphate dihydrate
CRISPR	clustered regularly interspaced short palindromic repeats
CRP	c-reactive protein
CVID	common variable immunodeficiency
Cys	cysteine
DADA2	deficiency of ADA2
DAMP	damage associated molecular pattern
ddNTP	dideoxynucleotides triphosphates
DIRA	deficiency of IL-1Ra
DITRA	deficiency of IL-36Ra
DMEM	Dulbecco's modified Eagle's medium
dNTP	deoxynucleotide

DSB	double strand break
dsDNA	double stranded DNA
DSS	dextran sulfate sodium
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ENA	extractable nuclear antibody
ENU	N-ethyl-N-nitrosourea
EOIBD	early onset inflammatory bowel disease
EOS	early onset sarcoidosis
ER	endoplasmic reticulum
ERK	extracellular-signal-regulated kinase
ExAC	exome aggregation consortium
FACS	fluorescence-activated cell sorting
FADD	Fas-associated protein with death domain
fathmm	functional analysis through hidden Markov models
FBS	fetal bovine serum
FCAS	familial cold autoinflammatory syndrome
FIIND	function to find domain
FKLC	familial keratosis lichenoides chronica
fl	full length
FMF	familial Mediterranean fever
FSC	forward scatter

gDNA	genomic DNA
GFP	green fluorescent protein
Gln	glutamine
Glu	glutamic acid
Gly	glycine
gnomAD	Genome Aggregation Database
GoF	gain of function
GPP	generalised pustular psoriasis
GS	grantham score
GSDMD	gasdermin D
HA20	haploinsufficiency of A20
HAMP	homeostasis altering molecular patterns
HD	hinge domain
HDR	homology directed repair
HEK	human embryonic kidney
HIDS	hyper-IgD syndrome
His	histidine
HLA	human leukocyte antigen
HLH	haemophagocytic lymphohistiocytosis
HOIL1	haem-oxidized IRP2 ubiquitin ligase 1
HOIP	HOIL-1L interacting protein
HREC	human research ethics committee
HRP	horseradish peroxidase
HSCT	haematopoetic stem cell transplant
HYDM1	recurrent hydatidiform mole-1

IBD	inflammatory bowel disease
ICE	interleukin-1 converting enzyme
IEC	intestinal epithelial cell
IFN	interferon
IFNAR	interferon receptor
IGV	integrated genomics viewer
IκB	inhibitor of κB
IKK	IκB kinases
IL	interleukin
IL-1Ra	interleukin-1 receptor antagonist
IL-1RAcP	interleukin-1 receptor accessory protein
Ile	isoleucine
iPSC	inducible pluripotent stem cells
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory factors
ISG	interferon stimulated genes
ISSAID	International Society for Systemic Autoinflammatory Diseases
IUIS	Internation Union of Immunological Societies
JAK	janus kinase
JMP	joint contractures, muscle atrophy, microcytic anemia and panniculitis-induced lipodystrophy
KO	knock-out
LB	lysogeny broth
LDH	lactate dehydrogenase

Leu	leucine
LoF	loss of function
LPS	lipopolysaccharide
LRR	leucine rich repeat
LUBAC	linear ubiquitin chain assembly complex
Lys	lysine
MAF	mean allele frequency
MAPK	mitogen activated protein kinase
MAS	macrophage activation syndrome
MAVS	mitochondrial antiviral-signalling protein
MDA5	melanoma differentiation-associated protein 5
MDP	muramyl dipeptide
MEF	mouse embryonic fibroblasts
Met	methionine
MGHA	Melbourne Genomics Health Alliance
MHC	major histocompatibility complex
MOI	multiplicity of infection
MSPC	multiple self-healing palmoplantar carcinoma
MSU	monosodium urate
MVK	mevalonate kinase
MWS	Muckle-Wells syndrome
MyD88	myeloid differentiation primary response 88
NAIP	neuronal apoptosis inhibitory protein
NATA	National Association of Testing Authorities
NEMO	NF-kappa-B essential modulator

NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	next generation sequencing
NHEJ	non-homologous end joining
NKJO	Nakajo-Nishimura syndrome
NLR	NOD like receptor
NLRC4-AID	NLRC4 associated autoinflammatory disorders
NLS	nuclear localization signals
NOD	nucleotide-binding oligomerization domain
NOMID	neonatal onset multisystem inflammatory disease
OMIM	Online Mendelian Inheritance in Man
ORAS	OTULIN-related autoinflammatory syndrome
OTU	ovarian tumour
PAAND	pyrin associated autoinflammation with neutrophilic dermatosis
PAM	protospacer adjacent motif
PAMP	pathogen associated molecular pattern
PAPA	pyogenic arthritis-pyoderma gangrenosum-acne
PBMC	peripheral blood mononuclear cells
PBST	phosphate buffered saline with tween
PCR	polymerase chain reaction
Phe	phenylalanine
PICF	patient information and consent form
PLAID	PLCG2-associated antibody deficiency and immune dysregulation

PLCG2	phospholipase C gamma2
PMA	phorbol myristate acetate
PolyPhen-2	polymorphism phenotyping v2
PRAAS	proteasome associated autoinflammatory syndrome
PRINTO	Paediatric Rheumatology INternational Trials Organisation
Pro	proline
PRR	pattern recognition receptor
PYD	pyrin domain
rAAV	recombinant adeno-associated virus
RIG	retinoic acid-inducible gene I
RIP	receptor-interacting protein
RLR	RIG-I like receptors
RPMI	Roswell Park Memorial Institute
RVIS	residual variation intolerance score
SAM	sequence alignment map
SAVI	STING associated vaculopathy with onset in infancy
SCAN4	syndrome of enterocolitis and autoinflammation associated with mutation in NLRC4
SCID	severe combined immune deficiency
Ser	serine
sgRNA	single guide RNA

SHARPIN	SHANK-associated RH domain interacting *protein*
shRNA	short hairpin RNA
SIFT	sorting intolerant from tolerant
sIL-1R	soluble IL-1 receptor
siRNA	short interfering RNA
sJIA	systemic onset juvenile idiopathic arthritis
SNP	single nucleotide polymorphoism
SOB	super optimal broth
SSC	side scatter
ssDNA	single stranded DNA
ssOligo	single stranded oligonucleotide
ssRNA	single stranded RNA
STAT	signal transducer and activator of transcription
STING	stimulator of interferon genes
sTNFR1	soluble TNF receptor 1
T-ALL	T-cell acute lymphoblastic leukaemia
T3SS	type three secretion system
TAB	TAK1 binding protein
TACE	TNF-alpha converting enzyme
TAK1	TGFβ-activated kinase 1
TALE	transcription activator-like effector
TALEN	transcription activator-like effector nuclease
TANK	TRAF associated NF-κB activator
TBK1	TANK-binding kinase 1

TBST	tris-buffered saline with tween
TcdB	toxin B from *Clostridium Difficile*
Th	T helper
Thr	threonine
TIR	toll/interleukin-1 receptor
TLR	toll like receptor
TNF	tumour necrosis factor
TNF-BP	TNF binding protein
TNFR1	TNF receptor
TOFIE	time of flight inflammasome evaluation
TRADD	TNFR1-associated death domain protein
TRAF	TNF receptor associated factors
TRAPS	Tumour Necrosis Factor Receptor Associated Periodic Syndrome
TRIM	tripartite motif-containing
Thr	threonine
Trp	tryptophan
Tyr	tyrosine
Ub	ubiquitin
Val	valine
VASP	variant analysis of sequenced pedigrees
VUS	variant of uncertain significance
WAS	Wiskott–Aldrich Syndrome
WCL	whole cell lysate

WEHI	Walter and Eliza Hall Institute of Medical Research
WES	whole exome sequencing
WGS	whole genome sequencing
WHD	winged helix domain
WT	wild type
XIAP	X-linked inhibitor of apoptosis protein
XLP	X-linked lymphoproliferative disease
ZFN	zinc-finger nucleases

1 INTRODUCTION

"But the curative force of nature, the most important element of which is the inflammatory reaction, is not yet perfectly adapted to its object"

Metchnikoff 1891 (Underhill, Gordon, Imhof, Nunez, & Bousso, 2016)

Inflammation, from the Latin *inflammatio* meaning 'set fire to', is a process that has been contemplated by many throughout history (Rivas, 2010). In the 1st century AD, the Roman Celsus noted the cardinal signs of inflammation, *rubor et tumor cum calore et dolore,* that are still recognized and assessed by clinicians (Rivas, 2010). The final sign, *functio laesa*, was added two centuries later by the Greek physician Galen who, ahead of scientific knowledge at the time, considered inflammation to be beneficial to the process of wound repair (Rather, 1971). This is in contrast to the 19th century German physician Virchow's belief that inflammation is inherently pathological (Heidland, Klassen, Rutkowski, & Bahner, 2006).

Inflammation is now accepted to be part of the response to trauma or infection, a balance of innate immune processes initiated in response to signal(s) such as damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs), and, importantly, ceasing once the signal(s) is removed (Janeway, 1989; Janeway & Medzhitov, 1998; Seong & Matzinger, 2004). These signals are sensed by pattern recognition receptors (PRRs) such as membrane bound toll-like receptors (TLRs) and C-type lectin receptors (CLRs) or cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) (H. Kumar, Kawai, & Akira, 2011). The majority of invertebrates depend solely on the innate immune system for their response to pathogens, with immunocompetence manifested by the ability to survey and neutralize threat. The innate

immune system is able to respond quickly and without previous exposure to a threat. This is distinct from the adaptive immune system, which is a relatively recent evolutionary development providing both immunological memory and specificity through an almost infinite variability of receptors due to gene rearrangement. The inflammatory process itself may cause pathology if it commences inappropriately or fails to terminate appropriately, as is the case with monogenic autoinflammatory disorders (AIDs).

The study of AIDs has provided insight into molecular mechanisms and pathways in inflammation and the innate immune system. Importantly, the study of these rare genetic disorders has broader implications than for the individual disorders studied, such as the identification of susceptibility alleles for complex phenotypes in multifactorial common diseases.

This literature review sets out to provide an introduction to the innate immune system in the context of AIDs, starting with an overview of the three major innate immune pathways implicated in disease pathogenesis. The definition and classification of AIDs will be examined with the aim of highlighting changes in our understanding in recent years. Past and present genetic sequencing techniques will be introduced and the clinical implications of this advancement will be explored in the context of AIDs. Achievements and issues surrounding the *in vitro* and *in vivo* modelling of monogenic human diseases will be considered to highlight limitations of current technology as well as future opportunities. Finally, the aims of this PhD and an overview of each chapter of the thesis will be provided.

1.1 Monogenic Autoinflammatory Disorders

The phrase '*autoinflammatory disease'* was proposed as an alternative to '*autoimmune disease'* by McDermott et al. in the paper identifying the genetic cause of Tumour Necrosis Factor (TNF) Receptor Associated Periodic Syndrome (TRAPS) (McDermott et al., 1999). This was considered a suitably representative term at the time, as patients with inherited periodic fever syndromes were thought to have innate immune dysregulation and lacked high titres of autoantibodies and self-reactive T cells (McDermott et al., 1999). Familial Mediterranean Fever (FMF) was the only genetically defined periodic fever syndrome prior to this publication and the clinical and biochemical features appeared to be well defined (F. F. Consortium., 1997; T. I. F. Consortium., 1997).

Since this initial description, advances in our knowledge of innate immune physiology have had a great impact on the understanding of AIDs and the pathways that are perturbed leading to disease. Although the classic periodic fever syndromes were considered to be predominantly IL-1β mediated, a number of conditions have since been described that affect nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and interferon (IFN) signalling, the resulting group of AIDs being quite heterogeneous. Considering these pathways separately is important as clinical presentation and therapeutic options vary appreciably.

1.1.1 The inflammasome and IL-1β pathway

Increased IL-1β from gain of function (GoF) mutations in *NLRP3* cause the archetypal periodic fever syndrome cryopyrin-associated periodic syndrome (CAPS) (Hoffman, Mueller, Broide, Wanderer, & Kolodner, 2001). The exquisite response in patients with CAPS to IL-1β antagonism (Hawkins, Lachmann, & McDermott, 2003) not only highlights the role of this cytokine in disease pathogenesis, but also the benefits of elucidating the key pathway involved

in disease. A number of AIDs, including CAPS, result from increased inflammasome formation and the downstream consequences of this, namely Caspase-1 activation and maturation of pro-IL-1β and pro-IL-18 to their respective active forms. As the focus of two results chapters (**Chapter 3** and **Chapter 4**) of this thesis is the evaluation of novel variants in inflammasome forming proteins, the discovery and function of inflammasomes will be explored here in detail.

1.1.1.1 The inflammasome

Early studies examining IL-1β documented a cytokine that existed as a high molecular weight precursor which was converted to an active low molecular weight protein (Auron et al., 1987; Bayne, Rupp, Limjuco, Chin, & Schmidt, 1986; Giri, Lomedico, & Mizel, 1985; Unanue & Kiely, 1977). Subsequently, Caspase-1, also known as IL-1β converting enzyme (ICE), was identified, purified and cloned from a number of human cell lines (Cerretti et al., 1992; Thornberry et al., 1992). A member of the inflammatory caspase family which includes Caspase-4 and -5 in humans, Caspase-1 is a heterodimer composed of two subunits, p10 and p20, derived from a single proenzyme and harbors autocatalytic activity required for its activation (Thornberry et al., 1992).

It was in an attempt to elucidate the mechanism of Caspase-1 activation that Tschopp, along with colleagues Martinon and Burns, introduced the term *inflammasome* in a seminal paper describing a high molecular weight platform involved in the processing of Caspase-1 and pro-IL-1β (Martinon, Burns, & Tschopp, 2002). There were a number of contemporary observations that enabled progression of this work. The first was the identification of a family of proteins known as NLRs. These are a cytosolic subset of PRRs that recognize PAMPs such as lipopolysaccharide (LPS) (Janeway, 1989), DAMPs such as monosodium urate (MSU) (Bianchi, 2007; Seong & Matzinger, 2004) and/or homeostasis-altering molecular processes

(HAMPs) such as modifications in Rho GTPases (Liston & Masters, 2017). Prior to the description of NLRs, human NOD1 had been shown to be involved in NF-κB signalling (**Section 1.1.2**) and its key domains identified as an N-terminal caspase activation and recruitment domain (CARD), a NOD and multiple C-terminal leucine rich repeats (LRR) important for structural framework and ligand recognition (Bertin et al., 1999; Inohara et al., 1999). Bertin and DiStefano compared the structure of NOD1 with PRRs in plants, noting conservation of the NOD and LRR domains, but not the CARD (Bertin & DiStefano, 2000). Using a BLASTP search of NOD and LRR domains in a mammalian protein database, the authors identified NLRP1 and described a domain coined PYRIN (PYD) due to homology with a domain in the protein pyrin. Importantly, they documented the presence of an N-terminal PYD in apoptosis-associated speck-like protein containing a CARD (ASC) and hypothesised that it may function as an adaptor protein, as it too has a C-terminal CARD. Subsequent work identified an interaction between pyrin and ASC (N. Richards et al., 2001) as well as NLRP3 and ASC (Manji et al., 2002).

The elaboration of the function of ASC was important work that preceded the description of the inflammasome (Masumoto et al., 1999; Masumoto et al., 2001). In an astute early observation using immunofluorescence, ASC was noted to form a single speck-like protein aggregate with a hollow center in cells dying in response to retinoic acid treatment (Masumoto et al., 2001). Alnemri and colleagues extended this work by transfecting human embryonic kidney (HEK) 293T cells with ASC, pro-Caspase-1 and pro-IL-1β (Srinivasula et al., 2002). Secreted IL-1β increased in a dose-dependent manner to increasing amounts of transfected ASC. Immunoprecipitation experiments performed on whole cell lysate (WCL) of cells co-transfected with ASC and Caspase-1 determined that an interaction between ASC and Caspase-1 existed, and that this was dependent on the CARD of the respective proteins. By separating

the CARD and PYD, and fusing each with an inducible FKBP12 oligomerization cassette, the authors determined that the CARD of ASC was an effector domain and that the PYD mediated oligomerization, and together they enabled autocatalysis of Caspase-1 by induced proximity.

In a series of overexpression experiments in HEK293T cells, Tschopp and colleagues explored the relationship between NLRP1, ASC, and the inflammatory caspases (Martinon et al., 2002). ASC interacted specifically with Caspase-1 leading to its cleavage, whereas NLRP1 associated with Caspase-1 and Caspase-5. Mechanical disruption of THP1 cells after LPS stimulation caused inflammatory caspase activation, resulting in the rapid cleavage of Caspase-1 and Caspase-5 and processing of IL-1β. The activation of Caspase-1 resulted in an increase in the size of NLRP1, Caspase-1, Caspase-5 and ASC as determined by gel filtration, suggesting that these exist in a multiprotein complex. Although the role of Caspase-5 in this complex has since been questioned, the identification of the inflammasome as a platform for Caspase-1 processing has contributed to a plethora of subsequent work in innate immunology. Most recently, the inflammasome-Caspase-1 complex has been described as a holoenzyme, a combination of enzyme and coenzyme, with the size of the platform and cell type affecting the initiation and self-termination of Caspase-1 activity (Boucher et al., 2018).

1.1.1.1.1 Priming

The process of IL-1β maturation by inflammasome formation and activation requires two distinct steps. The first, referred to as *Signal 1*, is the induction of pro-IL-1β transcription by TLR or NOD2 stimulation and subsequent NF-κB activation (**Section 1.1.2**) (Mariathasan et al., 2004; Marucha, Zeff, & Kreutzer, 1991). *Signal 1* is also vital for inducing adequate expression of NLRP3, which is present in low amounts in primary human monocytes but robustly induced with LPS or TNF stimulation (O'Connor, Harton, Zhu, Linhoff, & Ting, 2003). In the context of sterile inflammation, *Signal 1* is not sufficient to cause Caspase-1 cleavage and IL-1β processing, and a second *Signal* 2, such as particulate matter, is required to activate an inflammasome complex (Franchi, Eigenbrod, & Nunez, 2009).

1.1.1.1.2 Inflammasome forming proteins

Pyrin and NLRC4, two key inflammasome forming proteins, are the subject of **Chapter 3** and **Chapter 4** respectively, and will be addressed in detail there. Here, an overview will be provided of the remaining inflammasome proteins that have been linked with monogenic AIDs.

1.1.1.1.2.1 NLRP1

NLRP1, the first NLR shown to form an inflammasome, harbours a PYD, an LRR domain, a function to find domain (FIIND) and a C-terminal CARD (Hlaing et al., 2001), with autolytic processing important for oligomerisation (Finger et al., 2012). The search for a natural PAMP has been complicated by the differences between murine and human NLRP1. There is evidence that NLRP1 is required for control of *Toxoplasma gondii* (Witola et al., 2011). By sequencing 23 single nucleotide polymorphisms (SNPs) in a cohort of patients with congenital toxoplasmosis, two SNPs were determined to be associated with disease. In a short hairpin RNA (shRNA) knock-down human monocyte cell line model, NLRP1 was determined to be

important for the cleavage of pro-IL-1β in response to *T. gondii* infection. Further insight into the role of NLRP1 in humans may be gleaned from two reports of monogenic AIDs caused by mutations in *NLRP1* (Grandemange et al., 2017; Zhong et al., 2016). Zhong et al. investigated two dermatological conditions, multiple self-healing palmoplantar carcinoma (MSPC) and familial keratosis lichenoides chronica (FKLC), in four families with pedigrees suggesting dominant inheritance of disease (Zhong et al., 2016). Although a susceptibility locus had previously been identified for MSPC, whole exome sequencing (WES) was employed and four genetic variants were identified in NLRP1: p.Ala54Thr, p.Met77Thr, p.Ala66Val and p.Phe787_Arg843del. Interestingly, p.Met77Thr NLRP1 was previously identified through WES of a family with dominantly inherited corneal intraepithelial dyskeratosis (Soler et al., 2013). The substitution mutations documented in patients map to the PYD, and the deletion to the LRR domain. Transfection of mutant NLRP1 expressing constructs increased ASC speck formation in HEK293T cells stably expressing ASC-GFP when compared with either wild type (WT) NLRP1 or SNPs in NLRP1 taken from the 1000 Genome database. Using HEK293T cells reconstituted with components of the inflammasome, mutant NLRP1 resulted in increased ASC oligomerization and increased pro-IL-1β cleavage to its active form. Significantly, in immortalized keratinocytes expressing mutant NLRP1, spontaneous IL-1β production was documented that was abrogated when ASC was knocked down using small interfering RNAs (siRNAs), suggesting that this process was ASC-dependent. All of the patient mutations resulted in spontaneous oligomerization of NLRP1 as shown on Blue Native PAGE, whereas WT NLRP1 existed predominantly as a monomer. Together, these data suggest that the patient mutations result in increased NLRP1 activity.

A more systemic disease was documented by Grandemange et al. in three patients, two related, with mutations in *NLRP1* (Grandemange et al., 2017). The patients were initially considered

to have systemic onset juvenile idiopathic arthritis (sJIA), with febrile episodes as well as elevated C-reactive protein (CRP) both during and in between attacks. They all had skin dyskeratosis but had variable features of autoimmunity. Two patients had positive antinuclear antibody (ANA) levels, and one had evidence of haemolytic anaemia as well as thyroiditis. Two mutations were identified in domains other than the PYD and LRR domain. A homozygous p.Arg726Trp NLRP1 mutation was identified in the related patients, located in the linker region between the NOD and LRR domains. The second, a heterozygous p.Pro1214Arg NLRP1 mutation, is located in the FIIND. All patients had increased serum IL-18 levels when compared with healthy controls, and one had increased IL-1β levels. Although the mechanisms of NLRP1 activation, and the variability of disease presentations, were not explored, it is interesting to speculate as to whether the mutation in the FIIND results in increased cleavage of NLRP1 as it is located close to the proteolytic cleavage site.

1.1.1.1.2.2 NLRP3

The characterization of the protein encoded by *NLRP3,* originally identified in an attempt to generate a library of cDNA in haematopoietic stem/progenitor cells (Mao et al., 1998), was progressed significantly by the identification of mutations in *NLRP3* in patients with CAPS (Hoffman et al., 2001). CAPS encompasses a spectrum of clinical manifestations caused by GoF mutations in *NLRP3*, from familial cold urticarial syndrome (FCAS), Muckle-Wells syndrome (MWS) to neonatal onset multisystemic inflammatory disease (NOMID). Originally described in 1940 (Zip et al., 1993), FCAS is characterised by episodic fever as well as cold induced urticarial and conjunctival injection. Patients with MWS may have complications such as late onset sensorineural hearing loss and renal deposition of amyloid in addition to more persistent features of FCAS (Dode et al., 2002). Patients with NOMID are on the severe end of the CAPS spectrum, with a broad range of symptoms stemming from widespread

inflammation, with classic features including chronic aseptic meningitis as well as dermatological and articular manifestations. From the clinical manifestations described, it is understandable that these three conditions were originally considered to be distinct disease entities. However, subsequent genetic evaluation of symptomatic families determined the cause of all three syndromes to be heterozygous mutations in *NLRP3* (Aksentijevich et al., 2002; Feldmann et al., 2002; Hoffman et al., 2001). Having previously identified a locus for FCAS in four families (Hoffman, Wright, Broide, Wanderer, & Kolodner, 2000), Hoffman et al. subsequently sequenced over 80 exons in this region and identified mutations in exon three of *NLRP3* (Hoffman et al., 2001). There are now 205 variants in *NLRP3* included in the *Infevers* database (**Sections 1.2** and **5.1.5.1**) (Touitou, Hentgen, Kone-Paut, & French Reference Centre for Auto-Inflammatory, 2009), with most located in exon three of *NLRP3* encoding the NOD. Although not all of these variants have had their pathogenicity validated, there are key pathogenic mutations that have been described and a number with important genotype-phenotype correlations noted. A retrospective study of CAPS patients from the Eurofever Registry noted associations between p.Thr348Met NLRP3 with an early onset and chronic disease course, p.Asp303Asn NLRP3 with severe phenotype and both p.Arg260Trp and p.Ala439Val NLRP3 with milder phenotypes (Levy et al., 2015). Interestingly, two previously unreported variants in NLRP3 were also noted, p.Ile572Ser and p.Leu1016Phe. Although its pathogenicity has not been confirmed, the latter is located in the LRR domain, a feature that will be explored further in **Chapter 4** in the context of NLRC4. There is a proportion of patients in whom *NLRP3* mutations are not found despite symptoms of CAPS. This raises a number of issues including the classification of CAPS as a genetically defined disorder of *NLRP3* GoF mutations versus a clinical entity, as well as potential limitations of sequencing techniques that are employed to investigate the mutation negative population.

The role of increased inflammasome activity in CAPS was determined by Tschopp and colleagues (Agostini et al., 2004). Having shown that NLRP3 forms an inflammasome, the authors hypothesized that mutations in *NLRP3* could lead to spontaneous assembly, Caspase-1 activation and downstream IL-1β secretion. Monocytes purified from a patient harbouring heterozygous p.Arg260Trp NLRP3 secreted IL-1β when unstimulated and had further increase with LPS stimulation when compared with monocytes from healthy controls. Two CAPS mutations were modelled in mice, with embryonic or myeloid expression of either p.Arg350Val or p.Leu351Pro NLRP3, representing p.Arg352Val and p.Leu353Pro in human NLRP3 (Brydges et al., 2009). Expression of either CAPS mutation in mice resulted in a severe inflammatory phenotype and death in the neonatal period. Mice had evidence of inflammatory cell infiltrate in multiple tissues, as well as elevation of a number of serum cytokines including IL-1β, IL-18 and IL-6. Interestingly, treatment of CAPS mice with a murine IL-1 inhibitor resulted in only modest improvement in survival and no significant changes in weight. To further clarify this, CAPS mice were mated to mice lacking IL-1 receptor (IL-1R). These mice had improved survival, improved weight gain and decreased IL-6 compared with those with IL-1R expressed. IL-1β and IL-18 serum levels were unchanged, as expected because both cytokines are upstream of IL-1R.

NLRP3 has since been extensively studied and the 'natural' DAMPs and PAMPs documented to trigger NLRP3 inflammasome formation are many and varied (Dostert et al., 2008; Hornung et al., 2008; Orlowski et al., 2015; Shimada et al., 2012; Shio et al., 2009), including nigericin, a pore forming toxin derived from *Streptomyces hygroscopicus* (Mariathasan et al., 2006) as well as MSU and calcium pyrophosphate dihydrate (CPPD) crystals (Martinon, Petrilli, Mayor, Tardivel, & Tschopp, 2006). Such heterogeneous triggers made direct binding of specific ligands to NLRP3 unlikely and led to the consideration of a unifying mechanism of NLRP3

detecting or sensing changes in homeostasis. High on the list of likely candidates is alterations in cellular K^+ concentrations (Mariathasan et al., 2006; Pelegrin & Surprenant, 2006; Perregaux & Gabel, 1994). A seminal paper by Nunez and colleagues linked a decrease in intracellular K^+, or K^+ efflux, to NLRP3 activation (Munoz-Planillo et al., 2013).

1.1.1.1.2.3 NLRP12

Through the investigation of two families suspected of having CAPS, but without mutations in *NLRP3*, Jeru et al. identified truncating mutations in *NLRP12* as the cause of a disease termed familial cold autoinflammatory syndrome 2 (FCAS2) (Jéru et al., 2008). In a HEK293T cell NF-κB luciferase assay, activation of the NF-κB pathway was induced with transfection of p65 or TNF. Co-transfection of a plasmid expressing WT NLRP12 in this assay resulted in marked reduction in NF-κB activation. This decrease was abrogated when the mutant NLRP12 was transfected, suggesting that NLRP12 functions as a negative regulator of NF-κB activation. A subsequent publication on a patient with FCAS2 identified a point mutation in *NLRP12* that did not alter its inhibitory role in the NF-κB pathway, but resulted in increased ASC speck formation in an overexpression model (Jeru, Le Borgne, et al., 2011). An initial response to IL-1R antagonist (IL-1Ra) anakinra was noted in 2 patients, but the response was not sustained and serum TNF and IL-6 elevation persisted (Jeru, Hentgen, et al., 2011). Later work has complicated the understanding of mechanism of disease in FCAS2, with a variant p.Asp294Glu NLRP12 segregating with sensitivity to cold exposure, but not the inflammatory phenotype seen in the patients investigated and having no effect on monocyte NF-κB activity or IL-1β secretion (Borghini et al., 2011). The role of NLRP12 in health and disease has been explored in a number of murine models. Kanneganti and colleagues generated *Nlrp12*$^{-/-}$ mice that were healthy when unchallenged (Zaki et al., 2011). In response to a dextran sulfate sodium (DSS)-colitis model, *Nlrp12*$^{-/-}$ mice had evidence of ongoing inflammation when their WT

counterparts were recovering, with elevated serum levels of IL-1β, IL-6, TNF, IL-17, and IL-15. *Nlrp12*$^{-/-}$ mice had increased hyperplasia and high-grade colonic dysplasia, with higher NF-κB, extracellular-signal-regulated kinase (ERK) and signal transducer and activator of transcription (STAT) 3 signalling in bone marrow-derived macrophages (BMDMs). It is important to note that NLRP12 and ASC have only been shown to interact in overexpression models. Whether this NLR actually forms an inflammasome in a more physiological setting, and whether FCAS2 is indeed a disorder of inflammasome function, requires further clarification.

1.1.1.1.2.4 NLRP7

Similar to NLRP12, NLRP7 was also originally shown to have a regulatory function, in this case on Caspase-1 cleavage and IL-1β processing in a transiently transfected HEK293T model (Kinoshita, Wang, Hasegawa, Imamura, & Suda, 2005). In THP1 cells transfected with an empty vector or NLRP7, those expressing NLRP7 secreted less IL-1β in response to LPS. Various missense and truncating mutations in *NLRP7* have been associated with recurrent hydatidiform moles (HYDM1), a gestational trophoblastic disease (Murdoch et al., 2006; Qian, Deveault, Bagga, Xie, & Slim, 2007). Whilst the mechanism by which the mutant NLRP7 causes disease remains uncertain, a single publication showed that peripheral blood mononuclear cells (PBMCs) from patients with rare nonsynonymous *NLRP7* variants had reduced IL-1β response to LPS stimulation when compared with healthy controls (Messaed, Akoury, et al., 2011), whereas another showed an increase (Messaed, Chebaro, et al., 2011). More recently, NLRP7 in human primary macrophages has been shown to form an ASC-dependent inflammasome in response to bacterial acylated lipopeptides (Khare et al., 2012). Whether NLRP7 has cell specific pro-inflammatory or anti-inflammatory effects remains unclear, and research in this area is complicated by the lack of a murine homologue.

Whilst better characterised inflammasome forming proteins, such as absent in melanoma 2 (AIM2) and NLRP6 have been described in the literature, no monogenic disorders resulting from mutations in their respective encoding genes have been described to date. Their details are summarised in **Figure 1.1**.

1.1.1.1.2.5 Pyroptosis

Initially proposed by Cookson and Brennan in 2001 (Cookson & Brennan, 2001), the term pyroptosis has come to represent cell death resulting from activation of inflammatory caspases, namely Caspase-1. The original observation of a Caspase-1-dependent cell death occurred in the context of *Salmonella* infection of a macrophage-like cell line (Cookson & Brennan, 2001). The authors observed inactive Caspase-3, involved in apoptosis, in the infected macrophages, but cleavage of Caspase-1 on western blot of cell lysates. Cell death in response to apoptotic stimuli remained intact despite inhibition of Caspase-1, whereas the death of infected cells was prevented. Using osmoprotectant molecules that block pore formation, the lytic cell death seen in response to infection with *Salmonella* was prevented, as was the initial phase of cell swelling, as determined by forward and side scatter on flow cytometric analysis (Fink & Cookson, 2006). Pyroptosis was subsequently shown to be a means by which a host cell uses the inflammasome platform to clear pathogens independently of the secretion of IL-1β and IL-18 (Miao, Leaf, et al., 2010). In an attempt to elucidate possible effectors of cell death downstream of Caspase-1, a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 (**Section 1.4**) genome-wide screen was utilized and identified *Gsdmd,* encoding gasdermin D (GSDMD), as a candidate gene of importance (Shi et al., 2015). *Gsdmd*$^{-/-}$ inducible BMDMs were completely resistant to pyroptosis but remained sensitive to extrinsic and intrinsic apoptosis, suggesting specificity for pyroptotic cell death. GSDMD, a substrate for Caspase-1, requires cleavage for

activity. The cleaved GSDMD N-terminal fragment represented a new type of pore forming protein which caused loss of membrane integrity and cell death (Ding et al., 2016; X. Liu et al., 2016). Although the relationship between pyroptosis and release of IL-1β and IL-18 has been described (Cullen, Kearney, Clancy, & Martin, 2015; T. Liu et al., 2014), the presumed prerequisite of cell death for cytokine responses is being questioned as there is evidence that these responses may be uncoupled (K. W. Chen et al., 2014; Conos, Lawlor, Vaux, Vince, & Lindqvist, 2016). This notion will be explored further in **Chapter 4**. Most recently, the disconnect between cell death and cytokine response was highlighted by Kagan and colleagues using *Gsdmd*$^{-/-}$ immortalised BMDMs primed with LPS and stimulated with the NLRP3 agonist nigericin (Evavold et al., 2018). GSDMD was required for IL-1β secretion as well as lactate dehydrogenase (LDH) release, which was used as a measure of cell death. Interestingly, the osmoprotectant glycine almost completely abrogated LDH release but had no effect on IL-1β levels, suggesting that even when the cell lipid bilayer is intact, IL-1β is released in a GSDMD-dependent manner.

1.1.1.2 IL-1β pathway and regulation

IL-1β, a key member of the IL-1 family of cytokines, is secreted by monocytes and macrophages in particular (Dinarello et al., 1987; Munoz, Keusch, & Dinarello, 1987) and interacts with its receptor IL-1R through Ig-like domains (Schreuder et al., 1997; Vigers, Anderson, Caffes, & Brandhuber, 1997). This initiates a conformational change leading to the recruitment of the IL-1R accessory protein (IL-1RAcP) (Casadio et al., 2001; Greenfeder et al., 1995). IL-1RAcP is required for signalling downstream of IL-1R, as demonstrated by the lack of biologic response to IL-1β in *Il-1racp*$^{-/-}$ mice (Cullinan et al., 1998) as well as cell lines lacking constitutive IL-1RAcP expression (Wesche et al., 1997). The cytosolic Toll/IL-1R-like (TIR) domains of the complex associate with myeloid differentiation primary response gene

88 (MyD88) and interleukin-1 receptor-activated protein kinase (IRAK) 4, ultimately leading to the induction of a plethora of genes by activating NF-κB (Adachi et al., 1998; Brikos, Wait, Begum, O'Neill, & Saklatvala, 2007; Medzhitov et al., 1998; N. Suzuki et al., 2002), explored in **Section 1.1.2**.

The regulation of the IL-1β response is important given its potent pro-inflammatory effects. The expression of IL-1RII, either on the cell surface or a soluble version (sIL-1R), serves as a decoy receptor (Colotta et al., 1993). IL-1β binds to this receptor with higher affinity than to IL-1R and, as IL-1RII lacks a TIR domain, no downstream signalling is initiated. The extracellular domains of IL-1R and sIL-1R were hypothesised to serve as a mechanism for regulating IL-1β activity, however the affinity of this domain for a natural IL-1Ra is higher than for IL-1β. Given this property, it was unsurprising that healthy volunteers injected with intravenous LPS did not improve with the administration of sIL-1R (Colotta et al., 1993). In fact, the participants treated with sIL-1R had increased TNF and IL-1β in serum, as well as raised CRP levels. By contrast, IL-1Ra limits the biological activity of IL-1β by binding to IL-1R but not inducing the recruitment of IL-1RAcP thereby preventing downstream signalling.

Deficiency of IL-1Ra (DIRA) is a disease characterized by pustular dermatosis, multifocal sterile osteomyelitis as well as biochemical evidence of systemic inflammation. It is caused by homozygous truncation mutations in or complete deletion of *ILRN* encoding IL-1Ra (Aksentijevich et al., 2009; Reddy et al., 2009). All described disease causing mutations result in decreased *ILRN* mRNA expression. *In vitro* analysis of expression in HEK293T cells revealed that WT IL-1Ra could be detected in supernatant via western blot but IL-1Ra harboring mutations p.Glu77Ter or p.Asn52Lys-fsX25 were not secreted (Aksentijevich et al., 2009). Patients' peripheral blood leukocytes demonstrated significantly increased TNF, IL-8

and IL-6 responses following stimulation with IL-1β when compared with heterozygous carriers and healthy controls (Aksentijevich et al., 2009; Reddy et al., 2009). The prompt and complete clinical and biochemical response to anakinra in DIRA is not surprising as the therapy is essentially replacing the deficient protein in patients. Interestingly, however, it was the empiric treatment of the index case with anakinra and subsequent resolution of symptoms that initiated the search for a genetic cause of the disease (Aksentijevich et al., 2009).

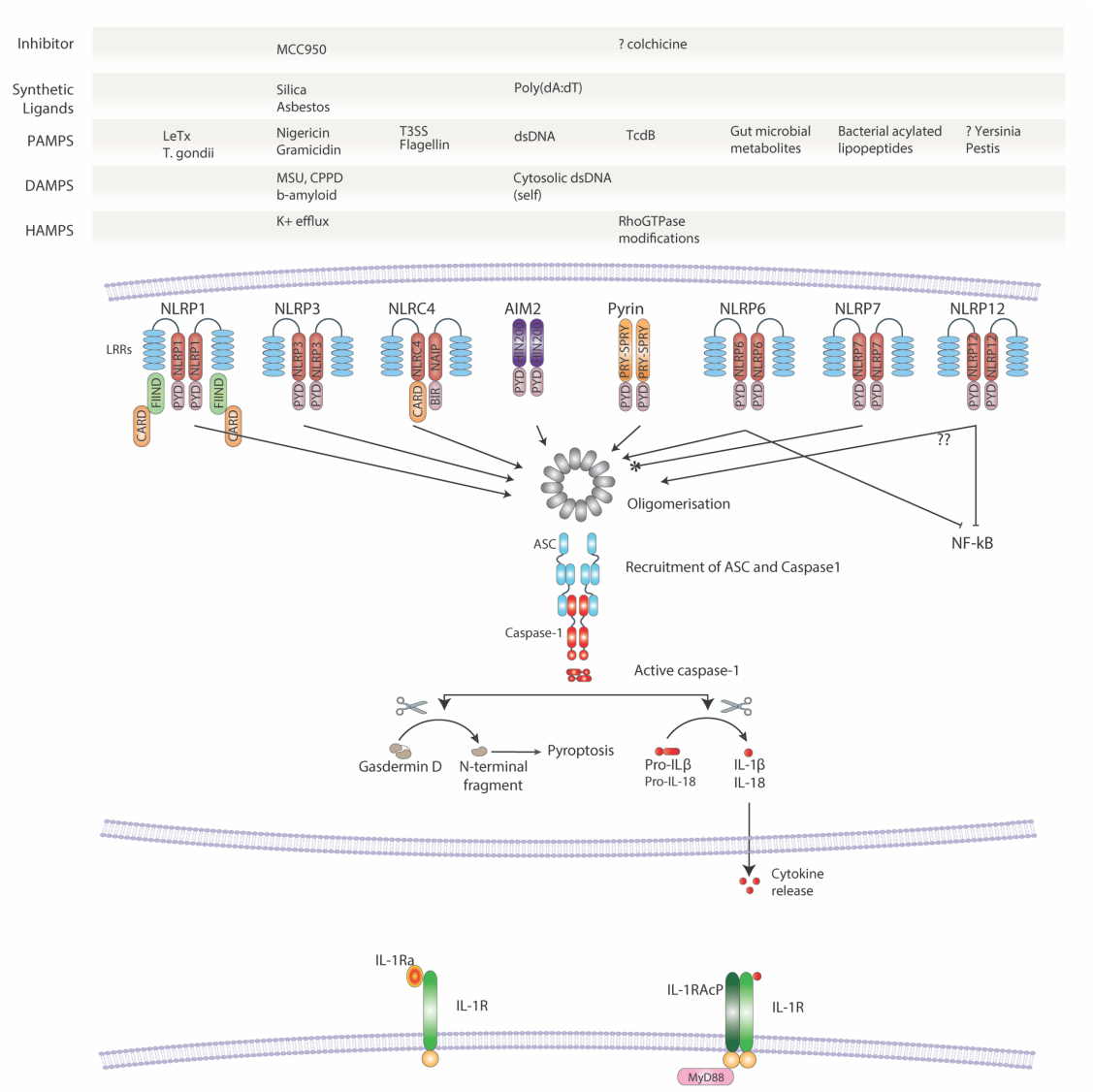


Figure 1-1 The inflammasome and IL-1β pathway.

Inflammasome forming proteins sense cytoplasmic alterations in the host environment. This initiates a conformational change in the receptor that then oligomerises, associates with ASC and serves as a platform for Caspase-1 cleavage. Active Caspase-1 cleaves gasdermin-D, pro-IL-1β and pro-IL-18 to their active forms leading to pyroptotic cell death and cytokine release. IL-1β binds to IL-1R and, with IL-1RAcP, initiates a MyD88-dependent signalling cascade. The binding of IL-1Ra to IL-1R does not result in the recruitment of IL-1RAcP and downstream signalling is not initiated. Adapted from (Broz & Dixit, 2016; Lamkanfi, 2011)

Abbreviations: AIM2 absent in melanoma 2, ASC apoptosis-associated speck-like protein containing a CARD, BIR baculoviral IAP repeat, CARD caspase activation and recruitment domain, CPPD calcium pyrophosphate dihydrate, DAMP danger associated molecular pattern, dsDNA double stranded DNA, FIIND function to find domain, HAMP homeostasis altering molecular patterns, IL-1R interleukin-1 receptor, IL-1Ra interleukin-1 receptor antagonist, IL-1RAcP IL-1 receptor accessory protein, LeTx anthrax lethal toxin, LRR leucine rich repeat, MyD88 myeloid differentiation primary response 88, MSU monosodium urate, NAIP, NF- κB nuclear factor κB, NLR NOD like receptor, PAMP pathogen associated molecular patterns, PYD pyrin domain, T3SS type 3 secretion system, TcdB *Clostridium difficile* toxin B.
* reports of both activating and inhibitory effects.

1.1.2 The NF-κB pathway

A recent development in the field of AIDs has been the description of conditions that are caused by altered ubiquitination within the NF-κB pathway. An overview of the NF-κB pathway will be presented here, followed by a description of the role of ubiquitination in its regulation.

NF-κB is a transcription factor family of five proteins that control the expression of genes involved in inflammation and cell survival (M. S. Hayden & Ghosh, 2008). The members of the family p50, p52, p65 (RelA), c-Rel, and RelB are encoded by *NFKB1*, *NFKB2*, *RELA*, *REL*, and *RELB*, respectively. These transcription factors translocate to the nucleus as homo- or heterodimers, binding to promoter regions in target genes and, through the recruitment of coactivators or corepressors, regulate transcription (G. Ghosh, van Duyne, Ghosh, & Sigler, 1995; Huang, Huxford, Chen, & Ghosh, 1997; Urban, Schreck, & Baeuerle, 1991). One of eight inhibitors of κB (IκB) proteins prevents nuclear localisation of the NF-κB dimer (Basak et al., 2007; Haskill et al., 1991; Hinz, Arslan, & Scheidereit, 2012; Hoffmann, Levchenko, Scott, & Baltimore, 2002; Rao et al., 2010; Read, Whitley, Williams, & Collins, 1994). By way of example, IκBα binds to the p65:p50 NF-κB heterodimer and prevents translocation to the nucleus as it possesses a nuclear export sequence (Beg et al., 1992). The activation of the NF-κB pathway requires the release of the dimer from the IκB protein. In order to achieve this, IκB is phosphorylated and ubiquitinated, prompting proteasomal-mediated degradation or processing (Alkalay et al., 1995; Z. Chen et al., 1995; S. Ghosh & Baltimore, 1990; Henkel et al., 1993; Scherer, Brockman, Chen, Maniatis, & Ballard, 1995). The IκB kinases (IKK), IKKα and IKKβ, along with IKKγ also known as NF-κB essential modulator (NEMO), mediate the phosphorylation of IκB proteins (DiDonato, Hayakawa, Rothwarf, Zandi, & Karin, 1997; Mercurio et al., 1997; Woronicz, Gao, Cao, Rothe, & Goeddel, 1997). The importance of the

IKKs is highlighted by the description of a number of monogenic immunodeficiencies resulting from mutations in their encoding genes. Homozygous loss of function (LoF) mutations in *IKBKB,* encoding IKKβ, results in severe combined B-cell and T-cell functional deficiency (Nielsen et al., 2014; Pannicke et al., 2013), whereas anhidrotic ectodermodysplasia with immunodeficiency is caused by mutations in *IKBKG,* encoding IKKγ, or *IKBA,* encoding IKKα (Courtois et al., 2003; Filipe-Santos et al., 2006).

The signalling cascade of the NF-κB pathway occurs downstream of a number of receptor classes, including TLRs, IL-1R (**Section 1.1.1**) and TNF-receptor (TNFR1). Importantly, none of these receptors possess kinase activity and all require the recruitment of multiple adaptor proteins to affect signalling after ligand binding. Separate from the NF-κB pathway, these receptors also signal through the mitogen-activated protein kinases (MAPK) pathway (McCain, 2013; Sabio & Davis, 2014). The MAPK pathway involves a series of kinase cascade signals that lead to the translocation of distinct transcription factors, further adding to the complexity and diversity of responses after ligand binding to receptor.

In the case of ligand binding to TNFR1, the canonical pathway proceeds initially with the cytoplasmic tails of the receptor complex recruiting TNFR1 associated death domain (TRADD) (H. Hsu, Xiong, & Goeddel, 1995), which then recruits receptor-interacting serine/threonine kinase 1 (RIP1) (H. Hsu, Huang, Shu, Baichwal, & Goeddel, 1996; H. Hsu et al., 1995). TNFR associated factor 2 (TRAF2) joins this complex through interaction with TRADD directly, or with the assistance of RIP1 (H. Hsu, Shu, Pan, & Goeddel, 1996; M. Rothe, Sarma, Dixit, & Goeddel, 1995). TRAF2 is important for the recruitment of cellular inhibitors of apoptosis (cIAPs) 1 and 2 (M. Rothe, Pan, Henzel, Ayres, & Goeddel, 1995), which are needed for the linear ubiquitin assembly complex (LUBAC) to associate (**Section**

1.1.2.1) (Emmerich et al., 2011; Haas et al., 2009; Mahoney et al., 2008). RIP1 also acts as a platform to facilitate the addition of transforming growth factor-β-activated kinase 1 (TAK1) and IKK, creating the large multiprotein Complex I (Sakurai, Shigemori, Hasegawa, & Sugita, 1998; Yamaguchi et al., 1995). The ubiquitination of RIP1 is considered important in this process as it enables association with TAK1 binding proteins (TABs) 2 and 3, which are ubiquitin chain sensory proteins, facilitating the recruitment of TAK1 to the complex (Takaesu et al., 2000; Zhang et al., 2011). With this proximity, TAK1 phosphorylates IKK which now, in its active state, is able to phosphorylate IκB (Takaesu et al., 2003; C. Wang et al., 2001). Complex II may also be formed, with TNFR1 recruiting first apoptosis signal (FAS) associating protein with death domain (FADD) leading to the initiation of a pro-apoptotic pathway (Chinnaiyan, O'Rourke, Tewari, & Dixit, 1995; H. Hsu, Shu, et al., 1996).

The pro-inflammatory effects of membrane bound and soluble TNF are mainly mediated through its interaction with TNFR1 encoded by *TNFRSF1A*. Mice with homozygous deletion of *Tnfrsf1a* had increased susceptibility to infection with *Listeria monocytogenes.* However, they were resistant to the lethal effects of LPS, with reduced serum IL-6 and TNF compared to WT mice (J. Rothe et al., 1993). TNFR1 is constitutively expressed in most cells at low levels, with increased expression induced by a number of signals including NF-κB activation (Loetscher et al., 1990; Schall et al., 1990; C. A. Smith et al., 1990). At rest, TNFR1 remains in the Golgi apparatus but is subsequently trafficked to the cell surface membrane (Storey, Stewart, Vandenabeele, & Luzio, 2002). Following ligand binding, TNFR1 undergoes rapid endocytosis, leaving TRADD free to initiate an alternative, pro-apoptotic pathway. TNFR1 expression on the membrane surface is also regulated by TNF-α converting enzyme (TACE)-directed cleavage of the receptor to a soluble form (sTNFR1) (Black et al., 1997). Originally isolated from urine and characterised as a TNF-binding protein (TNF-BP) with TNF inhibitory

properties (Olsson et al., 1989; Peetre, Thysell, Grubb, & Olsson, 1988; Seckinger, Isaaz, & Dayer, 1988, 1989), TNF-BP reduced the ability of TNF to bind to membrane bound TNFR1 (Engelmann, Aderka, Rubinstein, Rotman, & Wallach, 1989). Soon after its identification, the structural relationship between TNF-BP and TNFR1 was revealed and it was proposed that TNF-BP may indeed be a soluble form of TNFR1 (Engelmann, Novick, & Wallach, 1990). Dysregulation of this process, commonly known as 'shedding', was initially implicated in the pathophysiology of TRAPS, but other mechanisms have since been proposed.

TRAPS, an autosomal dominant periodic fever syndrome previously termed Hibernian fever, is caused by mutations in *TNFRSF1A* (McDermott et al., 1999). Characterised by long fever durations, periorbital oedema and poor response to colchicine, large pedigrees were used to locate a susceptibility locus (McDermott et al., 1998). Six heterozygous mutations in *TNFRSF1A* were identified in the original description of the genetic cause of the disease, and most disrupted extracellular disulphide bonds of the receptor (McDermott et al., 1999). Important steps were taken in the functional evaluation of these mutations at this time, including the assessment of sTNFR1 levels in patient compared with healthy controls. Patients symptomatic of disease and harbouring mutations in *TNFRSF1A* had lower sTNFR1 levels compared with mutation negative family controls at baseline, with 'pseudo-normalisation' during flares. It is possible that this result is due to experimental issues, such as reduced sensitivity of ELISA for the mutant sTNFR1. Interestingly, levels were not significantly different from asymptomatic family members carrying the mutation, suggesting that the levels were correlated with genotype but not necessarily phenotype.

Cell surface expression of TNFR1 was increased compared with healthy controls, but the affinity for TNF was unchanged. The ability of patient TNFR1 to shed in response phorbol

myristate acetate (PMA), which induces the metalloproteases that initiate receptor shedding, was impaired (McDermott et al., 1999), a finding that was later determined to be cell type specific (Huggins et al., 2004). A knock-in murine model expressing a mutant TNFR1 that is unable to shed demonstrated an improved ability to clear infection, but also spontaneous hepatitis and increased susceptibility to various experimental autoimmune models (Xanthoulea et al., 2004).

Defective shedding is not the only pathogenic mechanism of TRAPS that has been explored. The retention of TNFR1 in cytoplasmic aggregates with reduced surface expression was noted by Todd et al. in HEK293T overexpression experiments (Todd et al., 2004). Furthermore, Siebert et al. determined that TNF-induced apoptosis was decreased in TRAPS patient dermal fibroblasts, suggesting that these cells may remain viable and continue producing pro-inflammatory cytokines longer than fibroblasts from healthy controls (Siebert et al., 2005). A later paper used a murine model of TRAPS to show endoplasmic reticulum (ER) retention of the mutant TNFR1 and defective NF-κB signalling and apoptotic potential (Lobito et al., 2006). These conflicting observations may be due to experimental differences including overexpression models in human cells versus murine models. The ability for mutant TNFR1 to interact with WT TNFR1 has also been explored. In a HEK293T model using a HA-tagged WT TNFR1 construct and a doxycycline inducible V5-His-tagged mutant TNFR1 construct, a heterodimer between WT and mutant TNFR1 could form, resulting in NF-κB signalling (Yousaf et al., 2005). As a physical interaction between the two TNFR1s was later disputed (Lobito et al., 2006), Simon et al. revisited this in 2010 using heterozygous TRAPS-mutant *tnfrsf1a* knock-in mice to explore the functional relationship between WT and mutant TNFR1 (Simon et al., 2010). The authors noted that the misfolded mutant TNFR1 accumulated in the cytoplasm and resulted in activation of MAPK. The use of homozygous versus heterozygous

TRAPS-mutant *tnfrsf1a* knock-in mice demonstrated that the WT allele was required to maintain a cytokine response to LPS induced inflammation, and that without this allele, the homozygous TRAPS-mutant *tnfrsf1a* knock-in mice behaved similarly to *Tnfrsf1a* knock-out mice.

What is clear from the literature is that the pathophysiology of TRAPS is more complicated than defective shedding of a receptor leading to increased availability of unbound TNF for signalling. This is highlighted with the report of failure of etanercept, a dimeric fusion protein that binds TNF, to abate symptoms of TRAPS, as well as the administration of infliximab, a chimeric anti-TNF monoclonal antibody, resulting in a severe inflammatory reaction (Jacobelli, Andre, Alexandra, Dode, & Papo, 2007). Importantly, infliximab showed an inability to induce apoptosis in PBMCs from patients with TRAPS but was able to do so in healthy control PBMCs, a result not reproduced with etanercept (Nedjai et al., 2009). Patient PBMCs incubated in fresh media and infliximab demonstrated an increased IL-6, IL-8, IL-12, IL-1R and IL-1β cytokine responses when compared with healthy control PBMCs. Since this finding, caution has been advised on the use of this therapeutic agent in patients with TRAPS. IL-1β targeting therapy in the form of anakinra (Simon et al., 2004) and canakinumab (Gattorno et al., 2017) results in the rapid resolution of clinical and biochemical inflammation in patients with TRAPS. This suggests that IL-1β may be a key driver in the pathophysiology of disease and raises the possibility of cytoplasmic aggregates triggering inflammasome activation.

Mutations in *NOD2* have also been associated with an AID. When either NOD1 or NOD2 are activated, they oligomerise and recruit RIP2 through CARD-CARD interactions (Fridh & Rittinger, 2012; Nembrini et al., 2009; J. H. Park et al., 2007). RIP2 is ubiquitinated by cIAPs

(Bertrand et al., 2009; Reardon & Mak, 2009), and LUBAC is recruited by X-linked inhibitor of apoptosis (XIAP) (Damgaard et al., 2012), with TAK1 subsequently added to this complex (J. Y. Kim, Omori, Matsumoto, Núñez, & Ninomiya-Tsuji, 2008). TAK1 phosphorylates IKK as described in the TNFR1 pathway. GoF mutations in *NOD2* cause the eponymously named Blau Syndrome (BS) (Miceli-Richard et al., 2001). Initially described in 1985, the syndrome, characterised by iritis, rash and arthritis with histological evidence of non-caseating granulomatous changes, was noted to be dominantly inherited based on family pedigrees (Blau, 1985). Linkage analysis (**Section 1.3**) was used to identify the responsible locus of BS as chromosome 16q12 in a 74-member pedigree (Tromp et al., 1996). Through screening of each exon of *NOD2*, located at this locus, in four families with BS, three novel variants in *NOD2* were identified that segregated with disease (Miceli-Richard et al., 2001). This finding prompted consideration as to whether early onset sarcoidosis (EOS) has similar genetic origins. Although EOS and BS share clinical and histological findings, children with EOS lack a significant family history. A cohort of patients with EOS had all exons of *NOD2* sequenced and compared with 100 healthy controls (Kanazawa et al., 2005). Nine of the 10 patients sequenced had heterozygous mutations in *NOD2*. Four patients had an p.Arg334Trp mutation previously described in BS, and five were novel. These six mutations were tested in an NF-κB luciferase assay and displayed increased NF-κB activity when compared with WT NOD2 when overexpressed in HEK293T cells. Co-transfection of WT NOD2 with mutant NOD2 also showed increased NF-κB activity when compared with WT NOD2 alone, suggesting a dominant positive effect. This evidence suggests that constitutive induction of NF-κB activity by mutant NOD2 is part of the pathogenesis of BS and EOS.

1.1.2.1 Regulation of the NF-κB pathway

NF-κB translocation to the nucleus is involved in the transcription of over 150 genes, including the expression of cytokines, stress response genes, regulators of apoptosis and immune receptors (Pahl, 1999). The genes transcribed by activation of the NF-κB pathway are not identical between stimuli or cell type, suggesting that there are many determinants of gene expression other than NF-κB, including epigenetic factors, recruitment of pro-transcription adaptors, as well as differentially expressed regulators, depending on cell type and ligand specificity. This may go some way to explain why patients with CAPS, for example, have a different clinical and biochemical profile when compared with patients with BS, despite the fact that an increased IL-1β could be presumed to result in increased NF-κB signalling through the IL-1R.

Regardless, the consequences of dysregulation of the NF-κB pathway and the need for regulatory mechanisms are clear. The importance of ubiquitination in the NF-κB pathway has been recently elucidated. Ubiquitination is a post translational modification that involves the covalent attachment of ubiquitin (Ub), a 76-amino-acid protein, to a target protein through a series of enzymatic reactions (Hershko & Ciechanover, 1998; Hicke & Dunn, 2003; Kornitzer & Ciechanover, 2000; L. Sun & Chen, 2004). Three enzymes are involved in this process, Ub activating enzymes (E1), Ub conjugating enzymes (E2) and Ub protein ligases (E3) (Hershko & Ciechanover, 1998; Hicke & Dunn, 2003; Kornitzer & Ciechanover, 2000; L. Sun & Chen, 2004). Ub can form various linkages through attaching to one of seven lysines of another Ub, with important functional implications. Lys48 linked Ub chains, for example, usually signal proteins for 26S proteasomal degradation (Chau et al., 1989), whereas Lys63 linked chains are involved in a number of processes including protein kinase activation (Z. J. Chen & Sun, 2009). As mentioned earlier, the ubiquitination and proteasomal degradation of IκBα is required for

the translocation of NF-κB to the nucleus. Lys63-linked ubiquitination also plays a vital role in signalling, with TRAF6 functioning as an E3 ligase facilitating ubiquitination of the TAK1 kinase complex, with downstream phosphorylation and activation of IKK (L. Deng et al., 2000; C. Wang et al., 2001). Linear ubiquitination- the linking of the C-terminal tail of one Ub to the N-terminal methionine of another Ub- was first described in 2006 and has since been shown to play a key role in the modification of proteins in the NF-κB pathway (Kirisako et al., 2006). The only E3 ubiquitin ligase known to generate linear ubiquitin chains is the LUBAC. LUBAC was originally described as a complex comprising of the haem-oxidised IRP2 ubiquitin ligase 1L (HOIL1, also known as RBCK1) and the catalytic subunit HOIL1 interacting protein (HOIP, also known as RNF31), but was later shown to include SHANK-associated RH domain interacting protein (SHARPIN) (Ikeda et al., 2011; Tokunaga et al., 2011). Both HOIL1 and SHARPIN are important for the stability of this complex, but murine models suggest distinct roles. Genetic deletion of *Hoil1* in mice resulted in marked reduction in expression of HOIP in mouse embryonic fibroblasts (MEFs), as well as decreased NF-κB activation in response to stimulation with TNF or IL-1β (Tokunaga et al., 2009). These mice were, however, overtly normal. In contrast, LoF mutations in *Sharpin* resulted in an inflammatory skin phenotype, discussed further in **Chapter 5**. LUBAC has a demonstrable role in the ubiquitination of multiple substrates, including IKKγ, MyD88, IRAKs and RIPs 1 and 2 (Cohen & Strickson, 2017; Emmerich et al., 2013; Tokunaga et al., 2009). The role of LUBAC and linear ubiquitination in NLRP3 inflammasome assembly has also been determined in a paper that highlighted the cell type specific roles of LUBAC by comparing BMDMs with MEFs, recapitulating observations made in primary human cells (see below) (Boisson et al., 2012; Rodgers et al., 2014).

Mutations in two components of LUBAC have been associated with AIDs. Homozygous LoF mutations in *HOIL1* or *HOIP* have been described in patients with evidence of systemic inflammation, susceptibility to pyogenic infections, and amylopectinosis (Boisson et al., 2015; Boisson et al., 2012). The original description by Boisson et al. highlighted the importance of HOIL1 in maintaining the stability of LUBAC as well as promoting the association of IKKγ with TNF or IL-1 receptor signalling complexes (Boisson et al., 2012). The authors noted cell-type specific defects associated with HOIL1 deficiency. Fibroblasts and Epstein–Barr virus (EBV)-immortalized B cells from patients displayed impaired canonical NF-κB pathway activation in response to TNF or IL-1β as well as partial impairment of the response to TLR stimuli. The inflammatory phenotype was determined to originate from monocytes, with patient monocytes displaying hyper-responsiveness to IL-1β in terms of inflammatory cytokines produced compared with healthy control monocytes. The clinical and cellular phenotypes in the HOIL1-deficient patients overlap with those seen in a patient with a homozygous LoF mutation in *HOIP* (Boisson et al., 2015). Although no pathogenic mutations in *SHARPIN* have been described to date, given the striking similarities in presentation of HOIL1- and HOIP-deficient patients, it could be expected that patients would present in a similar manner. Having said this, the publication of a series of 10 patients from 8 families with polyglucosan storage myopathy harbouring either homozygous or compound heterozygous mutations in *HOIL1* (Nilsson et al., 2013) suggests that mutations in this gene, and potentially other components of LUBAC, may also present with a more limited clinical phenotype, and that much remains to be learnt about genotype-phenotype correlations in these disorders.

The importance, and indeed complexity, of ubiquitination in the NF-κB signalling pathway is highlighted by the description of patients with Behçet's like phenotype harboring mutations in *TNFAIP3* leading to haploinsufficiency of A20 (HA20) (Zhou, Wang, et al., 2016). A20, an

ovarian tumour (OTU) domain containing deubiquitinating enzyme, also has E3 ubiquitin ligase activity. A20 was initially observed to inhibit the NF-κB response to LPS stimulation when co-transfected into HEK293T cells with TLR4 (O'Reilly & Moynagh, 2003), and was later shown to replace Lys63-linked Ub chains on RIP1 with Lys48-linked Ub chains, resulting in RIP1 proteasomal degradation (Wertz et al., 2004). To date, reported pathogenic mutations in *TNFAIP3* have included truncating mutations caused by premature stop codon or frame shift mutations, mainly located in the OTU domain. Patients with heterozygous mutations have reduced expression of WT A20 compared with healthy controls, with mutant A20 not detected by western blot, suggesting instability of the truncated protein. In a HEK293T NF-κB luciferase assay, expression of WT A20 reduced the luciferase activity induced by TNF, whereas expression of mutant A20 failed to do so. PBMCs from these patients displayed evidence of increased NF-κB signalling with TNF stimulation on western blot assessment of phospho-IκBα and phospho-IKKα/IKKβ. The inflammatory cytokine response of patient PBMCs to LPS was increased when compared with control PBMCs. Interestingly, LPS stimulated PBMCs from patients showed NLRP3-dependent Caspase-1 cleavage and IL-1β secretion, suggesting that both the inflammasome and NF-κB pathways are perturbed in patients with HA20. Subsequent publications have expanded the clinical and cellular phenotype of patients with HA20 (Duncan et al., 2017; Kadowaki et al., 2017; Takagi et al., 2017). Heterozygous frame shift mutations in *TNFAIP3* have been reported in a patient with clinical and biochemical diagnosis of autoimmune lymphoproliferate syndrome (ALPS), as well as a patient with early onset complex autoimmunity. The patient with ALPS had evidence of increased NF-κB signalling in T cells in response to TNF stimulation (Takagi et al., 2017) illustrating the complexity of the innate/adaptive divide (**Section 1.2**).

Otulin-related autoinflammatory syndrome (ORAS) is the latest addition to the expanding group of AIDs caused by dysregulation of the NF-κB pathway (Damgaard et al., 2016; Zhou, Yu, et al., 2016). *OTULIN*, previously known as *GUMBY* or *FAM105B*, encodes a widely expressed OTU domain-containing deubiquitinating enzyme with specificity for linear ubiquitin (Keusekotten et al., 2013). Using HEK293T cells, Komander and colleagues determined that OTULIN directly interacted with LUBAC and that depletion of OTULIN via siRNA resulted in modification of HOIP with linear ubiquitin chains. Transient expression of LUBAC components resulted in the induction of linear ubiquitination and NF-κB activation as determined by western blot analysis of cell lysate and luciferase assay respectively. The co-expression of WT OTULIN abrogated the induction of ubiquitination by LUBAC, as well as NF-κB activation. The initial description of ORAS in three affected individuals in one large consanguineous kindred (Damgaard et al., 2016) was quickly followed by a report of a further three individuals each born to consanguineous parents (Zhou, Yu, et al., 2016). Currently, a total of three novel mutations in the OTU domain of *OTULIN* have been found in six patients experiencing early onset fever, lipodystrophy and panniculitis. Most have responded to TNF inhibition, although some patients remain steroid dependent. Zhou et al. used overexpression models to show that the mutations in *OTULIN* did not disrupt its binding to the LUBAC complex as determined by immunoprecipitation of SHARPIN and immunoblotting for OTULIN. However, two patient mutations showed significant defects in deubiquitinase function when co-transfected with substrates IKKγ, RIPK1, ASC and TNFR1, which were rescued when WT OTULIN was added. Functionally, TNF stimulated patient PBMCs and fibroblasts showed increased NF-κB pathway activation. In response to IL-1β, patient PBMCs had earlier, more sustained and increased linear ubiquitination of IKKγ. Damgaard et al. determined that, in a murine model, *Otulin* deficiency in myeloid cells was responsible for weight loss, increased serum neutrophil count, neutrophil infiltration into organs and an

increase in inflammatory cytokines, and that lymphocytes did not play a significant role (Damgaard et al., 2016). This phenotype was significantly abrogated with the administration of anti-TNF neutralizing antibodies, highlighting the role of this cytokine in the pathogenesis of ORAS. Although the *Otulin*-deficient mice show evidence of serum autoantibodies as determined by enzyme linked immunosorbent assay (ELISA), including extractable nuclear antigens (ENAs), double stranded DNA (dsDNA) and anti-Smith antibodies, there have been no reports of autoimmunity in patients so far.

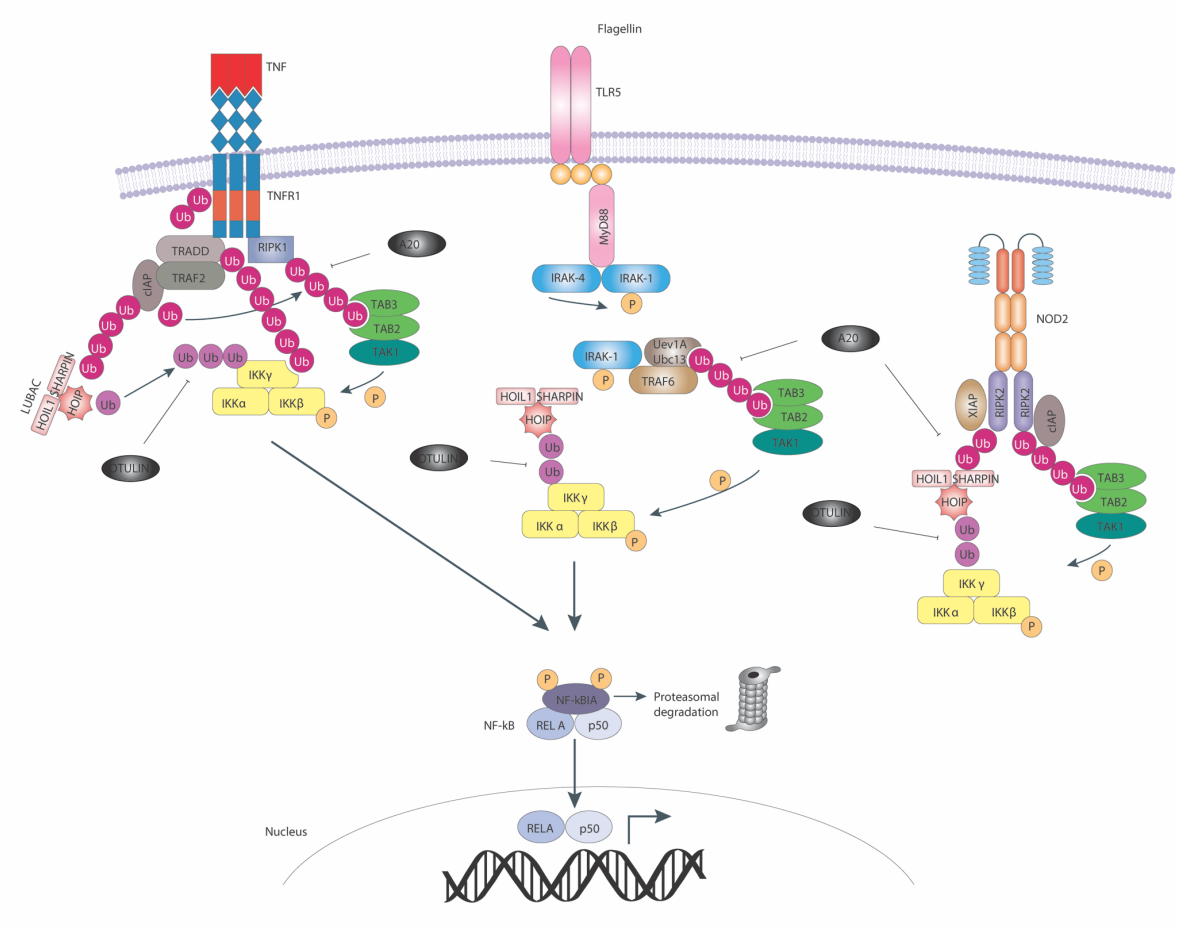


Figure 1-2 The canonical NF-κB pathway.

The canonical, or classic, NF-κB pathway is a series of protein interactions and post translational modifications that eventually result in the translocation of transcription factors to the nucleus to induce gene expression effecting a broad range of immunological processes. A detailed account of the pathway is given in the accompanying text. Adapted from (Gyrd-Hansen & Meier, 2010; Ozato, Shin, Chang, & Morse, 2008)

Abbreviations: cIAP cellular inhibitor of apoptosis protein, HOIL haem-oxidized IRP2 ubiquitin ligase 1, HOIP HOIL-1L interacting protein, IKK IkB kinases, IRAK interleukin-1 receptor-associated kinase, LUBAC linear ubiquitin chain assembly complex, MyD88 myeloid differentiation primary response 88, NOD nucleotide oligomerization and binding domain, RIP receptor-interacting protein, TAB TAK1 binding protein, TAK1 TGFβ-activated kinase 1, TLR toll-like receptor, TNF tumour necrosis factor, TRADD TNFR1-associated death domain protein, TRAF TNF receptor-associated factors, Ub ubiquitin, XIAP X-linked inhibitor of apoptosis protein.

1.1.3 The interferon pathway

IFNs, named for their ability to 'interfere' with viral replication, are a family of cytokines with well-described antiviral, antitumor and immunomodulatory activity (Parkin & Cohen, 2001). Type I IFNs (IFN-α, -β, -ω, -ε, -κ) are produced by most cells, whereas natural killer (NK), NKT and T cells are the primary sources of type II interferons (IFN-γ) (Hall & Rosen, 2010). Type I and type II IFN receptor components (IFNAR1/2 and IFNG1/2 respectively) are expressed on most nucleated cells, suggesting that both have the potential for widespread activity (Bigler et al., 2011). Type III IFNs are the most recently described group of IFNs, involved in antiviral defences of the epithelial surface of the respiratory and gastrointestinal tracts (Galani, Koltsida, & Andreakos, 2015; Lasfar, Zloza, & Cohen-Solal, 2015; Lazear, Nice, & Diamond, 2015; Wack, Terczynska-Dyla, & Hartmann, 2015). The net effect of the IFN pathway is activation (phosphorylation) of interferon regulatory factors (IRFs). IRFs are a group of 9 transcription factors with four positive regulators of type I IFN genes (Gonzalez-Navajas, Lee, David, & Raz, 2012).

Type I IFNs are encoded by 13 IFN-α genes and one IFN-β gene on chromosome 9 (Lopez de Padilla & Niewold, 2016). These act via a common receptor complex with both IFNAR1 and 2 required for downstream signalling. IFNAR2 binds to IFN with high affinity, but IFNAR1 has been shown to be necessary for signalling (Lopez de Padilla & Niewold, 2016). The binding of ligand to receptor activates Janus kinases (JAKs) which, through autophosphorylation and bound receptor phosphorylation, recruit the transcription factors STATs. The STATs are also phosphorylated and, after dimerising, translocate to the nucleus to regulate the transcription of a large number of interferon stimulated genes (ISGs) (Lopez de Padilla & Niewold, 2016). The importance of these signalling components is highlighted by the multiple reports of monogenic immunodeficiencies caused by mutations in JAKs or STATs. Homozygous mutations in *JAK3*

have been reported to cause autosomal recessive severe combined immune deficiency (SCID) (Macchi et al., 1995), and homozygous mutations in *TYK2* were originally identified in a patient with Hyper-IgE syndrome and susceptibility to mycobacterial disease (Minegishi et al., 2006). Patients with heterozygous and homozygous mutations in *STAT1* have susceptibility to mycobacterial infection (heterozygous) and lethal viral infection (homozygous) (Chapgier et al., 2006; Dupuis et al., 2001; Dupuis et al., 2003; Tsumura et al., 2012; van de Veerdonk et al., 2011). Homozygous intronic mutations in *STAT2* that affect splicing have been found in individuals with unusually severe viral infections (Hambleton et al., 2013). Finally, *de novo* dominant negative mutations in *STAT3* have also been associated with Hyper-IgE syndrome (Minegishi et al., 2007).

Type III IFNs are encoded by four *IFNL* genes and signal via IFN-λ receptor (IFNLR) 1 and IL-10RB (Wack et al., 2015). IFN-λ activates the same downstream pathway, and results in a similar gene signature/expression, as type I IFNs (Wack et al., 2015). Highlighting the similarities between type I and type III IFNs is evidence that shows IFN-β and IFN-λ1 promoters are predominantly activated by IRF3, while transcription of the IFN-α and IFN-λ2/3 relies more upon IRF7 activation (Osterlund, Pietila, Veckman, Kotenko, & Julkunen, 2007).

Both TLR-dependent and -independent pathways may be engaged to induce IFN production. TLRs are type 1 transmembrane PRRs (Gay & Gangloff, 2007) with an LRR on the luminal side and TIR domain in the cytosol (Gay & Gangloff, 2007). There are 10 TLRs encoded in the human genome, with 9 considered to be functional (Akira, Uematsu, & Takeuchi, 2006; De Nardo, 2015). TLR3, 4, 7/8 and 9 are involved in TLR-dependent IFN production, with all but TLR4 sensing nucleic acid of some description (Colonna, 2007; Uematsu & Akira, 2007).

TLR3, TLR 7/8 and TLR9 sense extracellular dsRNA (Alexopoulou, Holt, Medzhitov, & Flavell, 2001), ssRNA (Heil et al., 2004) and bacterial DNA rich in unmethylated CpG motifs (Hemmi et al., 2000; Takeshita et al., 2001) respectively, whereas TLR4 senses LPS (Medzhitov, Preston-Hurlburt, & Janeway, 1997; Poltorak et al., 1998). The nucleic acid sensing TLRs have been shown to move from the ER to endolysosomes in a process guided by the expression of UNC93B1 (Y. M. Kim, Brinkmann, Paquet, & Ploegh, 2008). Patients with UNC93B1 deficiency are susceptible to herpes simplex encephalitis, demonstrating the importance of transportation of TLRs to the appropriate compartment for sensing and activation (Casrouge et al., 2006; Conley, 2007). TLRs 7, 8 and 9 utilise a MyD88- and TRAF6- dependent pathway for IFN production in specific cell types such as plasmacytoid dendritic cells. The transcription factor IRF7 is recruited to the complex and, once activated, migrates to the nucleus, where it induces type I IFN gene expression. In contrast, TLR3 elicits IFNs via the adapter TRIF to signal through TRAF family member-associated NF-κB activator (TANK) binding kinase 1 (TBK1) and IRF3 (Akira & Hoshino, 2003; Akira & Sato, 2003; Akira, Yamamoto, & Takeda, 2003; Yamamoto et al., 2003).

Cytoplasmic sensing of nucleic acid and IFN production involves RIG-I, melanoma differentiation-associated gene 5 (MDA5) and cyclic GMP-AMP synthase (cGAS)- stimulator of interferon genes (STING), amongst other pathways (Kang et al., 2002; Yoneyama et al., 2004). Structurally, RIG-I and MDA5 share a DEAD box and a C-terminal regulatory domain, and both sense double stranded RNA. RIG-I recognises the 5'ppp or 5'pp motif on RNA, with a preference for <300 base pairs and strands that lack 7-methylguanoside (Kell & Gale, 2015; Yoneyama, Onomoto, Jogi, Akaboshi, & Fujita, 2015). Although ligand preference differs, they share a common downstream pathway, which involves interactions with membrane-bound mitochondrial antiviral signalling protein (MAVS). A prion-like aggregation of MAVS on the

mitochondrial membrane follows, providing a platform for the interaction with TRAF3 and TANK, which interacts with TBK1 and signals through IRF3. The role of MDA5 in the immune response and IFN release was highlighted in a mouse model published by Funabiki et al. in 2014 (Funabiki et al., 2014). Mice with a missense mutation in *Ifih1* leading to p.Gly821Ser MDA5 had increased unstimulated MDA5 signalling causing a constitutive IFN gene signature, as well as a lupus phenotype (Funabiki et al., 2014). Two case series, one of eleven patients (G. I. Rice et al., 2014) and another of six (Oda et al., 2014), were published soon after, describing patients presenting with an Aicardi-Goutières syndrome (AGS) phenotype harbouring heterozygous mutations in *IFIH1* associated with an IFN gene signature (**Section 1.2**) (Oda et al., 2014; G. I. Rice et al., 2014). Since this time there has been the description of GoF mutations in *IFIH1* and *DDX58* encoding MDA5 and RIG-I respectively resulting in atypical Singleton-Merten syndrome (Jang et al., 2015). This uncommon dominantly inherited condition is characterised by variable degrees of glaucoma, aortic calcification, skeletal abnormalities, dental anomalies and psoriasis. Patients with this condition also display an IFN gene signature (Jang et al., 2015; Rutsch et al., 2015).

STING, encoded by *TMEM173,* was originally identified as an adaptor protein residing on the ER that, through conformational changes, facilitated the activation of NF-κB and IRF3, as well as the production of type I IFN in a TBK1-dependent manner (Ishikawa & Barber, 2008; W. Sun et al., 2009). STING was subsequently characterised as an essential component of the host's response to cytoplasmic DNA (Ishikawa, Ma, & Barber, 2009). Although Vance and colleagues determined that STING binds directly to cyclic dinucleotides (CDNs) produced by bacteria, the means by which STING responded to cytoplasmic dsDNA was elucidated in two seminal papers published in *Science* in 2013 (Burdette et al., 2011; L. Sun, Wu, Du, Chen, & Chen, 2013; J. Wu et al., 2013). Cytoplasmic DNA, whether host or pathogen derived, may be

converted to an endogenous second messenger CDN, cyclic guanosine monophosphate-adenosine monophosphate (cGAMP), which is detected by the sensor cGAS (L. Sun et al., 2013; J. Wu et al., 2013). STING then binds to and is activated by cGAMP, leading to both an NF-κB and IFN-β response. The potential implications of these findings were noted at the time in a perspective piece by O'Neill, highlighting that the ability of STING to sense host DNA through its conversion to cGAMP by cGAS suggests that it may have a role in the pathogenesis of autoimmunity (O'Neill, 2013). Although the STING pathway has been shown to promote translocation of transcription factors IRF3 through TBK1 (Ishikawa & Barber, 2008), how it leads to NF-κB activation and translocation remains unclear.

Originally documented in six unrelated children, heterozygous GoF mutations in *TMEM137* were reported to cause an AID with an IFN gene signature, termed STING associated vaculopathy with onset in infancy (SAVI) (Y. Liu et al., 2014). Each child presented before the age of 8 weeks with evidence of peripheral vascular inflammation, nail dystrophy and paratracheal adenopathy. The majority of patients have evidence of interstitial lung disease. Transient low titre autoantibodies associated with vasculitis were present in three children. None of the children responded to glucocorticoids or disease modifying anti-rheumatic drugs. WES performed on one patient and both parents identified a candidate mutation in *TMEM137,* and subsequent Sanger sequencing in other patients identified a total of three novel *de novo* mutations in *TMEM137*. These mutations affected amino acid residues near the dimerization domain of STING, resulting in a more stable dimer when compared with WT STING. This idea was developed by Jeremiah et al. through an exploration of the 3D structure of STING, with the more stable dimer was predicted to behave in a similar manner to ligand bound STING (Jeremiah et al., 2014). Patients with GoF mutations in *TMEM137* expressed increased IFN-related genes at baseline, but diminished *IFNB1* transcription in response to cGAMP (Y. Liu

et al., 2014). At baseline, unstimulated patient PBMCs expressed constitutively high STAT1 phosphorylation in CD4+ T and CD19+ B cells. Patient monocytes failed to phosphorylate STAT1 in response to cGAMP stimulation. An important finding in this publication was the reduction in phosphorylated STAT1 expression in CD4+ T cell and CD19+ B cells in response to the JAK inhibitors tofacitinib, ruxolitinib and baricitinib. Each JAK inhibitor prevented the phosphorylation of STAT1 downstream of the IFNAR and reduced the transcription of IFN response genes. Work is currently underway to optimize dosing of JAK inhibitors for the treatment of patients with rare IFN mediated disorders, also known as interferonopathies (H. Kim et al., 2017). More recently, three novel mutations located distal to the dimerization domain and away from the cGAMP binding site were identified by Crow and colleagues (Melki et al., 2017). All mutations tested, including the previously described p.Val155Met, were dependent on phosphorylation of STING. Mutation of the phosphorylation site p.Ser366 STING to Alanine abolished the downstream phosphorylation of IRF3 as well as increased IFN-β luciferase activity.

An interesting addition to this field was made through the evaluation of the T cell phenotype of patients with SAVI (Cerboni et al., 2017). In this cell type, STING was determined to have antiproliferative activity that was distinct from and independent to interactions with TBK1 and IRF3. This report was quickly followed by a murine model of SAVI using p.Asn153Ser STING knock-in mice generated through CRISPR/Cas9 techniques (**Section 1.4**) (Warner et al., 2017). Heterozygous mice developed spontaneous inflammatory disease that mimicked certain features of SAVI, including ulcerative skin lesions. There was no evidence of pulmonary fibrosis, but lung histology suggested marked inflammatory infiltrate. Remarkably, breeding mutant STING mice to *Irf3*$^{-/-}$ mice did not rescue the inflammatory phenotype, raising questions, at least in the murine model, of the role of IRF3 in the inflammation associated with

SAVI. As STING also activates NF-κB, the potential role of this pathway in disease pathogenesis cannot be discounted.

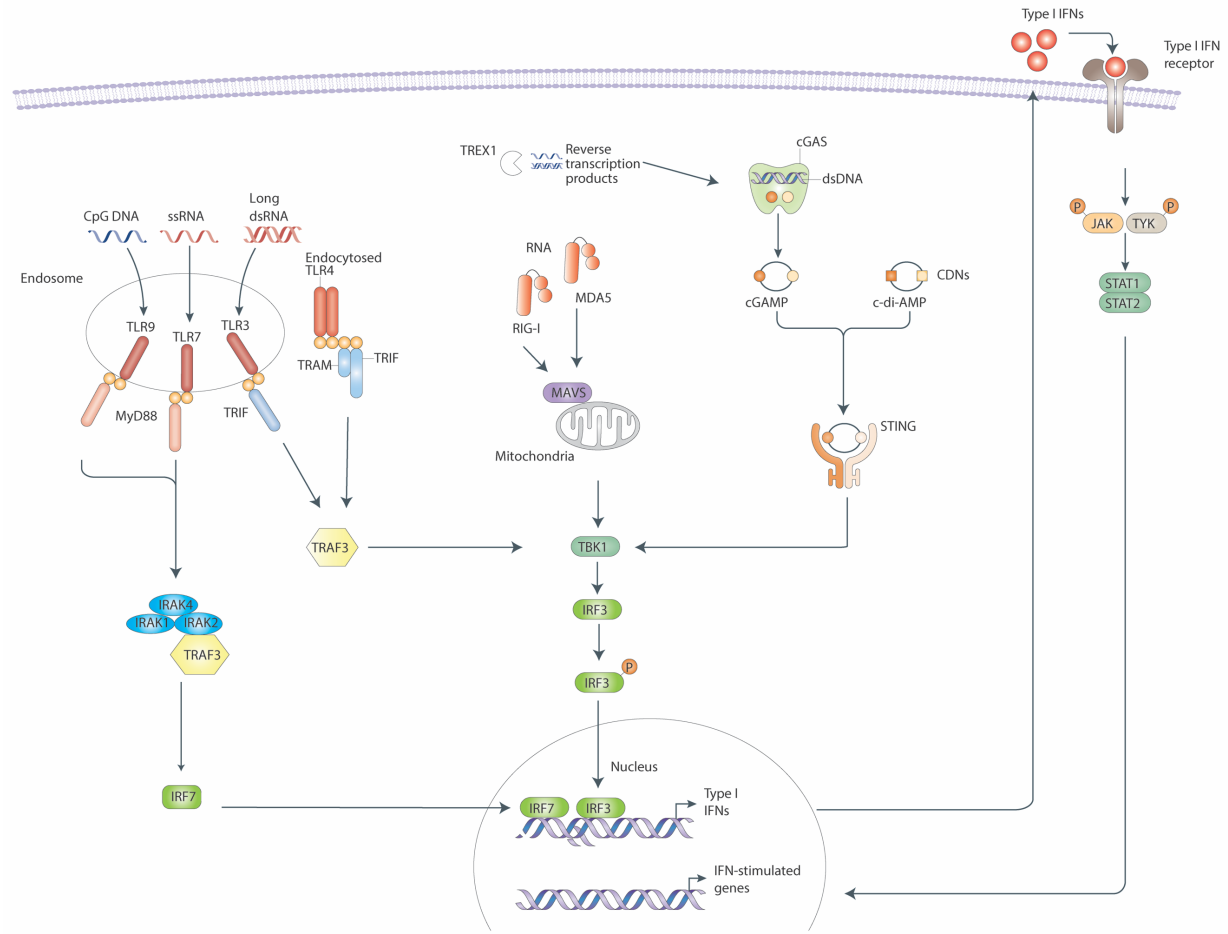


Figure 1-3 The type I interferon pathway.

Interferons are induced in response to stimulation of membrane bound TLRs or cytosolic sensors for nucleic acid such as RIG-I, MDA5 and cGAS-STING. Detailed description of pathways is outlined in **Section 1.3**. Loss of function in TREX1, the 3' repair endonuclease, results in Aicardi-Goutières Syndrome through a cGAS-STING-dependent pathway. Adapted from (Barber, 2015; O'Neill, Golenbock, & Bowie, 2013; Schlee & Hartmann, 2016)

Abbreviations: cGAMP cyclic guanosine monophosphate–adenosine monophosphate, cGAS cyclic GMP-AMP synthase, dsDNA double stranded DNA, IFN interferon, IRAK interleukin-1 receptor-associated kinase, IRF interferon regulatory factors, JAK janus kinase, MAVS mitochondrial antiviral-signalling protein, MDA5 melanoma differentiation-associated protein 5, MyD88 myeloid differentiation primary response 88, RIG-I retinoic acid-inducible gene I, ssRNA single stranded RNA, STAT signal transducer and activator of transcription, STING stimulator of interferon genes, TBK1 TANK-binding kinase 1, TLR toll like receptor, TRAF TNF receptor-associated factors, TRAM TRIF-related adaptor molecule, TRIF TIR domain-containing adaptor-inducing IFN-β, TYK tyrosine kinase 2.

1.2 Monogenic Autoinflammatory Disorders: Classification and Definition

In the two decades since the description of FMF, over 30 conditions have been added to the list of monogenic AIDs. The significant broadening of clinical features, pathway perturbations and genes involved bring into question the utility of the original definition of these disorders, and whether an alternative is required that better encapsulates the spectrum of immune dysregulation. Highlighting the complexity of this task, the list of conditions considered 'autoinflammatory' by the International Union of Immunological Societies (IUIS) is incongruent with the *Infevers* database, a registry of mutations associated with AIDs maintained by the International Society for Systemic Autoinflammatory Diseases (ISSAID) (**Figure 1.4**) (Bousfiha et al., 2018; Milhavet et al., 2008).

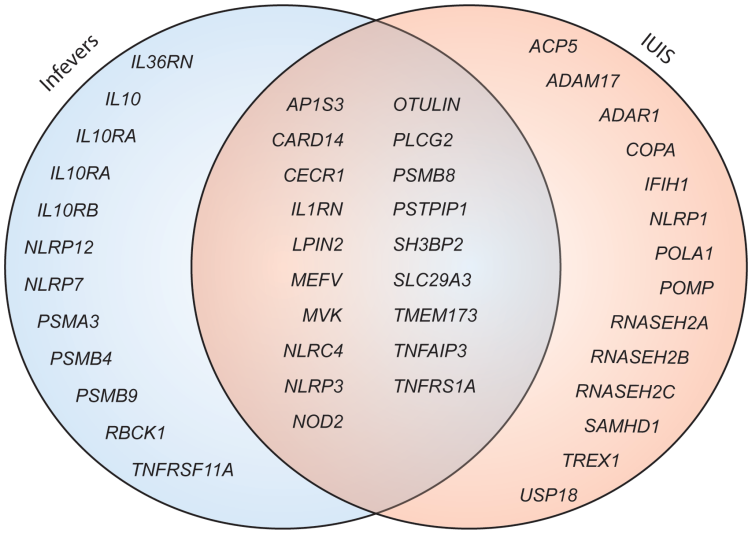


Figure 1-4 Genes involved in monogenic autoinflammatory disorders

Genes involved in monogenic autoinflammatory disorders according to International Society of Systemic Autoinflammatory Disorders (ISSAID) as listed in the *Infevers* database, compared with the International Union of Immunological Societies (IUIS).

The pathway model presented in **Section 1.1** has the advantage of highlighting possible targets for treatment downstream of an abnormal protein, as with JAK inhibitors for patients with SAVI (**Section 1.1.4**), as well as possible candidate genes for AIDs, such as *SHARPIN* (**Section**

1.1.3 and **Chapter 5**). There are, however, limitations to this classification. A clear example is the case of mutations in *TNFRSF1A* causing TRAPS (**Section 1.1.3**). Whilst TNFR1 is a key receptor in the NF-κB pathway, the disease is not necessarily caused by increased signalling through this pathway alone.

This classification also neglects the complex interaction between signalling pathways that exist. NF-κB translocation to the nucleus is important for the expression of pro-IL-1β and NLRP3, and the consequence of NF-κB dysfunction on inflammasome activation cannot be discounted. Key players in the regulation of NF-κB are also implicated in the regulation of NLRP3, as seen with A20 and the possible role of inflammasome activation in the inflammatory manifestations of HA20 (**Section 1.1.3**). Furthermore, although not yet shown in human cells, TAK1 has been shown to regulate NLRP3, with spontaneous NLRP3 activation documented in TAK1 deficient murine macrophages (Malireddi et al., 2018). The NF-κB and IFN pathways are also intimately linked, with a number of sensors leading to activation of both pathways. In the case of SAVI, literature to date suggests that the IFN pathway is dysregulated in this syndrome, but how these two pathways are uncoupled in the case of an overactive STING is unclear (**Section 1.1.4**). Furthermore, the role of NF-κB as a member of the IFN-β enhanceosome (Yoneyama et al., 2004), a multicomponent complex that optimises transcriptional activation of IFN-β, suggests that the pathways are closely connected.

An alternative classification strategy is based on the primary cytokine dysregulated, either increased or decreased, in patients with AIDs. This is of potential therapeutic benefit as the primary cytokine driving disease can be therapeutically targeted. An example of this is the treatment of patients with CAPS. In the original manuscript linking NOMID to mutations in *NLRP3,* cell lysates from unstimulated monocytes of a patient had high pro-IL-1β expression

as determined by western blot, and increased IL-1β mRNA in unstimulated PBMCs when compared with healthy controls (Aksentijevich et al., 2002). The empiric treatment of two patients with recombinant IL-1Ra and the rapid resolution of symptoms within hours, and inflammatory markers within days, highlighted the role of IL-1β in the disease pathogenesis (Hawkins et al., 2003). Having said this, the detection of IL-1β in serum of patients is difficult, with both patients and healthy controls having levels below the detection limit of currently available assays. Most publications looking at the IL-1β levels and response to treatment in patients with CAPS culture PBMCs and measure cytokine release over a 24 h period. The spontaneous secretion of IL-1β by CAPS PBMCs decreases with the initiation of IL-1β-targeted therapy (Goldbach-Mansky et al., 2006). From this it is clear that even without elevated serum levels, a therapeutic response to IL-1β neutralizing therapy suggests that this cytokine is important (Goldbach-Mansky et al., 2006; Lachmann et al., 2009). The response of patients with colchicine resistant FMF (Laskari et al., 2017) (**Chapter 3**), hyper immunoglobulin D syndrome (HIDS) (Arostegui et al., 2017) (**Chapter 3**) and TRAPS (Gattorno et al., 2017) to the neutralizing anti-IL-1β antibody canakinumab, suggests that IL-1β is a key cytokine in all of these disorders. Supporting this is evidence of increased expression of *IL1B* and *IL1R1* as determined by microarray in patients with TRAPS (Borghini et al., 2016). The gene expression profile of TRAPs moved towards the healthy control profile with canakinumab treatment (Gattorno et al., 2017). An interesting addition to the literature was a retrospective analysis by Savic and colleagues of patients with undifferentiated systemic AIDs who were treated with anakinra (Harrison et al., 2016). A total of 11 patients were identified over a 3-year period, and 9 responded completely to treatment with anakinra within 4-6 weeks of commencement. Although patients had undergone Sanger sequencing for *NLRP3*, *MEFV*, *TNFRSF1A*, and *NOD2* with no pathogenic mutations detected, the marked response to treatment suggests that genes in the IL-1β pathway could be further interrogated for variants

that may be causing disease. Conversely, patients could undergo a broader approach with WES or whole genome sequencing (WGS) (**Section 1.3**) and novel genes involved in the IL-1β pathway may be revealed.

Evaluation of the major cytokine/s involved in monogenic AIDs may point to distinctions between conditions within the same pathway described in **Section 1.1**. The GoF mutations in inflammasome forming proteins that lead to disease can be presumed to cause an increase in IL-1β processing and release. As will be seen in **Chapter 4**, mutations in *NLRC4* that result in an autoinflammatory phenotype are associated with markedly increased serum IL-18 levels in patients when compared with healthy controls and patients with CAPS. Treatment of these patients with IL-18 binding protein has shown promise, as will be described in detail in **Chapter 4**. This not only highlights the importance of identifying driver or dominant cytokines for possible therapeutic manipulation, but also the potential differences between inflammasome effector and regulatory mechanisms that results in one cytokine dominating over another.

There are also conditions that may involve pathways distinct from those presented in **Section 1.1**. Through the study of autosomal recessive generalised pustular psoriasis (GPP) in a number of multiplex families, Marrakchi et al. identified homozygous missense mutations in *IL36RN* causing deficiency in IL-36 receptor antagonist (IL-36Ra, DITRA) (Marrakchi et al., 2011). IL-36 is a member of the IL-1 family of cytokines. It acts via its receptor IL-36R in concert with IL1RAcP to signal to NF-κB through MyD88. The binding of IL-36Ra to IL-36R prevents the association of IL1RAcP and downstream signalling. Whilst there have been four case reports of the successful treatment of DITRA patients with anakinra therapy (Huffmeier, Watzold, Mohr, Schon, & Mossner, 2014; Podlipnik et al., 2017; Rossi-Semerano et al., 2013;

Tauber, Viguier, Le Gall, Smahi, & Bachelez, 2014), therapeutic benefit has also resulted from TNF inhibition (Fialova, Vojackova, Vanousova, & Hercogova, 2014; Matsumoto, Komine, Karakawa, Kishimoto, & Ohtsuki, 2017; Zangrilli, Papoutsaki, Talamonti, & Chimenti, 2008), IL-17 inhibition with secukinumab (Kostner et al., 2018) and IL-12/IL-23 inhibition with ustekinumab (Arakawa, Ruzicka, & Prinz, 2016; Bonekamp et al., 2017; Cherqaoui, Rossi-Semerano, Piram, & Kone-Paut, 2017). This suggests that these agents may be targeting cytokines that are downstream of IL-36 (Tauber et al., 2014). The possibility of developing a therapeutic agent that is specific for IL-36 has been explored. Mbow and colleagues characterised a mouse anti-human antibody (MAB92) with high affinity to the IL-36 receptor that blocks signalling through this pathway (Ganesan et al., 2017). Although highly specific for human IL-36R, the authors created MAB04, which cross reacts with murine IL-36R for *in vivo* studies. Importantly, MAB04 inhibited imiquimod- and IL-36- induced skin inflammation in mice.

Deficiency in regulatory cytokines have also been described, and the clinical course of these patients has been tumultuous. Homozygous mutations in *IL10, IL10RA* or *IL10RB*, leading to deficiencies in IL-10, IL-10Rα or IL-10Rβ respectively, have been reported to cause monogenic early onset inflammatory bowel disease (EOIBD) (Glocker et al., 2010; Glocker et al., 2009). IL-10 has regulatory effects on the inflammatory response which are mediated through STAT3, with IL-10 deficient mice developing chronic enterocolitis (Berg et al., 1995; Kuhn, Lohler, Rennick, Rajewsky, & Muller, 1993; Williams, Bradley, Smith, & Foxwell, 2004). PBMCs from patients with LoF of these proteins had higher proinflammatory cytokine responses to LPS stimulation, including IL-6, TNF and IL-1β, when compared with healthy controls (Glocker et al., 2009). Although multiple agents have been trialled in these patients including corticosteroids, azathioprine, methotrexate, cyclosporine A and anti-TNF therapy,

only mild clinical benefit has resulted (Engelhardt et al., 2013). A number of patients have undergone allogenic haemopoetic stem cell transplantation (HSCT) with marked improvement in their inflammatory bowel disease (Engelhardt et al., 2013; Kotlarz et al., 2012). Whilst recombinant human IL-10 replacement (rhuIL-10) in patients with IL-10 deficiency would seem to be a therapeutic option, there have been issues with the response to and side effects from rhuIL-10 in trials of patients with Crohn's disease (Buruiana, Sola, & Alonso-Coello, 2010). Furthermore, this option would not be effective in patient with mutations in IL-10RA or IL-10RB. At this point in time, HSCT is the only curative option.

A number of other conditions are presumed to result from dysregulation of a particular pathway because of their cytokine profile, but where little is known about the steps that lead to this alteration. Proteasome-associated autoinflammatory syndrome (PRAAS) is an autosomal recessive AID that encompasses conditions previously considered distinct entities: Nakajo-Nishimura syndrome (NKJO), joint contractures, muscular atrophy, microcytic anaemia, and panniculitis-induced lipodystrophy (JMP syndrome), as well as chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature syndrome (CANDLE). Three publications identified mutations in *PSMB8*, the β5i catalytic component of the immunoproteasome, as the cause of disease (Agarwal et al., 2010; Arima et al., 2011; Kitamura et al., 2011). Patients with homozygous LoF mutations in *PSMB8* experienced spontaneous febrile episodes with features of muscle weakness, lipodystrophy as well as neutrophilic and lymphocytic infiltrative skin nodules and evidence of cerebral calcification (Agarwal et al., 2010; Arima et al., 2011; Kitamura et al., 2011). Homozygous mutations were associated with poor proteasome assembly as well as reduced chymotrypsin-like activity and accumulation of ubiquitinated proteins in either EBV-transformed patient B cells or immortalised lymphoblastoid cell lines (Agarwal et al., 2010; Arima et al., 2011; Kitamura et al., 2011). In

these early papers, increased serum IL-6 was noted in all patients, but the role of IFN was only identified later (Y. Liu et al., 2012). Liu et al. noted an almost 80-fold increase in IFN-γ-inducible protein 10 (IP-10) in patients compared with healthy controls and CAPS patients, prompting whole blood microarray analysis to determine the gene signature of these patients. The IFN pathway was the most differentially regulated pathway in patients with PRAAS, further supported by stronger STAT1 phosphorylation in response to IFN-γ stimulation of patient monocytes when compared with healthy controls. These authors also highlighted patients with the clinical phenotype of PRAAS without *PSMB8* mutations, later explored by Goldbach-Mansky and colleagues (Brehm et al., 2015). Digenic mutations involving *PSMA3* or *PSMB4* and *PSMB8* or *PSMB9,* encoding constitutive proteasome subunits α7 and β7 or inducible subunits β5i and β1i respectively, were found in patients with the clinical diagnosis of PRAAS. One patient harboured a compound heterozygous mutation in *PSMB4*, and another patient a heterozygous mutation in *POMP*, encoding proteasome maturation protein. Similar to earlier reports, the mutant subunits were not efficiently assembled into the proteasome, resulting in reduced proteolytic activity. When compared with patients with homozygous *PSMB8* mutations, the chymotrypic proteolytic activity was less impaired, but deficiencies were noted in tryptic and caspase proteolytic activity. Similar to homozygous *PSMB8* mutations, there was inefficient clearing of ubiquitinated proteins and the presence of a type I IFN gene signature. Both siRNA models and proteasome inhibitors were used to recapitulate the IFN signature in PBMCs and fibroblasts. However, the mechanism/s by which proteasomal dysfunction leads to this response remains elusive. Classifying PRAAS by its IFN gene signature guides potential treatment considerations and also opens avenues for researchers to determine the role of the immunoproteasome in the IFN pathway.

The boundaries of what is classified as an AID are also being blurred. The strict definition of innate immune dysregulation without self-reactive T cells or high titres of autoantibodies is increasingly in question, especially when one considers interferonopathies such as AGS. AGS was originally described in the 1980s as a disorder of the central nervous system (CNS) associated with lymphocytosis on cerebrospinal fluid analysis and bilateral basal ganglia calcifications (Aicardi & Goutieres, 1984). The genetic causes of AGS are numerous, and all involve the processing of nucleic acid, either self or foreign, in the cytoplasm. LoF mutations in genes encoding deoxyribonuclease TREX1 (Crow, Hayward, et al., 2006), deoxynucleoside triphosphate triphosphohydrolase SAMHD1 (G. I. Rice et al., 2009), ribonuclease components RNASEH2A, RNASEH2B or RNASEH2C (Crow, Leitch, et al., 2006), or RNA-specific adenosine deaminase ADAR1 (G. I. Rice et al., 2012) have been identified in patients with AGS. The link between AGS and autoimmunity was initially made when Aicardi and Goutières speculated that the phenotype of two patients with infantile systemic lupus erythematosus overlapped AGS considerably. They hypothesised that the two may be either the same condition, or linked by an increase in IFN-α (Aicardi & Goutieres, 2000). The phenotypic link has subsequently been highlighted by a number of groups (De Laet et al., 2005; Ramantani et al., 2010), although the number of patients in a large cohort of 374 mutation confirmed AGS with clinically diagnosed lupus was low (Crow et al., 2015). An abnormal serum autoantibody profile was seen in a minority of patients with AGS in one cohort study (G. Rice et al., 2007), however another detected persistent ANA or autoantibodies against ENA, dsDNA and cardiolipin in the majority of their patients with mutation confirmed AGS (Ramantani et al., 2010). Subsequent work using multiplex autoantibody microarrays identified unique autoantibodies in patients with AGS (Cuadrado et al., 2015). Whether this condition, and indeed other interferonopathies, should be considered autoimmune or autoinflammatory is a matter of debate. AGS highlights that this distinction is not always clear.

Indeed, the spectrum of immune dysregulation and overlap between autoimmunity, autoinflammation and immune deficiencies has been seen in a number of recently described conditions. The brief discussion of deficiencies in HOIL1 or HOIP in **Section 1.1.2** highlighted cell specific manifestations, with fibroblasts and EBV-immortalized B cells demonstrating an attenuated NF-κB response, whereas the opposite was seen in monocytes. Classifying these as AIDs inherently fails to acknowledge the associated immunodeficiency, and vice versa. A similar problem concerns the conditions caused by mutations in *PLCG2* encoding phospholipase c gamma-2 (PLCγ2), PLCγ2-associated antibody deficiency and immune dysregulation (PLAID) and autoinflammation and PLAID (APLAID) (M. J. Ombrello et al., 2012; Zhou et al., 2012). PLCγ2 was linked to autoimmune and autoinflammatory disease initially through an N-ethyl-N-nitrosourea (ENU) mutagenesis screen (P. Yu et al., 2005). A heterozygous point mutation in PLCγ2 in mice led to spontaneous inflammation, arthritis and dermatitis with evidence of immune complex driven glomerulonephritis. Subsequently, by sequencing three families with dominantly inherited cold-induced urticaria, antibody deficiency and autoimmunity, in frame deletions in *PLCG2* were identified and shown to segregate with disease (M. J. Ombrello et al., 2012). These deletions affected the autoinhibitory C-terminal Src-homology 2 domain and resulted in constitutive phospholipase activity. Interestingly, and somewhat contradictory to the increased activity of PLCγ2, B cells and NK cells demonstrated reduced calcium flux and reduced phosphorylation of MAPK in response to stimulation with either IgM cross linking, or cross linking of activating receptors respectively. This was determined to be temperature specific, however, with increasing MAPK phosphorylation and cytosolic calcium in response to decreasing temperatures. This description was quickly followed by one of a family with a dominantly inherited autoinflammatory condition who had a missense mutation in *PLCG2*. Unlike the previous report, the patients had

no evidence of autoimmunity, but did have hypogammaglobulinaemia and markedly reduced class switched memory B cells in addition to inflammatory manifestations in the form of skin inflammation and granulomata, enterocolitis, bronchiolitis and uveitis (Zhou et al., 2012). Of the two patients described, neither had cold-induced symptoms. Increased baseline PLCγ2 activity was noted in an overexpression COS-7 cell model. Chae et al. progressed the understanding of the inflammatory manifestations of APLAID by showing that the increased activity of PLCγ2 and subsequent increase in inositol and release of Ca^{2+} from ER stores, previously established by Kurosaki et al., resulted in increased NLRP3 activation and IL-1β release when assessing PBMCs from patients compared with healthy controls (Chae et al., 2015; Kurosaki & Tsukada, 2000). It would be interesting to determine whether the same increase in NLRP3-driven IL-1β is seen when PBMCs from PLAID patients are examined, as their inflammatory phenotype was not as profound, and was temperature dependent. Furthermore, the partial response to IL-1β-targeted therapy (Zhou et al., 2012) suggests that there may be more than NLRP3 driving the inflammatory disease.

Clearly, more information is needed to tease out the different immunological consequences of mutations in *PLCG2*. This is, of course, not unique in AIDs. With the description of rare disorders and involvement of novel genes and mutations, one can expect the phenotypic spectrum to evolve as more cases are reported. In the case of deficiency of adenosine deaminase 2 (DADA2), homozygous LoF mutations in *CECR1* were found in patients with polyarteritis nodosa (Navon Elkan et al., 2014) as well as early onset stroke, vasculopathy and febrile episodes (Zhou et al., 2014). Although immunodeficiency and autoimmunity were not a major feature, IgM deficiency was noted in a number of patients (Zhou et al., 2014). Treatment of ten patients with TNF targeting therapy by Levy-Lahad and colleagues lead to significant clinical improvement, highlighting the role of this cytokine in disease pathogenesis (Navon Elkan et

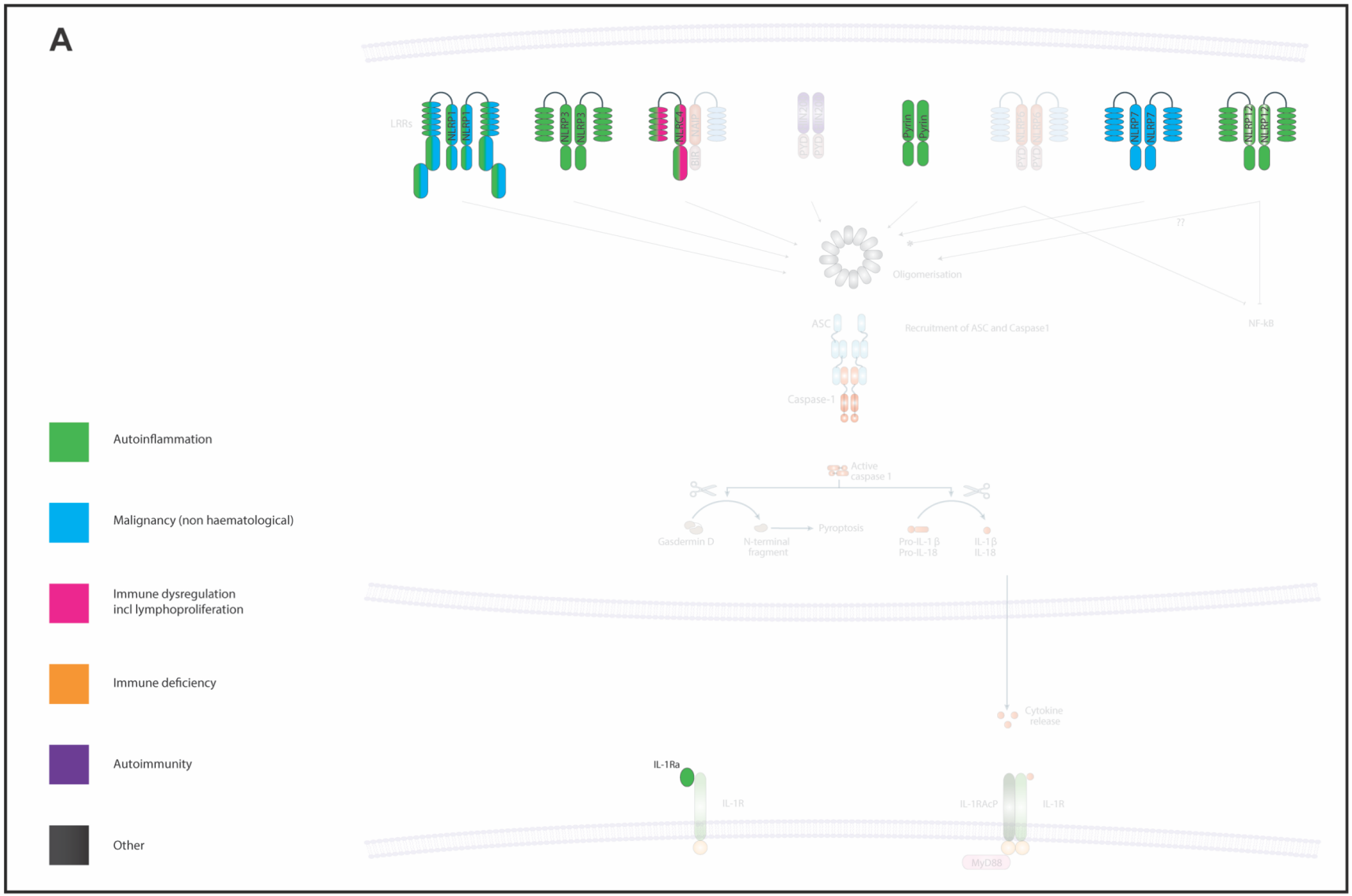
al., 2014). The response to TNF directed therapy has since been reproduced (A. Ombrello et al., 2015; Sahin et al., 2018). In a subsequent study of 48 patients with polyarteritis nodosa associated with livedo reticularis and/or strokes, Gattorno and colleagues performed Sanger sequencing of *CECR1* and determined that 15 patients harboured homozygous or compound heterozygous mutations (Caorsi et al., 2017). Since the time of the original description, there has been an expansion of the clinical phenotype of patients with DADA2, from cytopaenias and pure red cell aplasia (Ben-Ami et al., 2016), to lymphoproliferative disease (Alsultan, Basher, Alqanatish, Mohammed, & Alfadhel, 2017; L. Van Eyck, Jr. et al., 2015; L. Van Eyck, Liston, & Wouters, 2014), and combined immune deficiency, as well as common variable immune deficiency (CVID) (Schepp et al., 2016). Indeed, a cohort study of 181 patients with antibody deficiency diagnosed 11 patients with mutation and enzyme activity confirmed DADA2 (Schepp et al., 2017). An interesting finding in this group was that anti-TNF therapy resulted in an improvement in IgM levels in one patient, and there was an inverse correlation between CRP and IgG in another. Further complicating the potential mechanisms of this disease, patients with DADA2 have also been reported to have an IFN gene signature (Belot et al., 2014; Skrabl-Baumgartner et al., 2017). The teams investigated patients with features overlapping with AGS caused by mutations in *SAMHD1*. In each report, patients had enhanced IFN stimulated gene expression. These cases were treated with a range of immunosuppressive agents but had not been trialled on anti-TNF therapy. Given the reports of profound response in DADA2 patients to this therapy, it would be interesting to determine whether the interferon gene signature is abrogated with the use of anti-TNF therapy.

From the above discussion, it is apparent that there are significant barriers to a simple definition or classification criteria for what are considered a monogenic AIDs. As research progresses, the inflammatory component of disorders previously considered to be primarily of immune

deficiency or autoimmunity will become more apparent (**Figure 1.5**). Provided in **Appendix 2** is a summary of conditions listed as AID in the latest IUIS Expert Committee for Primary Immunodeficiency (2017) as well as the *Infevers* database, documenting the spectrum of immunological manifestations recognised to date.

Figure 1-5 Range of immunological manifestations in monogenic disorders of the IL-1β, NF-κB and IFN pathways.

The figures presented earlier have been adapted to highlight different immunological consequences of monogenic disorders involving the pathways explored in Section 1.1. For example, mutations in *TNFAIP3* encoding A20 result in autoinflammation manifestations, as well as autoimmunity and in one case report, an autoimmune lymphoproliferative syndrome (ALPS). This pictorial representation highlights the autoinflammatory dominant manifestations of conditions involving the IL-1β pathway (A), whereas both the NF-κB (B) and interferon pathways (C) have a significant number of monogenic disorders resulting in loss of function and immune deficiency.


A
Autoinflammation
Malignancy (non haematological)
Immune dysregulation
incl lymphoproliferation
Immune deficiency
Autoimmunity
Other
LRRs
NLRP1
NLRP3
NLRC4
CARD
NBD
PYD
NLRP6
NLRP7
NLRP12
Cyclin
Cyclin
Oligomerisation
??
ASC
Recruitment of ASC and Caspase1
NF-kB
Caspase-1
Active
caspase 1
Gasdermin D
N-terminal
fragment
Pyroptosis
Pro-IL-1 β
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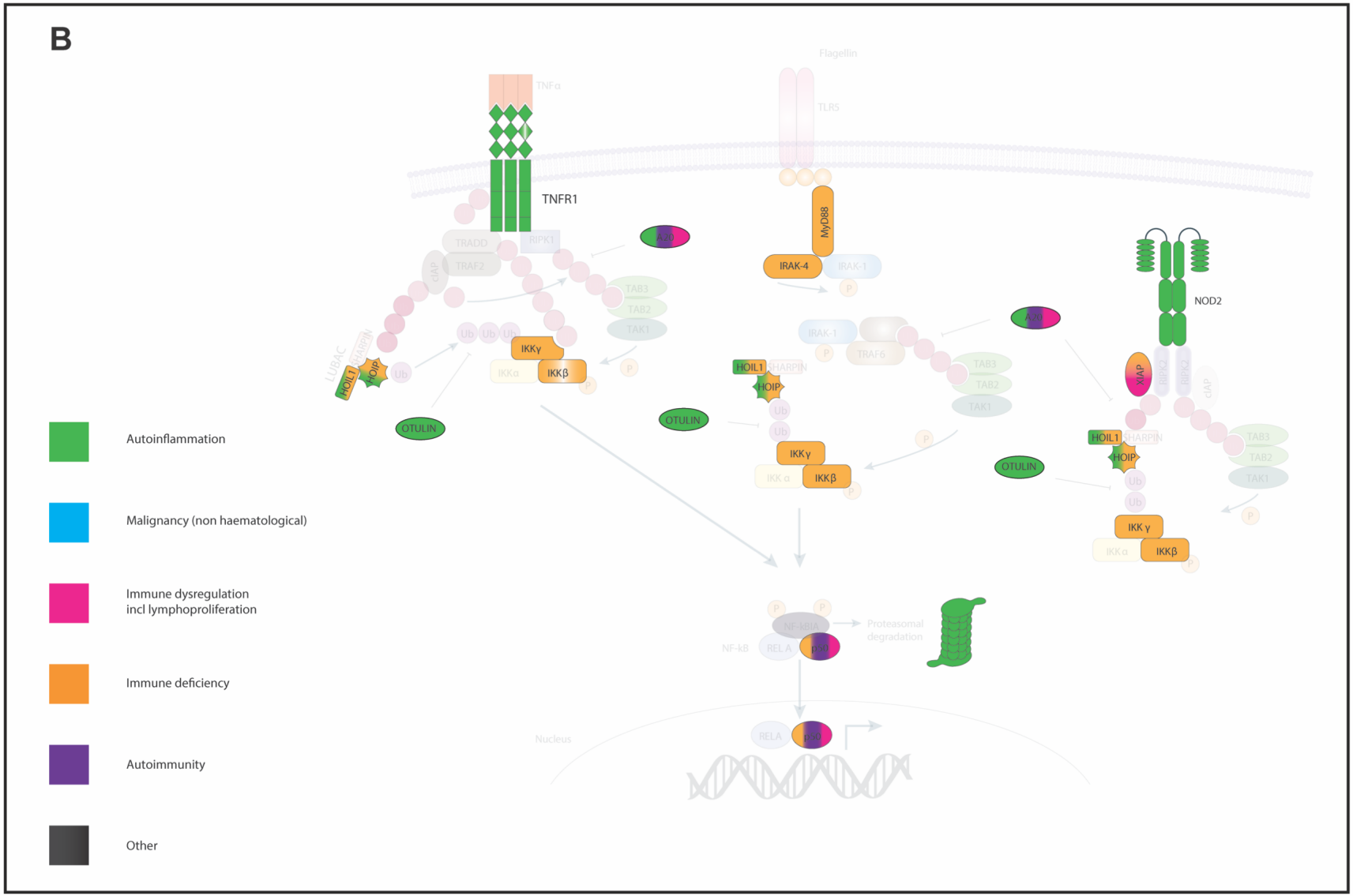
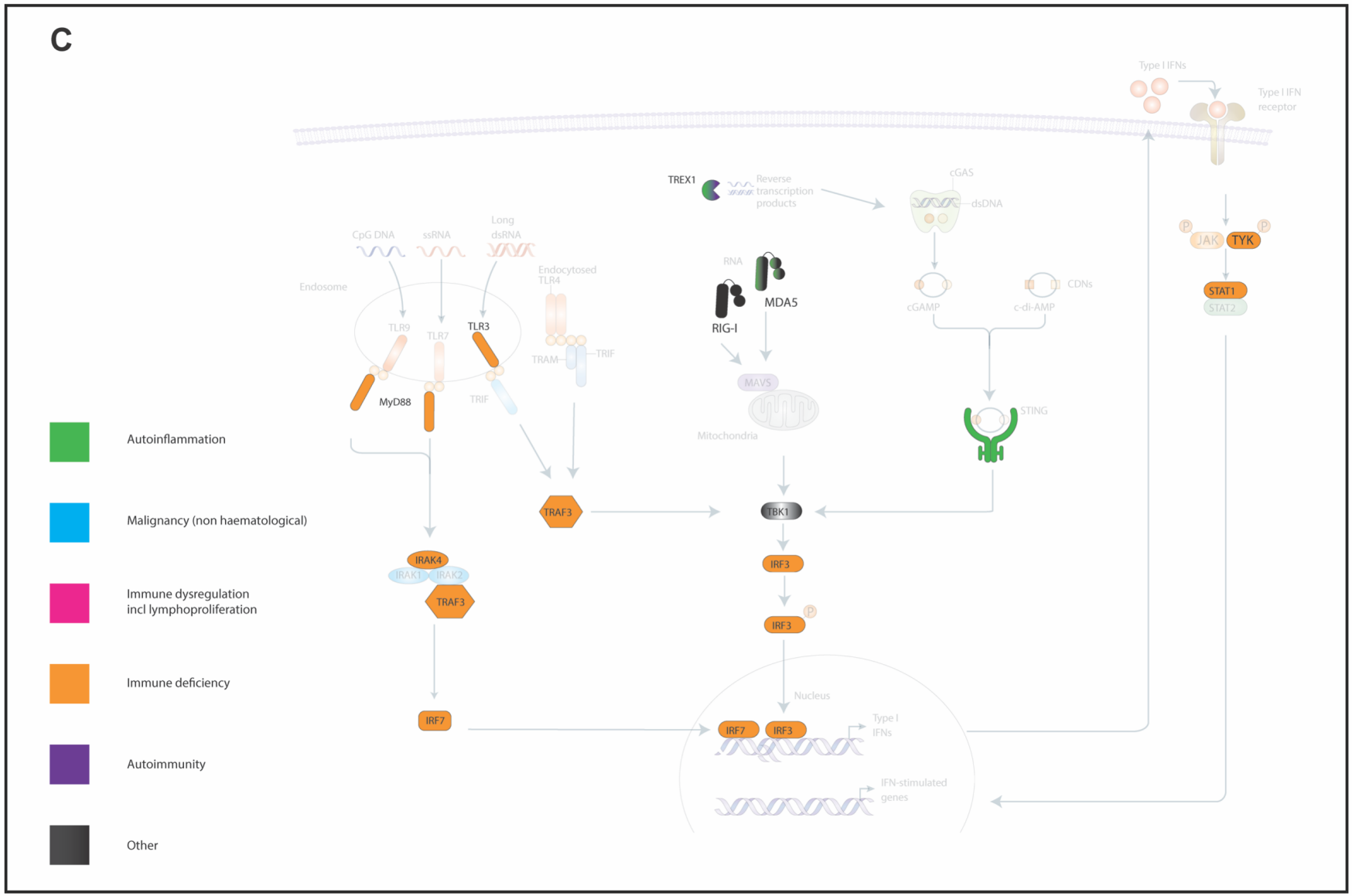
IL-1Ra
IL-1R
IL-1RAcP
IL-1R
MyD88


B
Autoinflammation
Malignancy (non haematological)
Immune dysregulation
incl lymphoproliferation
Immune deficiency
Autoimmunity
Other
TNFα
TNFR1
Flagellin
TLR5
MyD88
IRAK-4
IRAK-1
NOD2
A20
TRADD
TRAF2
RIPK1
cIAP
TAB3
TAB2
TAK1
TRAF6
LUBAC
SHARPIN
HOIL1
HOIP
Ub
IKKγ
IKKα
IKKβ
OTULIN
XIAP
RIPK2
NF-kB
NF-kBIA
REL A
p50
Proteasomal
degradation
Nucleus
RELA


C
Type I IFNs
Type I IFN receptor
JAK
TYK
STAT1
STAT2
TREX1
Reverse transcription products
cGAS
dsDNA
CpG DNA
ssRNA
Long dsRNA
Endosome
Endocytosed TLR4
TLR9
TLR7
TLR3
TRAM
TRIF
MyD88
TRIF
RNA
MDA5
RIG-I
MAVS
Mitochondria
cGAMP
CDNs
c-di-AMP
STING
TRAF3
TBK1
IRAK4
IRAK1
IRAK2
TRAF3
IRF3
IRF3
IRF7
Nucleus
IRF7
IRF3
Type I IFNs
IFN-stimulated genes
Autoinflammation
Malignancy (non haematological)
Immune dysregulation incl lymphoproliferation
Immune deficiency
Autoimmunity
Other

1.3 Genetic Sequencing Techniques In The Evaluation Of AIDs

"A knowledge of sequences could contribute to our understanding of living matter."

(Sanger, 1980)

The phenotypic heterogeneity of what is considered a monogenic AID, as well as the explosion in the number of newly described conditions, coincides with advances in genetic sequencing techniques. The gene mutated in FMF was determined to be *MEFV* using positional cloning methods in 1997, and since this time many more disease-causing genes have been recognized (**Figure 1.6**). Next generation sequencing (NGS) technology has been used in the description of these conditions since 2012, starting with the identification of *RBCK1* as the gene implicated in HOIL1 deficiency (Boisson et al., 2012).

The description of Sanger's chain-termination technique in 1977 (Sanger, Nicklen, & Coulson, 1977) was an important step in the rapid sequencing of DNA, which had until then been slow and dependent on converting DNA to RNA (Holley, Madison, & Zamir, 1964). Sanger's method determines the DNA sequence of a single stranded DNA template through a polymerase chain reaction (PCR) with a DNA polymerase, a primer, the four DNA nucleotides (dNTPs) and the addition of a small amount of fluoro-labelled dideoxynucleotides (ddNTP). In lacking a 3' hydroxyl group, the ddNTPs that attach during the PCR reaction are unable to facilitate the addition of further nucleotides and hence "terminate" the reaction. The result is multiple fragments of various lengths generated, depending on which cycle the ddNTP is added. Using capillary gel electrophoresis to generate a chromatogram, the fragments are separated based on size, which determines the position in the sequence, and the fluorophore detected determines the nucleotide.

The fragment length read by the Sanger technique is limited, and a 'shot-gun' approach was soon developed (S. Anderson, 1981; Staden, 1979), allowing for larger sequencing tasks, including the Human Genome Project (I. H. G. S. Consortium, 2004; Venter et al., 2001). In this method, DNA is initially fragmented by an endonuclease, fractionated and cloned in a phage vector. The cloned fragments undergo the chain-termination PCR technique and overlapping reads are subsequently aligned to determine an overall sequence for the original DNA (S. Anderson, 1981; Staden, 1979). A significant limitation to Sanger sequencing, in addition to the ability to detect only substitutions and small insertions or deletions, is the requirement of prior knowledge of the gene or locus of interest. A number of strategies have been employed to determine a possible locus of interest in the investigation of a genetic cause of disease. Once such approach is that of positional cloning through linkage analysis to detect the chromosomal location of disease causing genes in families with multiple affected individuals. This analysis uses the observation that during genetic recombination of maternal and paternal homologues, the inheritance of genes is not independent of the location on a chromosome, and that linked genes are inherited together (Sturtevant, 1925). Using genetic markers such as SNPs or DNA microsatellites, linkage analysis determines whether a clinical phenotype segregates with certain genetic markers (Pulst, 1999). Similarly, homozygosity mapping is used in the setting of rare disorders in consanguineous families. Genetic markers are used, but rather than segregation with disease, regions with homozygous markers are identified (Lander & Botstein, 1987). At its core, linkage and homozygosity analysis are able to determine the proximity of a disease-causing gene to an associated marker, providing information on where fine mapping and candidate gene analysis should be directed.

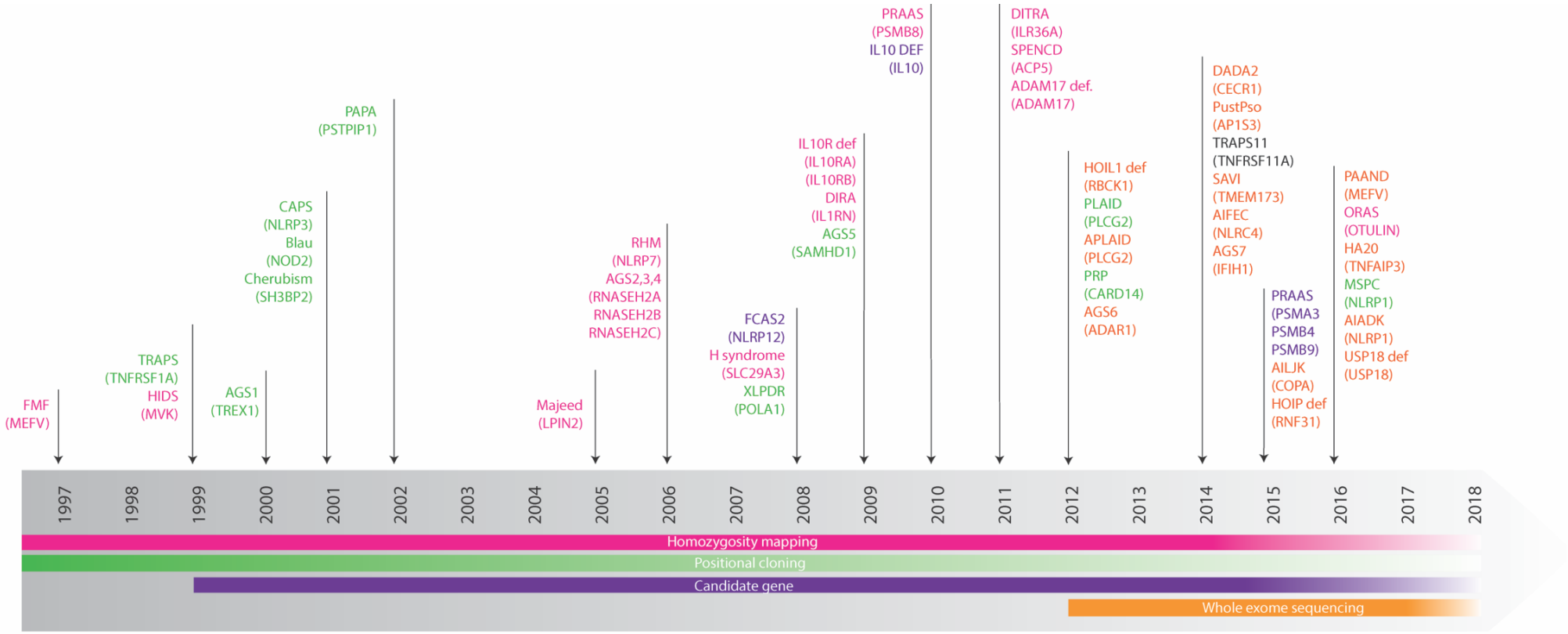


Figure 1-6 Timeline of monogenic autoinflammatory disorder discovery and genetic sequencing technique used.

Abbreviations: AGS Aicardi-Goutieres syndrome, AIADK autoinflammation with arthritis and dyskeratosis, AIFEC autoinflammation with infantile enterocolitis, AILJK autoimmune interstitial lung, joint, and kidney disease, APLAID autoinflammation and PLCG2-associated antibody deficiency and immune dysregulation, CAPS cryopyrin associated periodic syndromes, DADA2 deficiency in ADA2, DIRA deficiency of IL-1 receptor antagonist, DITRA deficiency of IL-36 receptor antagnosit, Dysreg dysregulation (including lymphoproliferation), EOIBD early onset inflammatory bowel disease, FCAS2 familial cold autoinflammatory syndrome 2, FMF familial Mediterranean fever, HA20 haploinsufficiency of A20, HIDS hyperIgD syndrome, HYDM1 hydatidiform molar pregnancy, MSPC multiple self-healing palmoplantar carcinoma, ORAS otulin related autoinflammatory syndrome, PAAND pyrin associated autoinflammation with neutrophilic dermatosis, PAPA pyogenic arthritis, pyoderma gangrenosum and acne, PLAID PLCG2 associated antibody deficiency and immune dysregulation, RAAS proteasome associated autoinflammatory syndrome, PRP pityriasis rubra pilaris, SAVI STING associated vasculopathy with onset in infancy, SPENCD spondyloenchondrodysplasia, TRAPS TNF receptor associated periodic syndrome, XLPDR x-linked pigmentary disorder, reticulate, with systemic manifestations. Adapted and updated from (Sarrabay, Barat-Houari, Annakib, & Touitou, 2015)

The now widely adopted NGS, also known as massive parallel or deep sequencing, is a broad term encompassing a number of different technologies that share the ability to generate and analyze millions of sequences per run. There are a large number of platforms on which NGS can be performed, and the specifics of the sequencing method varies depending on the instrument used (Goodwin, McPherson, & McCombie, 2016). In general, the sequencing process involves the preparation of a library of short DNA fragments through either enzymatic or sonication techniques. These short strands of DNA are then ligated to generic adapters *in vitro*. PCR amplification follows, performed using either emulsion PCR in oil-water emulsion micelles, or bridge PCR on a solid surface coated with complementary primers. Subsequent sequencing of the amplicon is performed by either pyrosequencing, sequencing by ligation or sequencing by synthesis. The large number of short reads generated from this process must then be aligned against a reference sequence. A plethora of software has been developed not only to align the reads, but to also determine where deviations from a reference sequence exist. Furthermore, considering that WES or WGS of an individual identifies 20,000 or 4,000,000 variants respectively, an appropriate filtering strategy must be employed to determine which of these variants are potentially pathogenic (**Chapter 5**) (Belkadi et al., 2015; Chou, Ohsumi, & Geha, 2012).

NGS has been employed in the diagnostic evaluation of patients with AIDs. Ceccherini and colleagues compared the performance of three NGS platforms in a pilot study interrogating 10 genes (*MEFV*, *MVK*, *TNFRSF1A*, *NLRP3*, *NLRP12*, *NOD2*, *PSTPIP1*, *IL1RN*, *LPIN2* and *PSMB8*) from 50 patients with genetically confirmed AIDs (Rusmini et al., 2016). The expected mutations were correctly called in most cases, although there was a failure to detect p.Val377Ile *MVK* in a number of patients due to low coverage. Additional variants were also noted, a number of which were false positives and detected on only one of the three platforms

used. Importantly, true positive incidental variants did not alter the clinical diagnosis or management of the patient. Taking a different approach, Nakayama et al. prospectively recruited patients with a clinical diagnosis of an AID prior to any genetic testing (Nakayama et al., 2017). Using a MiSeq platform developed in house, they sequenced 9 genes (*IL1RN, MEFV, MVK, NLRP12, NLRP3, NOD2, PSMB8, PSTPIP1,* and *TNFRSF1A*) in 108 patients. A total of 27 missense mutations were identified and confirmed with Sanger sequencing. Unfortunately, the authors did not outline any genotype-phenotype correlation, nor did they include positive controls to ensure that all pathogenic mutations were detected. A more robust addition to the literature was by Omoyinmi and colleagues with their development of a vasculitis and inflammation panel targeting up to 166 genes (Omoyinmi et al., 2017). Initially, 16 patient samples with known pathogenic mutations were analysed and the best performing pipeline carried over to the assessment of patients with unknown diagnosis. Pathogenic mutations were detected in 12% of patients, and likely pathogenic variants in 22%. Furthermore, the depth of coverage was sufficient to be able to detect a 3% somatic mosaicism in *NLRP3*.

Somatic mosaicism in *NLRP3* causing disease was first described in 2005 in a patient with a p.Tyr507Cys variant occurring at a frequency of 16.7% detected using Sanger sequencing (Saito et al., 2005). Somatic mosaicism in *NLRP3* has since been reported by multiple groups with a mutation frequency as low as 2.7% noted (Arostegui et al., 2010; Jimenez-Trevino et al., 2013; Lasiglie et al., 2017; Mensa-Vilaro et al., 2016; Nakagawa et al., 2015; Rowczenio et al., 2017; N. Tanaka et al., 2011; Zhou et al., 2015). Importantly, a recent study highlighted that NGS was able to detect somatic *NLRP3* mutations in 8 patients symptomatic of CAPS who had previously tested negative for mutations in *NLRP3* sequencing using Sanger techniques (Rowczenio et al., 2017). Retrospective review of the Sanger chromatogram identified small

peaks in only 3 of the 8 patients, each with an allele frequency of greater than 10%, suggesting that Sanger sequencing is not a sensitive technique for detecting low frequency somatic mosaicism.

Whilst the use of NGS panels for the diagnosis of AID in the clinical setting is increasing, the key limitation from a research perspective is the inability to discover new AID causing genes. In using WES or WGS, novel variants in genes known to cause disease, and also variants in novel genes, may be uncovered. The rationale for the use of WES is based on the finding that the majority of pathogenic variants causing Mendelian diseases that have been identified to date are located in protein-coding regions (Botstein & Risch, 2003; Cooper et al., 2010; Majewski, Schwartzentruber, Lalonde, Montpetit, & Jabado, 2011). Whilst WGS has the benefit of capturing introns and intergenic regions, and detecting copy number variants (Belkadi et al., 2015), a large volume of data must be interrogated and the bioinformatics analysis is complex. Both strategies raise the possibility of detecting an incidental finding that has implications for the health of the individual and their family. Furthermore, neither method negates the requirement for the validation of pathogenicity of a novel variant (**Section 1.4**).

1.4 Modelling of Monogenic Autoinflammatory Disorders

Modelling genetic findings experimentally is of great importance in determining the clinical significance of a novel variant. The modelling presented in this thesis has taken advantage of CRISPR/Cas9 gene editing techniques. CRISPR/Cas9 gene editing utilizes features of an adaptive immune response seen in bacteria and archaea.

Although the term was coined in 2002 by Jansen et al. (Jansen, Embden, Gaastra, & Schouls, 2002), a number of groups had described CRISPR arrays in bacteria from the late 1980s (Bult et al., 1996; Hermans et al., 1991; Ishino, Shinagawa, Makino, Amemura, & Nakata, 1987; Nakata, Amemura, & Makino, 1989; Nelson et al., 1999; Worning, Jensen, Nelson, Brunak, & Ussery, 2000), which were noted to be in close proximity to CRISPR-associated (Cas) genes (Jansen, Embden, et al., 2002; Jansen, van Embden, Gaastra, & Schouls, 2002). Subsequent work determined that the sequence in between CRISPR, the so-called spacer sequences, were of foreign or phage origin, introducing the idea that these may have a role in defense against invading pathogens (Bolotin, Quinquis, Sorokin, & Ehrlich, 2005; Mojica, Diez-Villasenor, Garcia-Martinez, & Soria, 2005; Pourcel, Salvignol, & Vergnaud, 2005). This concept that was promptly validated (Barrangou et al., 2007). The current understanding is that the incorporation of new spacer sequences into the host's DNA follows exposure to an invading phage (Marraffini & Sontheimer, 2010). When these spacer sequences are transcribed along with the repeats, they bind and activate the Cas protein. The complex acts as a guide that recognizes foreign DNA previously encountered, and the Cas protein initiates DNA cleavage.

CRISPR/Cas9 has now been adapted for applications involving mammalian cells (Jinek et al., 2012; Jinek et al., 2013; Sternberg, Redding, Jinek, Greene, & Doudna, 2014), with the Cas9 of *Streptococcus pyogenes* and simple guide RNAs optimised for this purpose (Heler et al.,

2015). Similar to its predecessors zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), CRISPR/Cas9 involves the generation of a double strand break (DSB) in genomic DNA, followed by repair through either non-homologous end-joining (NHEJ) or homology-directed repair (HDR). The specificity of the location of the DSB with CRISPR/Cas9 is determined by the design of the guide RNA, which is limited by the requirement of a canonical 5'-NGG-3' protospacer adjacent motif (PAM) in the complementary target DNA in order to be recognized (Anders, Niewoehner, Duerst, & Jinek, 2014). Without the PAM sequence, the guide/Cas9 complex dissociates and does not induce a DSB (Knight et al., 2015; Ma et al., 2016). With a short guide length and 5'-NGG-3' occuring in every 8 base pairs of the genome, there are usually multiple guide options for the deletion of a gene of interest. However, this also creates the possibility of targeting more than one location in the genome unintentionally. A number of off-target prediction algorithms exist and multiple groups have been working on methods to reduce the unwanted effects of this gene editing technique (Cho et al., 2014; Fu et al., 2013; Iyer et al., 2015; Kuscu, Arslan, Singh, Thorpe, & Adli, 2014; Pattanayak et al., 2013; Shen et al., 2014).

CRISPR/Cas9 techniques have improved the ability to create models of diseases caused by point mutations. Previously, creating point mutations in mice would require homologous recombination in embryonic stem cells, a lengthy and expensive process to generate a homozygous mouse strain (Hochheiser, Kueh, Gebhardt, & Herold, 2018). *In vivo* editing with CRISPR/Cas9 has allowed for genome editing of fertilized mouse eggs (Li et al., 2013; Shen et al., 2013). Briefly, plasmids with DNA encoding the editing tools, including the guide RNA and Cas9, are injected into the cytoplasm of a one-cell embryo, generating a target specific DSB. The subsequent repair of this break is mediated by NHEJ or HDR. The former often results in frame shift mutations and loss of function. HDR, on the other hand, can result in

substitutions, insertions or deletions if the one-cell embryo is co-injected with a single-strand oligonucleotide (ssOligo) that acts as a template. Soon after this technique was first published, the efficiency of gene disruption by frameshift mutations through NHEJ using this method was reported to be approximately 80-90% whereas introducing a point mutation through HDR is approximately 50-80% (Li et al., 2013; H. Yang, Wang, & Jaenisch, 2014).

Introduction of point mutations in human cells using CRISPR/Cas9 techniques has also been described. Early reports of CRISPR/Cas9 editing in HEK293T cells demonstrated NHEJ efficiency of up to 33% (Cho, Kim, Kim, & Kim, 2013; Cong et al., 2013; Mali et al., 2013; Pattanayak et al., 2013), but HDR efficiency of only 3-8% (Cong et al., 2013). Improved efficacy was noted through cell synchronisation techniques that control the timing of delivery of single guide RNA (sgRNA) and ssOligo to HEK293T cells, with HDR in up to 38% of cells (Lin, 2014). Various strategies have also been employed in an attempt to improve efficiency in cells that are difficult to nucleofect, a process by which components of the editing system are delivered to the nucleus of the target cell, including the use of a ssDNA template provided by recombinant adeno-associated virus (rAAV) (Kaulich & Dowdy, 2015; Vasileva & Jessberger, 2005). The process, however, remains less efficient than NHEJ and its widespread application in research is still limited.

A significant recent addition to the literature has been the description ‘base editors’, able to create point mutations in human cell lines without generating a DSB. In the initial descriptions, a catalytically dead Cas9 was fused to a cytidine deaminase enzyme, with the unit guided to a locus of interest with an sgRNA (Komor, Kim, Packer, Zuris, & Liu, 2016; Nishida et al., 2016). This complex allowed for a targeted C•G to T•A substitution in human and murine cell lines in up to 40% of total sequencing reads, with a maximum base editing yield possible of

50%. Subsequent base editors have included the adaptation of transfer RNA adenosine deaminase to edit DNA, allowing for A•T to G•C conversion (Gaudelli et al., 2017). The significance of this development in the potential for disease modelling and future gene editing was highlighted by the correction of the *c.G845A HFE* mutation implicated in hereditary haemochromatosis in an immortalised-lymphoblastoid cell line (Gaudelli et al., 2017). Despite the difficulty with which these cells are transfected, an efficiency rate of 28% was noted, with no off-target effects. Similar to the process of CRISPR/Cas9 with HDR, this technique is not yet used widely. However, significant advances in a short period of time suggests that either may become a routine method for modelling diseases caused by point mutations.

Given the reduced efficiency in creating point mutations in human cell lines when compared with gene deletion, the modelling presented in this thesis has relied primarily on the use of CRISPR/Cas9 gene editing techniques to delete the gene of interest in THP1 cells through NHEJ, followed by lentiviral transduction of cells with a gene harboring the mutation of interest (**Section 2.3.1**). THP1 cells are spontaneously immortalized-monocyte-like cells, which were initially derived from the peripheral blood of a child with acute myeloid leukaemia (Tsuchiya et al., 1980). Whilst intended as a model of primary human peripheral blood monocytes, THP1 cells have well recognized limitations. In addition to the inherent issues of a cell line, THP1 cells differ from primary cells in the response to stimulation. For example, peripheral blood monocytes produce greater inflammatory cytokines in response to LPS stimulation than THP1 cells (Schildberger, Rossmanith, Eichhorn, Strassl, & Weber, 2013). Furthermore, the use of a single cell line limits the capacity to investigate tissue specific or cell specific effects of a variant.

In contrast, *in vivo* murine models have provided great insight into disease pathology given genetic homologies between humans and mice and the ability to create transgenic, knock-out and knock-in mice. For example, the initial murine model of CAPS published in 2009 by Hoffman and his team recapitulated the IL-1β-mediated inflammation (Brydges et al., 2009). Furthermore, a number of teams have used murine models to explore the skeletal consequences of CAPS (Bonar et al., 2012; Snouwaert et al., 2016). More recently, the generation of *Nlrp3* mutant mice on *Il1b/Il18*, *casp-1*/*casp-11* or *Tnf*-deficient backgrounds raised the possible role of TNF in CAPS disease pathology (McGeough et al., 2017). There are, however, shortcomings here too. The recent attempt to model LPS-responsive and beige-like anchor protein (LRBA) deficiency using *Lrba*$^{-/-}$ mice, which in humans causes a range of manifestations including autoimmunity, hypogammaglobulinaemia, organomegaly and chronic diarrhea (Alkhairy et al., 2016), failed to recapitulate the clinical or immunological phenotype (Burnett, Parish, Masle-Farquhar, Brink, & Goodnow, 2017; Gamez-Diaz et al., 2017). Furthermore, as will be highlighted in **Chapter 3**, differences between murine and human pyrin led to many years of work predicated on pyrin as anti-inflammatory, rather than an inflammasome forming protein. Modelling of disorders that act through the pyrin pathway, such as Pyogenic Arthritis, Pyoderma gangrenosum and Acne (PAPA) syndrome caused by mutations in *PSTPIP1* (**Chapter 3**) may thus be similarly problematic. Additionally, humanized mouse models, using immunodeficient mice engrafted with human hematopoietic cells, are useful in the study of hematopoiesis, but have been limited in the investigation of the innate immune system due to quantitative and functional deficiencies of a number of cells including monocytes and macrophages (Rongvaux et al., 2014).

An alternative method of modelling AIDs is the use of patient derived induced pluripotent stem cells (iPSCs). This method of reprogramming somatic cells to pluripotency (Takahashi &

Yamanaka, 2006) allows for indefinite propagation as well as differentiation to a variety of human cell types that would previously have been unobtainable (R. H. Anderson & Francis, 2018; Avior, Sagi, & Benvenisty, 2016; Grskovic, Javaherian, Strulovici, & Daley, 2011). Saito and colleagues utilized this method in the investigation of AIDs involving NLRP3 (T. Tanaka et al., 2012) and NLRC4 (Kawasaki et al., 2017). Two patients with somatic mutations in *NLRP3* had both WT and mutant NLRP3 iPSC lines generated (T. Tanaka et al., 2012). The WT iPSC lines served as a comparator, with the authors able to determine that only macrophages differentiated from mutant NLRP3 iPSC lines showed abnormal IL-1β secretion. A subsequent publication generated iPSC lines from a patient suspected of CAPS but without pathogenic mutations in *NLRP3* (Kawasaki et al., 2017). Heterogeneous responses to LPS stimulation in the iPSC clones prompted WES, with clones having a robust response to LPS possessing a mutation in *NLRC4*. Subsequent deletion of *NLRC4* using CRISPR/Cas9 techniques in the mutant clones abrogated the enhanced response to stimulation, indicating that the mutation was likely pathogenic. As the frequency of the mutation was later determined to be approximately 63%, WES would most likely have identified this as a candidate variant of interest. Furthermore, despite its promise, the generation of a cell line from patient samples demands expertise, as well as specific ethical considerations. It also requires access to patient samples which may be difficult in the case of critically ill patients who succumb to disease prior to genetic evaluation.

Thus, the choice of modelling technique is complicated and represents a balance of benefits and costs. The availability of THP1 monocyte-like cells and the efficiency of CRISPR/Cas9 gene deletion through NHEJ has proved very useful. As will be seen in this thesis, this method has allowed for the validation of pathogenicity of two novel variants, as well as the opportunity to further explore the pathogenic mechanisms of AIDs.

1.5 Thesis Outline

Although significant progress has been made in the field of AIDs, sequencing techniques and *in vitro* modelling of disease-associated mutations, patients and families would benefit from a streamlined approach to genetic and immunological evaluation. This thesis sets out to demonstrate a method of *in vitro* CRISPR/Cas9 validation of the pathogenicity of novel variants that may be uncovered through WES of patients with suspected AIDs.

After a detailed description of the laboratory techniques used throughout the thesis in **Chapter 2**, the following two chapters will present cases of patients with evidence of inflammatory disease and novel variants in genes known to cause monogenic AIDs. **Chapter 3** focuses on a single family with a dominantly inherited suppurative dermatological condition caused by a previously unreported variant in *MEFV*. **Chapter 4** details the case of two unrelated children with macrophage activation syndrome (MAS) caused by a *de novo* variant in *NLRC4*. Each of these chapters has an accompanying manuscript. The final result chapter, **Chapter 5**, will document the establishment of a national registry for patients with AIDs, the Australian Autoinflammatory Diseases Registry (AADRY), and summarise the genetic evaluation of the first twenty index cases with suspected AIDs who have tested negative to currently available diagnostic techniques. An overall discussion proceeds in **Chapter 6**, where plans for future projects are explored.

2 METHODS

2.1 General reagents

Oligonucleotides were synthesised by Integrated DNA Technologies (Boronia, Australia). Restriction enzymes and buffers were purchased from New England Bioscience (Genesearch, Arundel, Australia) unless otherwise stated. Culture media, LG Agar, Super Optimal Broth (SOB), and Lysogeny Broth (LB) were prepared by the Walter and Eliza Hall Institute of medical research (WEHI) media department unless otherwise stated and were stored at 4°C in the dark.

2.2 Cell culture

Human THP1 (Tsuchiya et al., 1980) and HEK293T (Graham, Smiley, Russell, & Nairn, 1977) cell lines were grown at 37°C in a humidified atmosphere of air with 10% CO_2. THP1 cells were maintained in Human Tonicity Roswell Park Memorial Institute medium (HT RPMI, 1% (w/v) RPMI-1640, 0.2% (w/v) $NaHCO_3$, 0.011% (w/v) $C_3H_3NaO_3$, 0.1% (w/v) streptomycin, 100u/mL penicillin) supplemented with 10% (v/v) Foetal Bovine Serum (FBS, Sigma 12003C). HEK293T cells were maintained in Dulbecco's Modified Eagle's medium (DMEM, 1% (w/v) D-glucose, 0.11% (w/v) sodium pyruvate, 0.1% (w/v) streptomycin, 100u/mL penicillin). Trypsin- ethylenediaminetetraacetic acid (EDTA) solution (Sigma 59430C) was used for dissociation of adherent HEK293T cells.

2.3 Molecular techniques

2.3.1 Generation of knock-out cell lines

THP1 cells had a number of genes knocked out and were reconstituted with either WT constructs or had mutant genes overexpressed to model novel variants and known pathogenic mutations. CRISPR gene editing techniques were used to generate KO cell lines. The use of

CRISPR in the modelling of human diseases was discussed in **Chapter 1**, and outlined in **Figure 2.1** is the general process used throughout this thesis. THP1 cells stably expressing Cas9 with an mCherry reporter were generously supplied by Dr. Marco Herold (WEHI).

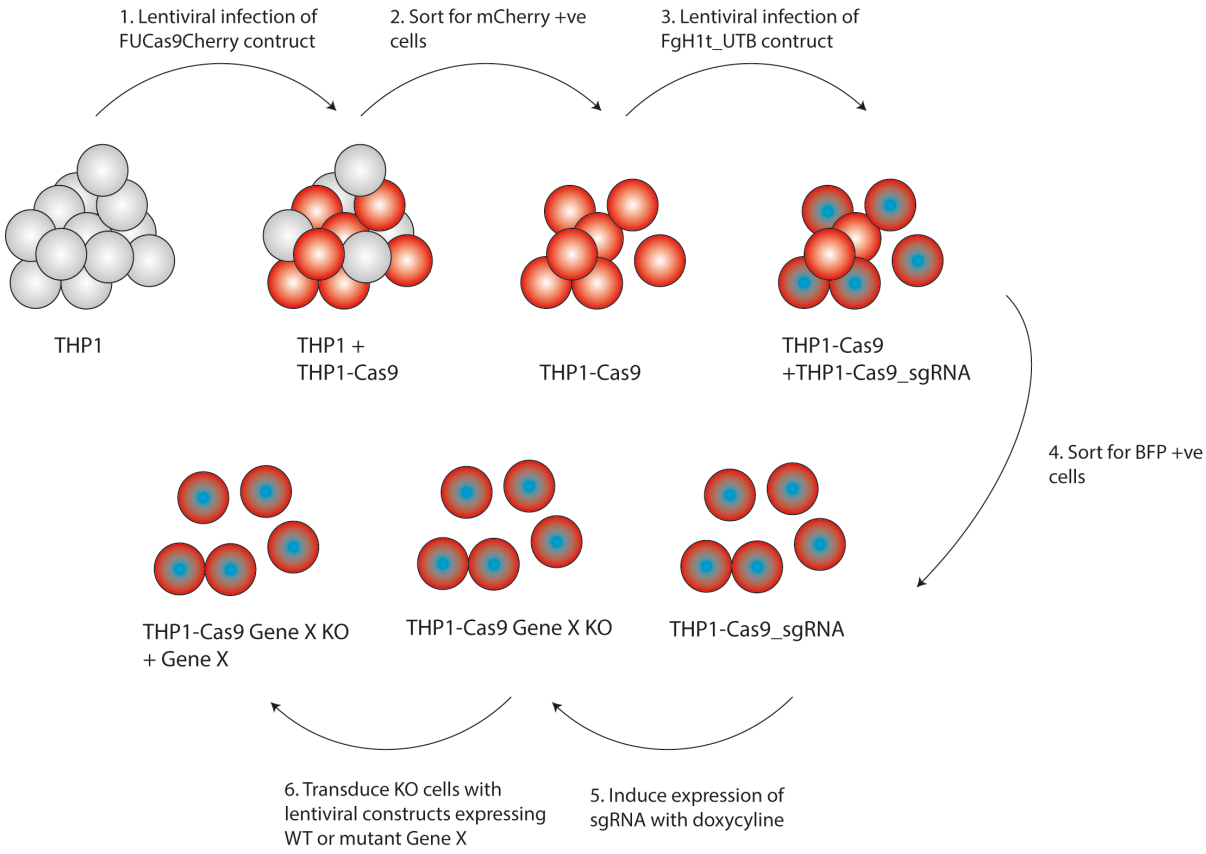


Figure 2-1 Schematic approach to the generation of THP1 cells expressing both wild type or mutant gene of interest.

Abbreviations: BFP blue fluorescent protein, KO knock-out, sgRNA single guide RNA, WT wild type.

2.3.1.1 Generation of FgH1t_UTB sgRNA construct

2.3.1.1.1 sgRNA sequence design

sgRNAs (**Table 2.6**) were designed against the human genome hg19 with the aid of an online CRISPR Design Tool (Massachusetts Institute of Technology, Massachusetts, http://crispr.mit.edu). High scoring guides were chosen to reduce the impact of off-target effects (P. D. Hsu et al., 2013).

2.3.1.1.2 Annealing of sgRNA oligonucleotides

5' and 3' oligonucleotides were annealed according to the following PCR thermocycling protocol:

Table 2-1 Oligonucleotide annealing polymerase chain reaction protocol

Reaction mix		PCR Protocol
100uM 5' oligonucleotide	3µL	95° 5mins
100uM 3' oligonucleotide	3µL	→grad (1°/sec)
3M NaCl	0.5µL	70° 20mins
1M MgCl2	2µL	→grad (1°/sec)
1M TRIS pH 7.5	2µL	40° 20mins
TE	9.5µL	→grad (1°/sec)
		25° 20mins
		→grad (1°/sec)
		4° infinity

2.3.1.1.3 Digestion of backbone

FgH1t_UTB (provided by Dr. Marco Herold, WEHI) was digested using the *Bsm*BI restriction enzyme in combination with NEBuffer 3.1 (B7203S) overnight at 55°C.

Table 2-2 Enzymatic digestion reaction

Digestion mix	Volume
FgH1t_UTB	2µL
NEB buffer 3.1	2µL
BSA (10x)	2µL
***Bsm*BI**	2µL
Nuclease free H_2O	10µL

2.3.1.1.4 Agarose gel electrophoresis and DNA fragment purification

Agarose gel was prepared (1% (w/v) agarose in Tris-acetate-EDTA buffer) and electrophoresis was used to visualize the digested fragment. Gel purification of fragment was performed using gel extraction kit (BioBasic B5654) according to manufacturer's instructions.

2.3.1.1.5 Ligation of sgRNA into digested backbone

The annealed sgRNA oligonucleotides were ligated into the digested FgH1t_UTB vector overnight at 4°C.

Table 2-3 Ligation reaction

Ligation mix	Volume
Digested vector	1µL
Annealed sgRNA oligonucleotide (1/1000)	3µL
	1µL
Ligase buffer (10x)	1µL
T4 ligase	1µL
Nuclease free H_2O	3µL

2.3.1.2 Generation of chemically competent bacterial cells

Chemically competent DH10β *E. coli* were prepared by culturing bacteria in LB (1% tryptone, 0.5% yeast extract, 1% NaCl, 2.5mM NaOH) in a shaking incubator set to 37°C and 230rpm until an optical density of 0.6 at 600nm was reached. Bacteria were pelleted by centrifugation at 4500rpm for 10 minutes and resuspended in 300mL of cold 0.1M $MgCl_2$. Bacteria were again pelleted and resuspended in 60mL of cold 0.1M $CaCl_2$. Bacteria were distributed into 1mL aliquots, incubated on ice for 1h and then pelleted by centrifugation at 13,000rpm for one minute. Each pellet was resuspended in 500uL of cold 15% (v/v) glycerol and 0.1M $CaCl_2$. Bacterial suspensions were divided into 100uL aliquots and stored at -80°C.

2.3.1.3 Transformation of bacterial cells

DNA constructs were introduced into chemically competent bacterial cells using heat shock transformation. Competent cells were thawed on ice. Then 5uL of each DNA construct was mixed with 50uL of competent cells in separate 1.5 mL tubes. The mixtures were incubated on ice for 15 mins and then placed in a dry heat block set to 42°C for 45 secs. The tubes were returned to ice for a further 2 minutes. 950µL of SOB (2% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5mM KCl, 5M NaOH to pH 7) supplemented with 0.4% (v/v) glucose was added to each tube. The tubes were incubated at 37°C for 60 minutes on a dry heat block. Transformed cells were centrifuged at 13,000 rpm for 1 minute after which 900uL supernatant removed from each tube. Each pellet was resuspended in remaining 100uL of supernatant. The bacterial suspensions were plated on individual $10cm^2$ petrie LG Agar plates (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 0.2% (w/v) D-Glucose, 10mM Tris Buffer, 1mM (v/v) MgCl2, 1.5% (w/v) agar, 100µg/ml Ampicillin) and incubated overnight at 37°C.

2.3.1.4 Small scale plasmid DNA preparation

Single bacterial colonies were inoculated in 10mL of LB supplemented with 100µg/ml Ampicillin (Roche *ROAMP*) and incubated in a shaker at 37°C overnight. DNA was extracted according to manufacturer instructions using either Wizard *Plus* miniprep DNA purification system (Promega A1460) or GET Plasmid DNA miniprep kit (Astral Scientific786-362).

2.3.1.5 Large scale plasmid DNA preparation

Single colonies were inoculated in 300mL of LB supplemented with 100µg/ml Ampicillin and incubated in a shaker at 37°C overnight. DNA was extracted according to manufacturer's instructions using GeneJET Plasmid Maxiprep kit (ThermoFisher K0492).

2.3.1.5.1 Sequencing

Sanger sequencing of plasmid DNA was performed through Australian Genome Research Facility (Melbourne). Approximately 1µg of plasmid DNA was combined with 10pmol of sequencing primer (**Table 2.6**) and the total volume made up to 12µL with nuclease free H_2O.

2.3.1.6 Lentiviral infection of THP1-Cas9 cell line

CRISPR sgRNA guide DNA was transfected into $3x10^6$ HEK293T cells seeded in a $10cm^2$ dish the previous day using Lipofectamine® 2000 (ThermoFisher 11668019) transfection reagent and Opti-MEM™ (ThermoFisher 31985070) as per manufacturer's guidelines. Third generation lentiviral packaging constructs were used in the following ratio:

Table 2-4 Lentiviral cDNA construct ratios.

Construct	Amount
FgH1t_UTB (sgRNA vector)	10µg
pMDL (lenti packaging vector)	5µg
RSV-REV (lenti packaging vector)	2.5µg
VSVg (lenti envelope vector)	3µg

After 24h, the media on the HEK293T cells was aspirated and replaced with fresh RPMI. Viral supernatant was collected 48h after the initial transfection and was filtered through a 0.45µm

filter. If not used immediately, viral supernatant was stored at -80°C. $1x10^6$ THP1-Cas9 cells were plated in 500µL and 3mL of viral supernatant was added per well of a 6-well plate. Polybrene (Sigma 107689) was added to a final concentration of 8µg/mL. The plate/s were centrifuged for 3h at 2200rpm at 32ºC and then incubated overnight at 37ºC. Media on the cells was changed after 24h and replaced with fresh RPMI.

2.3.1.7 Selection of cell population by flow cytometry

After 48h of rest, a flow cytometer with sorting capacity was used to select for cells with both FUCas9Cherry and FgH1t_UTB (expressing BFP) integration. Cells were sorted on an Aria W flow cytometer through a 100µm nozzle using the 405nm and 561nm lasers to excite the BFP and Cherry fluorophores, respectively. Cells were then pelleted, and resuspended in warm RPMI supplemented 20% FCS.

2.3.1.8 Doxycycline-induced expression of sgRNA construct

Expression of sgRNA constructs in cells was achieved with doxycycline (Alfa Aesar J60579) treatment at a concentration of 1µg/ml for 72h. Cells were allowed to rest for a further 72h in fresh RPMI media. WCL was obtained for western blot analysis of protein expression to confirm KO as described in **Section 2.9**.

2.3.2 Generation of plasmids and expression vectors

Lentiviral constructs were generated through PCR amplification of cDNA using the primers flanked with a 5' *Age*I restriction enzyme sequence (ACCGGT-) and 3' *Bam*HI restriction enzyme sequence (-GGATCC), allowing for ligation in to a digested pFUGW backbone (Sanjana, Shalem, & Zhang, 2014). cDNA was amplified by primers flanked with 5' and 3' *Mlu*I restriction sites (-ACGCGT) to allow for ligation in to a digested pEF_BOS_Flag

(provided by Dr. Dominic De Nardo, WEHI) construct. cDNA was amplified by primers flanked with 5' *Bam*HI (-GGATCC) and 3' *Not*I restriction sites (-GCGGCCGC) to allow for ligation in to a digested pEF_BOS_mCitrine (provided by Dr. Dominic De Nardo, WEHI) construct. Primer sequences are listed in **Table 2.6**.

2.3.2.1 PCR amplification

All reaction components were thawed on ice and mixed prior to use. The appropriate volumes of the following were combined to make a final concentration of: 1x Phusion® HF Buffer (New England BioLabs B0518S), 200µM deoxynucleotide solution mix (dNTP, New England Biolabs N0447S), 0.5µM forward primer, 0.5µM reverse primer, 250ng template DNA, 1unit Phusion® DNA Polymerase (New England BioLabs M0530S). The protocol for template amplification was adjusted depending on the melting temperature of the primers used and the length of the amplicon. The general protocol is shown below. If initial attempts were unsuccessful, a temperature gradient was performed to determine the optimal annealing temperature.

Table 2-5 Phusion® PCR thermocycler protocol

Step	Temp	Time
Initial Denaturation	98°C	30 seconds
30 Cycles	98°C	5 seconds
	Tm°C	15 seconds
	72°C	30 seconds per kb
Final Extension	72°C	5-10 minutes

Agarose gel electrophoresis and DNA extraction was performed on fragments of the correct size as previously described (**Section 2.3.1.1.4**).

2.3.2.2 Restriction enzyme digestion

pFUGW backbone and corresponding insert DNA was digested using *Age*I-HF (R3552) and *Bam*HI-HF (R3136) restriction enzymes with CutSmart® Buffer (B7204S) for 2h at 37°C. pEF_BOS_FLAG backbone and corresponding insert DNA was digested using *Mlu*I-HF (R3198) restriction enzyme with CutSmart® Buffer for 2h at 37°C. pEF_BOS_mCitrine backbone and corresponding insert DNA was digested using *Bam*HI_HF with CutSmart® Buffer for 2h at 37°C followed by *Not*I (R0189) with NEBuffer 3.1 at 37°C for a further 2h. The digestion mix used in each reaction was: 5µL buffer, 5µl of 10x BSA, 0.5µL of each restriction enzyme, 1µL DNA and nuclease free H_2O to a final volume of 50µL. Agarose gel electrophoresis and DNA extraction was performed on fragments of the correct size as previously described (**Section 2.3.1.1.4**).

2.3.2.3 Ligation of cDNA into a vector backbone

The T4 DNA Ligase Buffer (B0202S) was thawed at room temperature. The ligation reaction was set up on ice. A ratio of 1:5 of vector to insert was used for ligations. The amount of vector and insert for each reaction was calculated using the online NEBioCalculator™, according to the equation: required mass insert (g) = desired insert/vector molar ratio x mass of vector (g) x ratio of insert to vector length. The vector and insert DNA was added to 2µL of T4 DNA Ligase Buffer (10x), 1µL T4 DNA Ligase (M0202) and nuclease free H_2O to a total volume of 20µL. The reaction was mixed by pipetting, incubated at room temperature for 10 minutes and heat inactivated at 65°C for 10 minutes. Chemi-competent cells were transformed, single colonies selected for small scale DNA production and sequencing undertaken as described (**Sections 2.3.1.3** and **2.3.1.4**).

2.3.3 Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuickChange Lightning Kit (Agilent Technologies 210519-5) according to manufacturer's instructions. Mutations were introduced to constructs using the oligonucleotides listed in **Table 2.6**.

2.3.4 Transduction of knock-out THP1 cell lines

THP1-Cas9 cells with the gene of interest knocked out using CRISPR/Cas9 techniques were reconstituted using lentiviral vector transduction. The generation of lentiviral vector containing cDNA of interest is described elsewhere (**Section 2.3.2**). Virus was generated using a similar method to that used in **Section 2.3.1.6**, with third generation lentiviral packaging vectors used in the same ratios and the pFUGW substituted for the FgH1t_UTB. Virus was harvested and stored at -80°C until needed.

For generation of stable cell lines expressing WT or non-pathogenic variants, THP1-cas9 cells were plated at $1x10^6$ cells per well of a 6-well plate with 3.5 mL of virus cells and 8μg/mL polybrene. Cells underwent centrifugation for 3h at 2200rpm at 32ºC and were then incubated overnight at 37ºC. After 24h, cells were washed with sterile phosphate buffered saline (PBS) and resuspended in fresh RPMI. After a further 24h of rest, cells with successful integration of vector were selected with blasticidin (ThermoFisher A1113903) at 6μg/mL for 7 days. Protein expression was determined through generation of WCL and western blotting as described below (**Section 2.9**).

Attempts were made to generate stable THP1 cell lines expressing known or potential pathogenic mutations in *MEFV* and *NLRC4*. The above method was used and blasticidin selection undertaken. Marked cell death was noted. Optimisation was undertaken with titration

of volume of virus used and subtraction of polybrene to reduce infection efficiency. Despite this, stable cell lines were not able to be generated. As a result, lentiviral transduction was undertaken prior to each experiment. For pyrin experiments (**Chapter 3**), $6x10^6$ THP1 cells were infected per condition, $1x10^6$ cells seeded per well in a 6-well plate with 3.5mL of virus and 8μg/mL polybrene. Centrifugation was undertaken for 3h at 2200rpm at 32ºC. Cells were incubated overnight at 37ºC. The following day, cells were washed in PBS and reseeded in fresh RPMI. After a further 24h, live and dead cells were separated using Ficoll density gradient centrifugation (GE Healthcare17-1440-03). Live cells were seeded for experiments. For NLRC4 experiments (**Chapter 4**), $2x10^6$ THP1 cells were infected per condition, again with $1x10^6$ cells per well in a 6-well plate. Only 1mL of viral supernatant was added to each well, supplemented with 2.5mL RPMI to make a total of 3.5mL per well, and 8μg/mL polybrene. The remainder of the transduction method was as per pyrin experiments. WCL was generated at the time of the experiment to determine protein expression by western blotting (**Section 2.9**).

2.4 In vitro analysis of cytokines and cell death

Cytokine release and cell death were analysed on THP1 cells transiently or stably expressing variants of interest. For experiments in **Chapter 3**, THP1 cells were plated at a density of $5x10^5$ cells per 100μL per well in a 96 well flat bottom plate (ThermoScientific 442400) in RPMI. After 24h, the plate was centrifuged at 400g for 5 minutes. A total of 75μL of supernatant was harvested from each well for cytokine assessment using ELISA (**Section 2.4.2**) and the remaining 25μL with cells used for cell death analysis (**Section 2.4.3**).

THP1 cell experiments in **Chapter 4** were conducted with or without priming. For priming, THP1 cells were plated at a density of $5x10^5$ cells per 80μL RPMI per well in a 96 well flat bottom plate and 20μL of synthetic triacylated lipopeptide (Pam3CSK4, InvivoGen tlrl-pms)

added to make a final concentration of 1ng/mL. Stimulation of THP1 cells with type three secretion system (T3SS) protein PrgI were conducted using retroviral infection of cells with pMXsIG_PrgI_GFP (A/Prof. Edward Miao, UNC School of Medicine, North Carolina) 3h after priming. A titration of virus was added to cells as detailed in the relevant figure legends. Retrovirus was produced prior to experiment as detailed below (**Section 2.4.1**) and stored at -80°C in 1mL aliquots if not used immediately.

2.4.1 Retrovirus production

Similar to generation of lentivirus detailed in **Section 2.3.1.6**, the T3SS PrgI retroviral construct was transfected into $3x10^6$ HEK293T cells seeded in a $10cm^2$ dish the previous day, again using both Lipofectamine® 2000 transfection reagent and Opti-MEM™ as per manufacturer's guidelines. Retroviral packaging constructs were used in the ratios outlined below. The remainder of the process is as per **Section 2.3.1.6**.

Table 2-6 Retroviral cDNA construct ratio

Construct	Amount
pMXsIG_PrgI_GFP	10µg
Gag-pol	5µg
VSVg	500ng

2.4.2 Enzyme linked immunosorbent assay

Supernatants were assessed for cytokine presence by ELISA for IL-1β and IL-18 using DY201 and DY008 kits, respectively (R&D Systems). Manufacturers guidelines were followed with the exception of increasing the highest concentration of the standard curve to 10,000pg/mL.

2.4.3 Propidium iodide staining

After the supernatant from THP1 experiments was harvested for cytokine assessment , 25uL of supernatant and all cells remained in each well. A total of 125µL fluorescence-activated cell sorting (FACS) buffer (PBS with 1% FBS and 0.1mM EDTA) was added to each well, with propidium iodide (Sigma P4170) added to a final concentration of 1µg/ml. The final volume in each well was 150µL. Propidium iodide uptake was assessed using a flow cytometer with plate reading capacity. Settings of the plate reader were as follows: forward scatter (FSC) 198V, side scatter (SSC) 210V, YG610/20 330V, sample flow rate 3µL/sec, sample volume 50µL, mixing volume 100µL, mixing speed 180µL/sec, 2 mixes and wash volume 400µL. Gating strategy for assessment of live cells is shown in Figure 2.2.

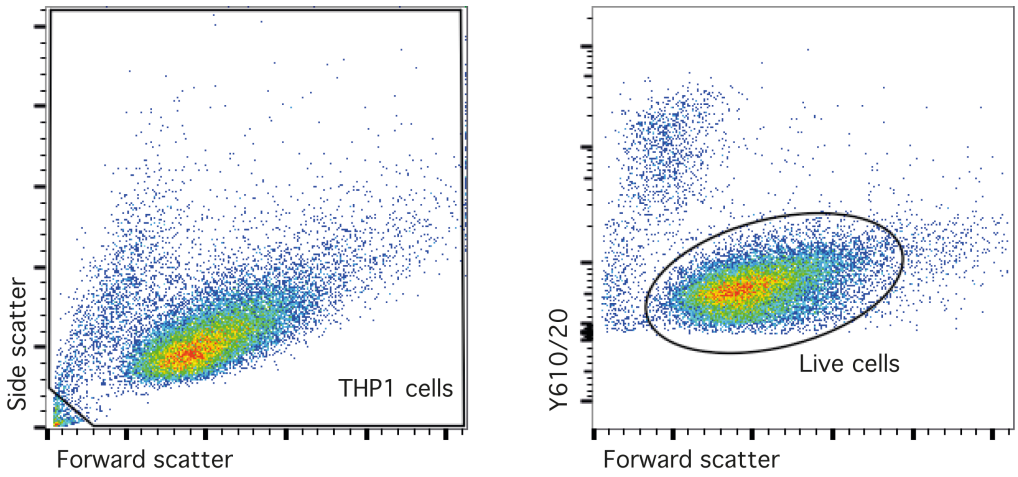


Figure 2-2 Gating strategy for assessment of cell viability

2.5 Immunofluorescence microscopy

Colocalisation of ASC and pyrin was documented using immunofluorescence microscopy. Ibidi 8 well chamber slides (ibidi GmbH) were coated with 4.6ug/cm^2 RetroNectin® (Takara Bio T100B) for 2h at room temperature. RetroNectin® was then aspirated and wells blocked in 2% bovine serum albumin (BSA) in PBS for 30min at room temperature. 1x10^5 HEK293T

cells were then seeded in each well. The following morning, cells were transfected with mCherry-MEFV and GFP-ASC constructs. After 24h, cells were visualized with a Zeiss LSM 780 Confocal microscope using the 40x oil objective. Images were viewed and processed using FIJI software (NIH).

2.6 Quantification of ASC speck formation by flow cytometry

Flow cytometry for quantification of ASC speck formation, also known as Time of Flight Inflammasome Evaluation (TOFIE), was used as a surrogate marker of inflammasome activation (Sester et al., 2015). The redistribution of ASC from a diffuse cytosolic protein to a single speck upon inflammasome formation, when tagged with a fluorescence protein, can be measured by flow cytometry using area versus width. For TOFIE experiments in **Chapter 3** HEK293T cells were transfected with 5ng of GFP-ASC and 25ng mCherry-MEFV constructs. Cells were harvested 16 h post transfection with trypsin and ASC speck formation was quantified using the gating strategy shown below (**Figure 2.3**).

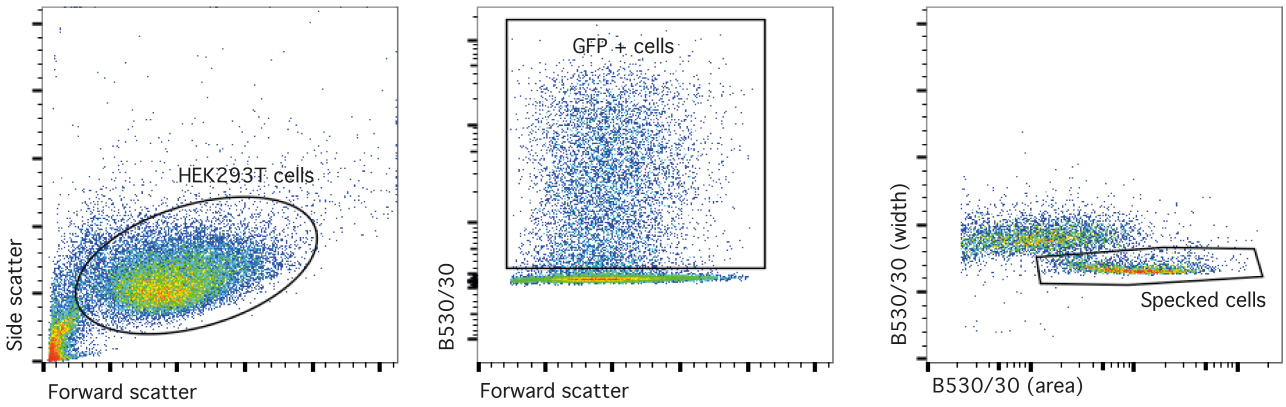


Figure 2-3 Gating strategy for ASC speck assessment.

For TOFIE experiments in **Chapter 4**, HEK293T cells were transfected with 5ng GFP-ASC and 10ng pCR3_NLRC4_VSV and ASC speck formation quantified at the same time point as

above. The expression of NLRC4 was determined via western blot (**Section 2.9**). TOFIE experiments looking at response to T3SS were conducted in HEK293T cells stably expressing ASC-RFP (provided by Dr. De Nardo, WEHI). These cells were transfected with 10ng pCR3_NLRC4_VSV along with variable amounts of pMXsIG_PrgI_GFP and pCS2_hNAIP_myc (provided by Dr. Feng Shao, National Institute of Biological Sciences, Beijing).

2.7 Immunoprecipitation

2.7.1 GST Immunoprecipitation

The interaction between pyrin and 14-3-3 proteins, as well as pyrin and PSTPIP1, was achieved through immunoprecipitation of GST-tagged pyrin using Glutathione Sepharose 4B beads (GE Healthcare 17-0756-05). $3x10^6$ HEK293T cells were seeded in $10cm^2$ plates overnight and transfected using Lipofectamine® 2000 with 5µg of WT or mutant pcDNA3.1_MEFV_GST with or without 2.5µg peBOS_PSTPIP1_flag. The assessment of 14-3-3 binding to pyrin in setting of stimulation with *Clostridium difficile* toxin B (TcdB), the cells were treated with 5µg/ml TcdB (Abcam ab124001) 16h before harvesting. After 48h, media was aspirated from plates and cells washed with cold PBS on ice. Cells were lysed in 700µL 1% NP40 lysis buffer (1% NP40 (Sigma-Aldrich, CA-630), 10% Glycerol, 20nM Tris-HCl, 150mM NaCl, 1mM EGTA, 10mM NaPPi, 5mM NaF, 1mM Na_3VO_4, 1mM PMSF) supplemented with protease inhibitors (Roche, cOmplete 1353B20). Cells were scraped, collected in Eppendorf tubes, and kept on ice for 30 minutes for lysis. WCL was clarified by centrifugation for 15 minutes at 15,000 rpm at 4°C. During this time, 40µL per condition of Glutathione Agarose 50% bead slurry was washed twice in lysis buffer and resuspended in original volume of lysis buffer. WCL control samples were prepared by taking 60µL of lysate and adding 20µL of 4x sample buffer. This was boiled at 95°C for 10 minutes and stored at -20°C until western blot. Samples

for immunoprecipitation were prepared on ice by transferring 600µL of lysate to new Eppendorf tubes and adding 40µL washed Glutathione Agarose beads per sample. Samples were incubated at 4°C on a rotator for 2h. Beads were washed using pulse spins of 13,000 rpm at 4°C and removal of lysis buffer. 1mL of fresh lysis buffer was added and beads resuspended and mixed by inversion. This wash step was repeated three times, and after the final pulse, the lysate was removed by aspiration with a gel loading tip. The beads were resuspended in 40µL of 2x sample buffer and sample heated at 95°C for 10 minutes. The tubes were spun down and stored at -20°C for western blot.

2.7.2 GFP Immunoprecipitation

Tissue culture experiments were again performed in HEK293T cells seeded at the same density as above. Cells were transfected with 5µg pEF_BOS_NLRC4_mCitrine, with or without 5µg pCR3_NLRC4_VSV. After 48 h, cells were washed then lysed with 1% NP40, and WCL controls and samples for immunoprecipitation were prepared as described above. As the mCitrine tag on pEF_BOS_NLRC4_mCitrine is recognized by antibodies targeting GFP, 2µg of Anti-GFP antibody (Invitrogen A11122) was added to each immunoprecipitation sample and then incubated at 4°C on a rotator for 2 h. 40µL per sample of Protein A coupled agarose beads (Roche Protaa-ro) was washed twice in lysis buffer and added to the immunoprecipitation sample. Samples were incubated at 4°C on a rotator for a further 2 h. Beads were subsequently washed as described above and resuspended in 40µL 2x sample buffer. Samples were heated at 70°C for 10 minutes, then spun down and stored at -20°C until western blot.

2.8 **Western blot assay**

2.8.1 Preparation of whole cell lysate for western blot assay

WCL not prepared for the immunoprecipitation (described above) were generated as follows: $5x10^5$ cells were washed 1x in cold PBS and pelleted by centrifugation. Cell pellets were resuspended in 150µL RadioImmunoprecipitation Assay (RIPA) buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 0.1% SDS and 0.5% deoxycholate) supplemented with protease inhibitors (Roche Biochemicals 11697498001). Lysates were incubated on ice for 30 minutes and centrifuged at 13,000 rpm for 20 minutes for pelleting of debris. Clarified lysate was combined with SDS-PAGE sample buffer (50mM Tris pH 6.8, 2% (v/v) SDS, 10% (v/v) glycerol, 0.01% (v/v) bromophenol blue, 0.05% (v/v) 2-mercaptoethanol) and were then boiled at 90-100°C for 5 minutes prior to immunoblotting.

2.8.2 SDS-PAGE

Proteins were resolved on 4-12% SDS-PAGE (Novex™) gels in MES running buffer (NuPAGE NP0002-02), followed by transfer on to nitrocellulose membranes. Membranes were blocked with either 5% skim milk+0.1%Tween/PBS (PBST) or 3% BSA+0.1%Tween/tris-buffered saline (TBST) at room temperature for 1h and then probed overnight at 4°C with primary antibodies (**Table 2.7**). Membranes were subsequently washed in either PBST or TBST at least 3 times before the incubation with appropriate Horseradish Peroxidase (HRP) conjugated antibodies (**Table 2.7**) for 1h at room temperature. The secondary antibodies were diluted in the same solution as primary antibody of interest. Membranes were washed three times before being visualized using enhanced chemiluminescence (ECL) reagent (Millipore WBKLS0500).

2.9 **Human sample processing**

2.9.1 DNA extraction from human whole blood samples

Whole blood was collected from participants in EDTA tubes by an accredited pathology service with same day transport at room temperature. DNA was extracted using NucleoBond CB20 (Macherey Nagel 740507) as per manufacturer's guidelines and stored at -20°C until required.

2.9.2 DNA extraction from human saliva

DNA was obtained from saliva using Oragene DNA collection kits for adults (DNA Genotek OG500) or children (DNA Genotek OG575). DNA was extracted using prepIT extraction kit (DNA Genotek PDLB00206) following manufacturer's instructions.

2.9.3 DNA quantification

Genomic DNA (gDNA) was quantified using Agilent 2200 TapeStation (Agilent) as per manufacturer's instructions to ensure that an amount of at least 1µg and concentration of at least 20ng/µl was available for WES.

2.10 **Whole exome sequencing**

WES was performed by Macrogen using the Hiseq 4000 platform and Illumina Sureselect XT V5 library. The curation process is detailed in **Chapter 5**.

2.11 **Statistical analysis**

Unless otherwise specified, statistical analysis of data was performed using unpaired two-tailed *t* tests with Prism software (GraphPad). Data throughout the thesis is typically presented as mean +/- SEM *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Table 2-7 Primer sequences

Primer	Sequence (5' → 3')
Amplification	
MEFV_Lenti_blast_F	CACAGGACCGGTTCTAGAGCGCTGCCACCATGGCTAAGACCCCTAGTGAC
MEFV_Lenti_blast_R	TGCGCCGGATCCGTCAGGCCCCTGACCAC
NLRC4_Lenti_blast _F	GCGGATCCAGCAGTTACTAGTTTAAAAGC
NLRC4_Lenti_blast_R	CACAGGACCGGTTCTAGAGCGCTGCCACCATGAATTTCATAAAGGACAATAG
SHARPIN_F	CCTGAACCCACATTCTGGCA
SHARPIN_R	TAGGTGGAAGCTGCAGCAAG
CRISPR sgRNA	
NLRC4 Exon2_1	TCCCTCAGCTGCTCCACGCGGTGA
NLRC4 Exon2_1	AAACTCACCGCGTGGAGCAGCTGA
NLRC4 Exon2_2	TCCCAGAAGGAGACTTGGACGATT
NLRC4 Exon2_2	AAACAATCGTCCAAGTCTCCTTCT
Sequencing	
FgH1t_UTB	CAGACATACAAACTAAAGAAT
MEFV Forward	GCTGTATCATTGTTCTGGGC
MEFV Reverse	GCTGTGTTCTTCCCTCC
NLRC4 Forward	CAAAGTGTGAAAAACACCACTGAGC
NLRC4 Forward	GTGTTTTTTGACTTTAGTAC
NLRC4 Forward	AGGACTTGAATGGACAAAGTC
Lenti_Blast_Fwd	GTCTTGAAAGGAGTGGGAATTGG
Lenti_Blast_Rev	CATTGACACCAGTGAAGATGCG
SHARPIN_Rev	AGGAAGGACAGGACCAGCTG
Mutagenesis	
MEFV p. E244K	TAGAAATGGTGACCTTAAGGCTTCTAGGTCGCATC
MEFV p. E244K	GATGCGACCTAGAAGCCTTAAGGTCACCATTTCTA
MEFV p.M694V	GGTACTCATTTTCCTTCACCATTATCACCACCCAGTAG
MEFV p.M694V	CTACTGGGTGGTGATAATGGTGAAGGAAAATGAGTACC
MEFV p. S242R	GAAATGGTGACCTCAAGCCTTCTAGGTCGCATCTT
MEFV p. S242R	AAGATGCGACCTAGAAGGCTTGAGGTCACCATTTC
MEFV p. E244P	GATGCGACCTAGAAGCCTTCCGGTCACCATTTCTACAG
MEFV p. E244P	CTGTAGAAATGGTGACCGGAAGGCTTCTAGGTCGCATC
MEFV p. E244D	CGACCTAGAAGCCTTGATGTCACCATTTCTACAGG
MEFV p. E244D	CCTGTAGAAATGGTGACATCAAGGCTTCTAGGTCG
MEFV p. E244R	AGATGCGACCTAGAAGCCTTAGGGTCACCATTTCTACAGG
MEFV p. E244R	CCTGTAGAAATGGTGACCCTAAGGCTTCTAGGTCGCATCT
MEFV p. R39R	GGAGCACTCCAGAATCCCCCGGAGC
MEFV p. R39R	GCTCCGGGGGATTCTGGAGTGCTCC
MEFV p. I666I	CAGGCTCCCAGTATCCATGCTGTCTTGTCTCC
MEFV p. I666I	GGAGACAAGACAGCATGGATACTGGGAGCCTG
NLRC4 p.W655C	TATCTTTGTTCTTCAACTGCAAGCAGGAATTCAGGACTC
NLRC4 p.W655C	GAGTCCTGAATTCCTGCTTGCAGTTGAAGAACAAAGATA
NLRC4 p.T177A	TCGCTGCAGCAGAGCGGACTTGCCTTTGC
NLRC4 p.T177A	GCAAAGGCAAGTCCGCTCTGCTGCAGCGA
NLRC4 p.S171F	TTGCCTTTGCCAAATTCCCCTTCAATGATGCAGGGG
NLRC4 p.S171F	CCCCTGCATCATTGAAGGGGAATTTGGCAAAGGCAA
NLRC4 p.W655A	GAGTCCTGAATTCCTGCTTCGCGTTGAAGAACAAAGATACAG
NLRC4 p.W655A	CTGTATCTTTGTTCTTCAACGCGAAGCAGGAATTCAGGACTC
NLRC4 p.W655S	AGTCCTGAATTCCTGCTTCGAGTTGAAGAACAAAGATAC
NLRC4 p.W655S	GTATCTTTGTTCTTCAACTCGAAGCAGGAATTCAGGACT
NLRC4 p.S533A	TTTTCACACTTTGCAAAGCTTCCTGTCTCCAGAGAGG
NLRC4 p.S533A	CCTCTCTGGAGACAGGAAGCTTTGCAAAGTGTGAAAA

Mutagenesis	
NLRC4 p.E600A	ACTTGCACAATTGGGCAAATGTGCAAAGAAGTCAAATAAGTAATCG
NLRC4 p.E600A	CGATTACTTATTTGACTTCTTTGCACATTTGCCCAATTGTGCAAGT
NLRC4 p.E600G	ACTTGCACAATTGGGCAAATGTCCAAAGAAGTCAAATAAGTAATCG
NLRC4 p.E600G	CGATTACTTATTTGACTTCTTTGGACATTTGCCCAATTGTGCAAGT
NLRC4 p.C605A	TGACTTCTTTGAACATTTGCCCAATGCTGCAAGTGCCCTG
NLRC4 p.C605A	TGACTTCTTTGAACATTTGCCCAATGCTGCAAGTGCCCTG
NLRC4 p.C605S	TTTGAACATTTGCCCAATAGTGCAAGTGCCCTGGA
NLRC4 p.C605S	TCCAGGGCACTTGCACTATTGGGCAAATGTTCAAA
NLRC4 LRR1	CAGTTACTAGTTTAAAAGCACCTGTAGCAACACTGGCAGCAGCATCATCAAATTGCCACCCAACAAGCC
NLRC4 LRR1	GGCTTGTTGGGTGGCAATTTGATGATGCTGCTGCCAGTGTTGCTACAGGTGCTTTTAAACTAGTAACTG
NLRC4 LRR2	CTTCTTGCAGAAAAGTTAACTTGGATAACACTGCGGCAAGTTTTGCGACTAATGCTGGATCAGGTAGAAATTCTTTAG
NLRC4 LRR2	CTAAAGAATTTCTACCTGATCCAGCATTAGTCGCAAAACTTGCCGCAGTGTTATCCAAGTTAACTTTTCTGCAAGAAG
NLRC4 p.V341A	TTGCACAAGTGATGACCGCAAAGAGAGGGGTCTTC
NLRC4 p.V341A	GAAGACCCCTCTCTTTGCGGTCATCACTTGTGCAA
NLRC4 p.T337S	TGAGGAATCTCATGAAGTCCCCTCTCTTTGTGGTC
NLRC4 p.T337S	GACCACAAAGAGAGGGGACTTCATGAGATTCCTCA
NLRC4 p.Q657A	TTTGTTCTTCAACTGGAAGGCGGAATTCAGGACTCTGGAG
NLRC4 p.Q657A	CTCCAGAGTCCTGAATTCCGCCTTCCAGTTGAAGAACAAA

Table 2-8 Antibody details

Antigen	Clone name	Species	Conjugate	Product code	Source	Dilution
Primary antibodies						
14-3-3τ		Mouse IgG_1	Nil	sc-59414	Santa Cruz	1:500
14-3-3ε		Rabbit IgG	Nil	orb6357	Biorbyt	1:1000
Actin		Goat IgG	Nil	sc-1616	Santa Cruz	1:5000
ASC		Rabbit IgG	Nil	sc-22514	Santa Cruz	1:500 WB, IF
Caspase-8	1C12	Mouse IgG_1	Nil	9746	Cell Signaling Technology	1:500
GFP		Rabbit IgG	Nil	A11122	Life Techonology	1:500
GST		Goat IgG	Nil		WEHI	1:1000
IL-1β		Goat IgG	Nil	AF-401	R&D	1:1000
NLRC4	D5Y8E	Rabbit IgG	Nil	12421	Cell Signaling Technology	1:500
p10 Caspase-1		Rabbit IgG	Nil	sc-514	Santa Cruz	1:200
p20 Caspase-1		Mouse IgG	Nil		Adipogen	1:500
Pan-14-3-3		Rabbit IgG	Nil	sc-629	Santa Cruz	1:500
pSer 14-3-3 binding motif		Rabbit IgG	Nil	9601	Cell Signaling Technology	1:500
PSTPIP1	1D5	Mouse IgG2b kappa	Nil	H00009051-M02	Abnova	1:500
Pyrin		Rabbit IgG	Nil	AL196	AdipoGen	1:500 WB 1:100 IF
Secondary antibodies						
Goat IgG		Donkey	HRP		Santa Cruz	1:10000
Mouse IgG		Goat	HRP	NA931	Amersham	1:10000
Rabbit IgG		Goat	Alexa Fluor647		Invitrogen	1:1000
Rabbit IgG		Donkey	HRP	NA934	Amersham	1:10000

3 A NOVEL PYRIN-ASSOCIATED AUTOINFLAMMATION WITH NEUTROPHILIC DERMATOSIS MUTATION FURTHER DEFINES 14-3-3 BINDING OF PYRIN AND DISTINCTION TO FAMILIAL MEDITERRANEAN FEVER

3.1 Introduction

Pyrin-Associated Autoinflammation with Neutrophilic Dermatosis (PAAND) is a recently described monogenic AID that is clinically distinct from FMF and is caused by a mutation in the 14-3-3 binding motif of pyrin. This chapter explores a previously undescribed p.Glu244Lys pyrin mutation at the +2 position of a 14-3-3 binding domain of pyrin in a family initially suspected of suffering from PAPA syndrome subsequently diagnosed with PAAND. *In vitro* validation of pathogenicity as well as exploration of the mechanism of increased pyrin activity, seen with p.Glu244Lys pyrin compared with WT is detailed in the published manuscript presented in **Section 3.2** (Moghaddas et al., 2017).

Provided in this section is a detailed review of the current understanding of the role of pyrin in response to pathogens and the mechanisms of pyrin activation. Following this is a description of the pathophysiology of pyrin-associated diseases as a number will be considered in the manuscript and discussion section (**Sections 3.2** and **3.3**) and selected background information is necessary.

3.1.1 Pyrin

The gene encoding pyrin, *MEFV,* was independently characterised by two teams in the pursuit of identifying the cause of FMF, the most common monogenic AID. The International FMF Consortium used positional cloning to identify a candidate locus on chromosome 16p and subsequently the novel gene, with 10 exons encoding a 781-amino acid protein, they termed

pyrin (T. I. F. Consortium., 1997). The French FMF Consortium also identified this gene, having previously determined a candidate 250-kb region by linkage disequilibrium and haplotype analysis (T. F. F. Consortium, 1996), and named the gene marenostrin (F. F. Consortium., 1997). Since this time the gene/protein nomenclature of *MEFV*/pyrin has been adopted.

Pyrin, also known as TRIM20, is a member of the tripartite motif (TRIM) family of proteins (Weinert, Grutter, Roschitzki-Voser, Mittl, & Grutter, 2009) (**Figure 3.1**). The TRIM proteins are generally characterised by an N-terminal zinc finger RING domain, one or two B-box domains, a coiled-coiled domain and may have one of several distinct C-terminal domains (Reymond et al., 2001). Pyrin differs from this archetype, harbouring an N-terminal pyrin domain (PYD) (T. I. F. Consortium., 1997) that lacks the ubiquitin E3 ligase activity of a RING domain (Deshaies & Joazeiro, 2009), but shares a C-terminal B30.2 domain present in approximately 50% of all TRIM proteins (Reymond et al., 2001). The PYD domain is a feature of a number of regulators of apoptosis and inflammation (Chu, Gangopadhyay, Dorfleutner, & Stehlik, 2015; Fairbrother et al., 2001). Specifically, in this case it mediates the association between pyrin and ASC (Chae et al., 2003), explored further in **Section 3.1.2**.

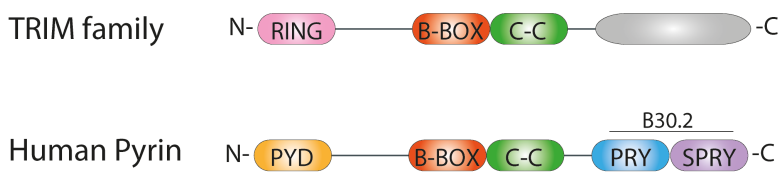


Figure 3-1 Schematic structure of TRIM protein pyrin.

Pyrin shares a B-Box domain and coil-coiled (C-C) domain common to tripartite motif (TRIM) family proteins but contains an N-terminal pyrin domain (PYD) rather than the usual RING domain with ubiquitin E3 ligase activity.

Through northern blot analysis, pyrin RNA was determined to be highly expressed in peripheral blood but not significantly in a number of other tissues tested including the spleen and thymus (T. I. F. Consortium., 1997). Further analysis with reverse transcription PCR revealed high expression in granulocytes, activated monocytes and synovial fibroblasts (Centola et al., 2000; Matzner et al., 2000; Tidow et al., 2000). Work by Touitou and colleagues determined five splice variants, with a number being regulated by nonsense mediated decay in a cell-type specific manner (Grandemange, Soler, & Touitou, 2009).

Originally hypothesized to be a transcription or nuclear DNA binding factor due to the presence of nuclear localization signals (NLSs), the cellular localization of pyrin has been explored. Using an overexpression model with a GFP-tagged pyrin expression construct, COS-1 cells were transiently transfected and viewed with fluorescent microscopy (Tidow et al., 2000). Discrete perinuclear cytoplasmic distribution of pyrin was noted. Exclusive cytoplasmic localization in a COS-7 overexpression model was also seen, and a yeast two-hybrid assay using B30.2 domain as bait determined a possible Golgi interacting protein (X. Chen et al., 2000). Subsequent work has since determined that pyrin does not usually colocalise with the Golgi apparatus but may be perinuclear in specific cell types (Mansfield et al., 2001).

Two distinct transcripts were identified in the attempt to clone human *MEFV* from peripheral blood leukocytes, one full length (fl), and one isoform lacking the entire exon 2 (d2) (Papin et al., 2000). Stable expression of pyrin-fl-GFP in CHO cells resulted in diffuse cytoplasmic staining, whereas pyrin-d2-GFP localized predominantly in the nucleus, confirmed by western blot analysis of fractionated cellular extracts (Papin et al., 2000). Interestingly, this localization was not dependent on the NLSs in pyrin. Furthermore, the finding has not been independently

reproduced. Since this publication, subsequent work by the same group has shown that the addition of ASC changes the localization of both pyrin-fl-GFP and pyrin-d2-GFP to a discrete cytoplasmic speck (Cazeneuve, Papin, Jeru, Duquesnoy, & Amselem, 2004). Whilst progress has been made in elucidating the function of cytoplasmic pyrin, the role of nuclear pyrin-d2 remains to be determined. Given that FMF mutations did not appear to change the localization of pyrin (Cazeneuve et al., 2004), it is difficult to presume that this has a role in the mechanism of disease.

An astute observation that colchicine was an effective therapy for patients with FMF, and that colchicine disrupts microtubules, prompted consideration of a relationship between pyrin and the cytoskeleton (Mansfield et al., 2001). Colocalization of pyrin and β-tubulin was confirmed by immunofluorescence microscopy of cells transfected with GFP-tagged pyrin then treated with paclitaxel to stabilize microtubules. The implications of this relationship would take over a decade to recognize (**Section 3.1.3**).

3.1.2 Previous pyrin model

Much of the initial work on the role of pyrin in health and disease was undertaken in murine models, and, although difficult to foresee at the time, the differences between murine and human pyrin resulted in conclusions that were later determined to be inaccurate. As shown in **Figure 3.2**, murine pyrin lacks a B30.2 domain. The B30.2 domain contains an evolutionarily ancient SPRY domain plus an N-terminal PRY domain (Rhodes, de Bono, & Trowsdale, 2005), with loop regions important for binding interactions (Henry, Ribouchon, Offer, & Pontarotti, 1997; Masters et al., 2006; Woo et al., 2006). Although structure suggests its importance in protein-protein interactions, the binding partners to the B30.2 domains are often unknown, as

is the case with pyrin. Importantly, this domain is where the majority of FMF-associated mutations reside (**Figure 3.2, Section 3.1.4.1**).

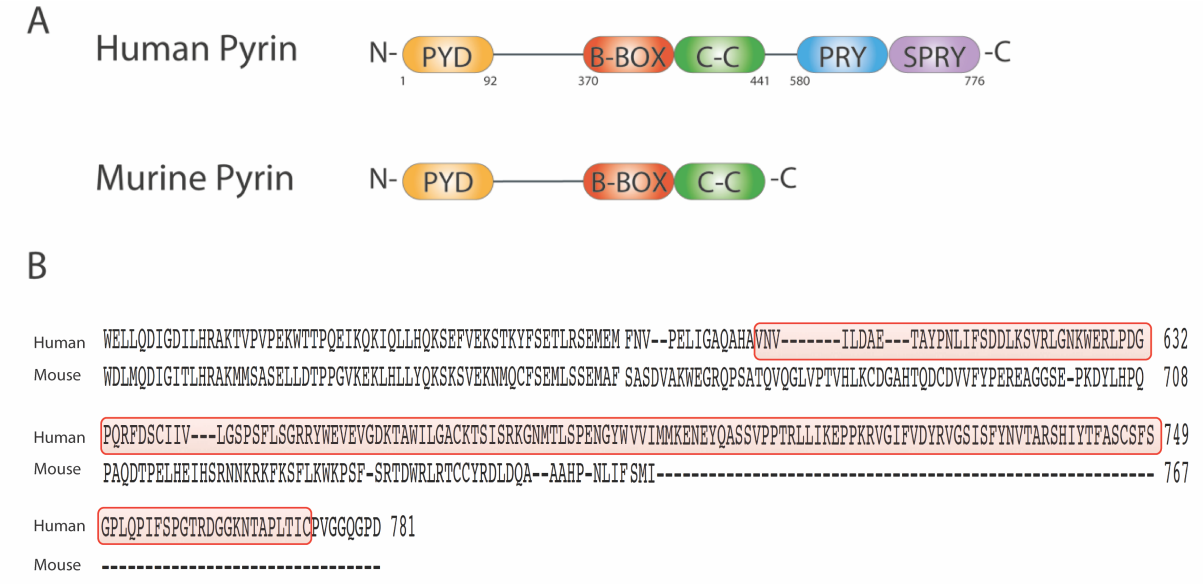


Figure 3-2 Comparison of murine and human pyrin.

(A) Schematic representation of human pyrin domains. Most familial Mediterranean fever mutations are located in the PRY-SPRY, or B30.2 domain. (B) Murine pyrin lacks the B30.2 domain (highlighted in red) as seen in the amino acid sequence alignment.

A number of earlier publications suggested that pyrin plays an inhibitory role in the production of IL-1β (Chae et al., 2011; Chae et al., 2003; Chae et al., 2006; Chae et al., 2008; Hesker, Nguyen, Kovarova, Ting, & Koller, 2012). In 2003, Chae et al. published results from mice expressing a truncated hypomorphic pyrin expressing only the PYD (Chae et al., 2003). The authors made a number of observations, including more severe hypothermia and increased IL-1β production in the pyrin-truncation mice compared with WT in response to LPS stimulation. They also showed an interaction between the PYD of mouse pyrin and mouse ASC in an *in vitro* model using co-transfection of expression constructs in PT67 cells and subsequent immunoprecipitation. The authors hypothesised that pyrin binds and sequesters ASC, or at least competes with other proinflammatory molecules for access to ASC (Chae et al., 2003).

Interestingly, a subsequent group noted a normal response to LPS but an increase in IL-1β secretion from peritoneal macrophages of *Mefv*$^{-/-}$ mice compared with WT mice in response to NLRP3 and NLRC4 stimuli (Hesker et al., 2012).

Later work by Chae et al. described that the C-terminal B30.2 domain of pyrin was responsible for an ASC-independent interaction between pyrin and Caspase-1, and suggested that there may be a role for pyrin in an inhibitory feedback loop to maintain homeostasis (Chae et al., 2006). Although immunoprecipitation was performed on lysates from THP1 cells expressing endogenous pyrin and Caspase-1, western blot analysis of ASC was not shown and it is possible that this interaction was indeed dependent on ASC. Further experiments performed were in PT67 cells, which do not express ASC, however these cells were then transfected with pyrin and Caspase-1 expression constructs with expression levels likely to be increased compared with endogenous and hence difficult to interpret.

In contrast to this, a seminal paper by Alnemri and colleagues noted the potential proinflammatory role of pyrin (J. W. Yu et al., 2006). This group used a HEK293T reconstitution system to show that human pyrin was able to induce ASC oligomerisation and ASC-dependent Caspase-1 activation, but not NF-κB, and that it did not play a role in NLRP3 interaction with ASC. It was the first paper to suggest pyrin itself forms an inflammasome, with later papers reporting that pyrin promotes ASC clustering (Zhang et al., 2015).

The development of pyrin deficient mice and mice with the human FMF mutant B30.2 domain knocked in signified a change in the accepted role of pyrin (Chae et al., 2011). Discussed further in **Section 3.1.4.1**, mice expressing a fusion pyrin protein with an N-terminal murine PYD, BB and CC domain and a C-terminal human B30.2 domain were used to model FMF. A

severe IL-1β inflammatory phenotype was seen in mice harbouring homozygous FMF mutations which was determined to be both ASC- and Caspase-1-dependent but NLRP3-independent.

3.1.3 Current model

3.1.3.1 Sensor of Rho GTPase inactivation

Interesting developments in the physiological role of pyrin have come to light in recent years, namely the role pyrin plays in detecting proteins that have undergone modification by pathogens, rather than detecting pathogens directly (Xu et al., 2014), data which has since been independently reproduced (Y. H. Park, Wood, Kastner, & Chae, 2016). Shao and colleagues used a toxin produced by *C. difficile,* TcdB, to elegantly show that pyrin inflammasomes are triggered by sensing the inactivation of Rho GTPases (Xu et al., 2014). Taking the earlier observation that TcdB and TcdA induce inflammasome-mediated intestinal inflammation (Ng et al., 2010), the authors stimulated BMDMs from WT and *Asc*$^{-/-}$ mice with TcdB and confirmed that the IL-1β response was indeed ASC-dependent. Importantly, stimulation of BMDMs from *Nlrp3*$^{-/-}$, *Nlrc4*$^{-/-}$ and *Aim2*$^{-/-}$ mice with TcdB resulted in similar levels of IL-1β compared to WT mice. Co-transfection of expression constructs containing cDNA for pyrin and RFP-ASC in a HEK293T model followed by stimulation with TcdB resulted in ASC speck formation that was not seen with co-transfection with other inflammasome forming proteins. Using both an siRNA knock-down model in BMDMs as well as TALEN generated pyrin deficient mice, pyrin was determined to be necessary for TcdB-induced Caspase-1 cleavage and IL-1β secretion. Rho GTPases function as molecular switches and may exist in two states, an active GTP-dependent 'switch I' state and an inactive GDP-dependent 'switch II' state. A number of bacteria such as *C. difficile* and *Burkholderia cenocapacia* can modify the switch function through a number of post translational events such as adenylylation and deamination,

and hence mediate inactivation of Rho GTPases. Shao and colleagues determined that pyrin does not sense this post translational modification event directly, but rather the downstream events resulting from switch I modification.

3.1.3.2 14-3-3 binding is mediated through phosphorylation of pyrin by PKN, a downstream effector of Rho GTPases.

The linking of 14-3-3 to pyrin was originally noted by Jeru et al. in a yeast two-hybrid screen with subsequent confirmation by immunoprecipitation (Jeru et al., 2005). Briefly, the family of 14-3-3 dimeric proteins are involved in modulation of protein interactions (Aitken, 2006). Initially shown to be involved in the activation of tyrosine and tryptophan hydroxylases in the brain (Perkins & Vogel, 2015), the seven isoforms of 14-3-3 protein are involved in a number of signalling and cell cycle processes (Aitken, Ellis, Harris, Sellers, & Toker, 1990; Toker, Ellis, Sellers, & Aitken, 1990). The mechanisms by which they achieve such a broad range of activities includes: providing structural stability of the target protein conformation given the rigidity of their own structure; competitive or non-competitive binding to ligand sites on target protein; and acting as a scaffold protein (A. J. Smith, Daut, & Schwappach, 2011). The interaction of 14-3-3 is generally regulated by the phosphorylation of the target protein (Aitken, 2006). The basis of the classic 14-3-3 binding motif RSX(pS/T)XP is a phosphorylated Serine or Threonine (Obsilova, Nedbalkova, et al., 2008; Obsilova, Silhan, Boura, Teisinger, & Obsil, 2008).

Jeru et al. initially showed a phosphorylation and exon-two-dependent interaction between pyrin and the 14-3-3τ and 14-3-3ε isoforms (Jeru et al., 2005). Of the serine sites in exon two, the authors demonstrated that the phosphorylation of p.Ser242 was necessary for 14-3-3 binding, and that substitution of serine by an alanine residue abolished the interaction. In

contrast to this, p.Ser208 substitution to alanine reduced the interaction between pyrin and 14-3-3 but did not completely terminate it, suggesting that p.Ser242 was the dominant residue required for the interaction. Although Jeru et al. provided evidence in overexpression models that the interaction between 14-3-3 and pyrin mediated cellular localisation, significant progress in the understanding of the physiological role of pyrin has transpired since this time and this idea has not been revisited.

Through the study of a dominantly inherited AID, we provided key insights into the interaction between 14-3-3 and pyrin (Masters et al., 2016). PAAND, discussed further in **Section 3.1.4.4**, resulted from a heterozygous *c.C726G* transition in *MEFV* encoding p.Ser242Arg pyrin. In an overexpression HEK293T model, GST-tagged pyrin was transfected into cells followed by immunoprecipitation for GST. Western blot showed the binding of 14-3-3 to WT pyrin, and the phosphorylation of the binding motif, was lost when cells were treated with TcdB. This suggested the phosphorylation of pyrin was important for 14-3-3 binding, and that the loss of binding corresponded with pyrin activation.

With the above evidence that pyrin is phosphorylated in its inactive state, and Shao and colleagues' work showing that pyrin senses an event downstream of RhoA inactivation, Chae and colleagues explored this further through siRNA interrogation of RhoA effector kinases (Y. H. Park et al., 2016). Pyrin-dependent inflammasome activation was seen in protein kinases *Pkn1* and *Pkn2* knock-down BMDMs primed with LPS. Endogenous pyrin of LPS-primed BMDMs was found to interact with the C-terminal kinase domain of PKN1 in immunoprecipitation experiments, suggesting that PKN1 phosphorylates pyrin, facilitating 14-3-3 binding. A schematic representation of pyrin activation is presented in **Figure 3.3.**

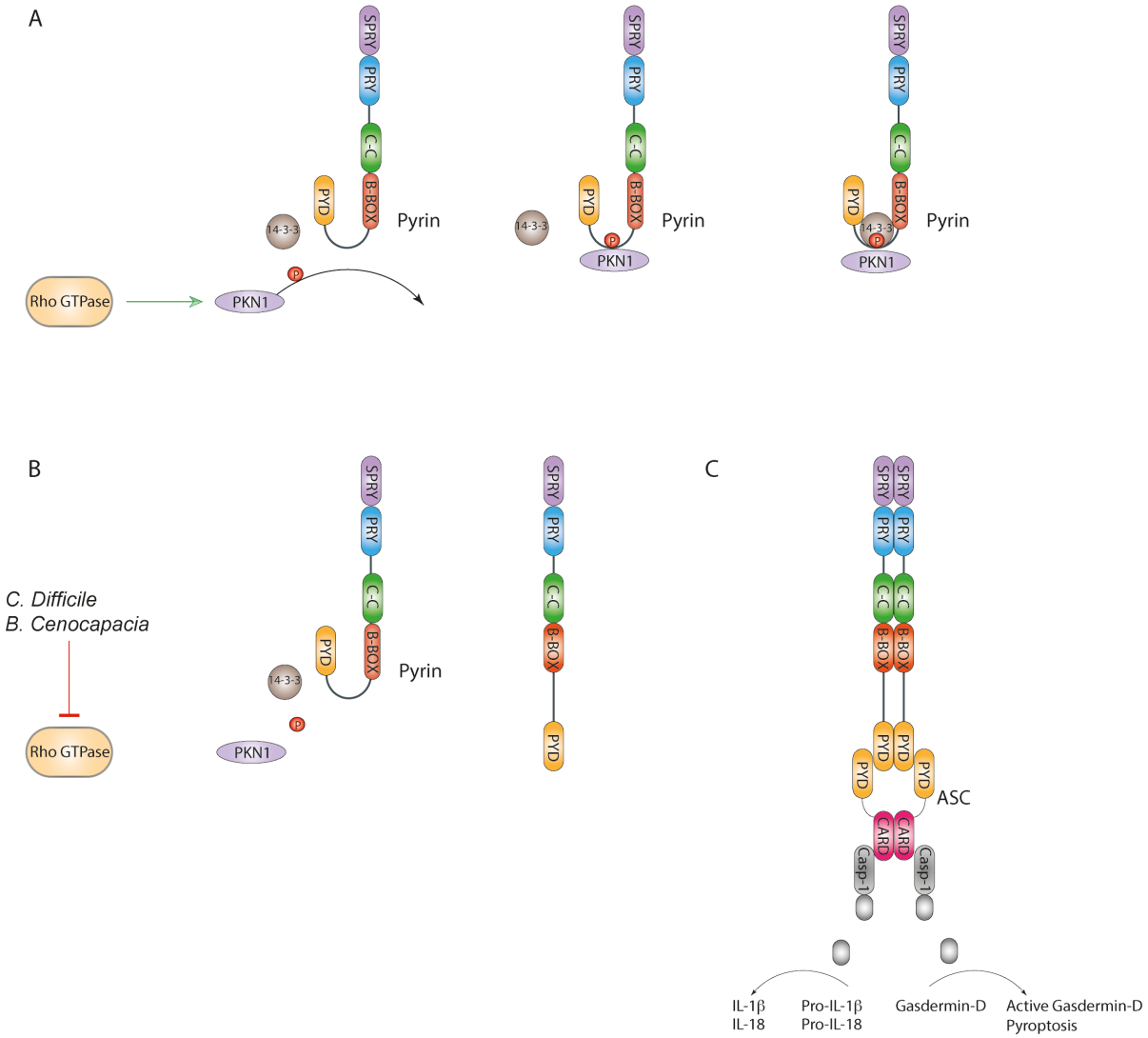


Figure 3-3 Pyrin senses Rho GTPase modifications through changes in its interaction with effector kinases.

(A) Inactive pyrin is phosphorylated at p.Ser208 and p.Ser242 by Rho GTPase effector kinases PKN1 and PKN2. This allows for 14-3-3 binding to pyrin. (B) Inactivation of Rho GTPases from, for example, *Clostridium difficile Toxin B* (TcdB) results in loss of PKN1 activity and loss of phosphorylation of pyrin. 14-3-3 dissociates from pyrin. Pyrin alters conformation to an active state. (C) Active pyrin oligomerises and associates with ASC through its PYD and subsequently pro-Caspase-1. Active Caspase-1 cleaves pro-IL-1β and pro-IL-18 to their respective active forms. Gasdermin-D is also cleaved by Caspase-1, and creates pores in the cell membrane leading to an inflammatory form of cell death, pyroptosis.

3.1.4 Pyrin-associated diseases

Given the progress made in the elucidation of the pyrin pathway, pyrin-associated disorders are reviewed here with specific focus on the recent advances in understanding of disease pathogenesis.

3.1.4.1 FMF

FMF was the first described and genetically defined AID, characterised by periodic fever and recurrent serositis (Estren & Fazekas, 1947; Siegal, 1964). The seminal murine model of FMF was generated by Kastner and colleagues in 2011 through the creation of mice harboring either WT or one of three FMF-associated mutant B30.2 domains through the replacement of murine *Mefv* exons 7-10 with human *MEFV* exons 7-10 (Chae et al., 2011). The authors successfully produced $Mefv^{M680I/M680I}$, $Mefv^{M694V/M694V}$ and $Mefv^{V726A/V726A}$ mice, with all homozygous mutant mice developing spontaneous inflammation with features of growth retardation, arthritis, dermatitis, lymphadenopathy and splenomegaly. This inflammation was abrogated when FMF mice were crossed with $Il1r^{-/-}$ mice, suggesting that the disease seen was IL-1-mediated. Supporting these findings is the clinical response of colchicine refractory FMF patients to IL-1β targeted therapy including canakinumab and anakinra (Ben-Zvi et al., 2017; Laskari et al., 2017). Despite FMF being considered an autosomal recessive disease, the hypothesis that it is indeed a GoF of pyrin that results in disease is supported by the lack of disease in the $Mefv^{-/-}$ mice, as well as the release of IL-1β from macrophages differentiated from FMF patients' PBMCs when stimulated with LPS alone.

A fascinating observation that treatment of serum-starved Swiss 3T3 cells with colchicine results in rapid activation of RhoA was made in 1999 (Ren, Kiosses, & Schwartz, 1999). Park et al. explored this role in the context of pyrin by immunoprecipitation using Rhotekin-RBD

beads that bind active Rho proteins (Y. H. Park et al., 2016). Colchicine was able to increase active RhoA in a dose dependent manner in BMDMs treated with LPS and *C. botulinum* C3 toxin, a known pyrin activator. Furthermore, and possibly more relevant given the exquisite response to colchicine seen in the majority of patients with FMF, a reduced amount of active RhoA was seen in BMDMs of mice harbouring $Mefv^{V726A/V726A}$ compared with WT BMDMs, and this was increased with exposure to colchicine. Using immunoprecipitation assays, Park et al. demonstrated reduced PKN1 binding to pyrin in $Mefv^{V726A/V726A}$ and $Mefv^{M694V/M694V}$ mice when compared with those from $Mefv^{B30.2/B30.2}$ and $Mefv^{M680I/M680I}$ mice as well as reduced 14-3-3 binding to pyrin in all mutants compared with $Mefv^{B30.2/B30.2}$ BMDMs. Complicating the interpretation of this data is the finding that BMDMs from $Mefv^{B30.2/B30.2}$ mice had significantly less 14-3-3 and PKN1 bound to pyrin when compared with WT BMDMs. Having said this, the data from healthy controls and patients with FMF supports that seen in the murine model. Park et al. assessed 14-3-3 binding through immunoprecipitation of endogenous pyrin from macrophages differentiated from PBMCs from FMF patients treated *ex vivo* with IFN-γ and LPS, observing that they have reduced 14-3-3 binding to pyrin when compared with healthy controls. This data suggests that the pharmacological benefit of colchicine in FMF may be via increasing RhoA activity to activate PKN1 leading to pyrin phosphorylation, 14-3-3 binding and hence reduced pyrin activation.

Interesting work by Park et al. demonstrated that PBMCs of patients with FMF required priming with IFN-γ the stimulation with LPS to show IL-1β secretion (Y. H. Park et al., 2016). Using monocytes from patients with p.Met694Val homozygous FMF, Jamilloux et al. determined that there was no IL-1β secretion without stimulation. However, using priming and TcdB stimulation, the authors demonstrated that monocytes from homozygous p.Met694Val pyrin patients responded with greater IL-1β secretion than heterozygous carriers of

p.Met694Val pyrin, and both more than that seen from healthy control monocytes suggesting a gene-dose effect.

3.1.4.2 HIDS

Despite having been defined clinically and genetically for some time, the link between pyrin and HIDS, an AID caused by LoF mutations in *MVK* encoding mevalonate kinase (MVK) (Drenth et al., 1999; Houten et al., 1999), was only described recently. MVK plays an important role in the production of isoprenoids from acetate, involved in a number of cellular processes including cholesterol synthesis (Miziorko, 2011). Park et al. noted observations in the literature that the membrane localization of RhoA was dependent on a post translational modification called geranylgeranylation, and that the necessary substrate for this process was a product of the mevalonate pathway (Y. H. Park et al., 2016). The authors hypothesized that reduced geranylgeranylation as a result of reduced substrate, for example, could lead to a change in localization of RhoA, decreased function and hence decreased inhibition of pyrin. PBMCs from HIDS patients were stimulated with LPS, and IL-1β release in the supernatant assessed by precipitation and immunoblot. IL-1β release was seen in all patients. Co-treatment with PKN1 activators arachidonic acid or bryostatin reduced this significantly, suggesting that the inflammatory disease seen in these patients is due to reduced PKN1 activity, likely from reduced geranylgeranylation of RhoA.

3.1.4.3 PAAND

The investigation of four pedigrees, including a three-generational pedigree with a presumed dominantingly inherited inflammatory condition, led to the description of a new pyrin related disorder, PAAND, in 2016 by our group in collaboration with others (Masters et al., 2016). The phenotype of patients was distinct from FMF, with pustular acne, pyoderma gangrenosum,

recurrent fever, myalgia, arthralgia and elevated acute-phase reactants seen in most patients. The serositis often associated with FMF was absent. Using WES of two trios in one pedigree, a single missense mutation in *MEFV* encoding p.Ser242Arg pyrin was found and segregated with disease. In a HEK293T overexpression system, co-transfection of ASC-GFP and p.Ser242Arg pyrin was associated with increased ASC speck formation when compared with WT pyrin, as determined by immunofluorescence microscopy and flow cytometry. CRISPR/Cas9 gene editing techniques were used to generate *MEFV* deficient THP1 cells. These cells were subsequently reconstituted with WT or p.Ser242Arg pyrin, with cells expressing p.Ser242Arg pyrin displaying significantly increased Caspase-1-dependent IL-1β release when treated with LPS when compared to cells with WT pyrin. Mass spectrometry analysis of WT and p.Ser242Arg pyrin revealed binding of 6 of the 7 isoforms of 14-3-3 to WT pyrin that was absent from p.Ser242Arg pyrin. By transfecting HEK293T cells with GST-tagged pyrin performing GST immunoprecipitations, endogenous 14-3-3 was shown to be bound to WT pyrin but not p.Ser242Arg pyrin, in a pattern that was similar to WT pyrin treated with TcdB. We noted that within the 14-3-3 binding motif is a phosphorylated Serine, which p.Ser242Arg substitution abolishes, causing loss of 14-3-3 binding.

Although predominantly a description of a clinical syndrome, investigation of pathophysiological mechanism presented by Masters et al. prompted more detailed evaluation of this pathway leading to recent advances discussed in **Section 3.1.3.2**.

3.1.4.4 PAPA syndrome

The phenotype of patients with PAAND overlaps significantly with those experienced by patients with PAPA syndrome, an autosomal dominant condition caused by mutations in

PSTPIP1 (Wise et al., 2002). Importantly, the interaction of pyrin with PSTPIP1 is thought to be key to the pathophysiology of PAPA syndrome (Shoham et al., 2003; Wise et al., 2002).

PSTPIP1 is a tyrosine-phosphorylated F-BAR domain containing protein associated with cortical actin cytoskeleton and actin stress fibres (Spencer et al., 1997). Initial work by Spencer et al. demonstrated that PSTPIP1 is a substrate for PTP-PEST, a tyrosine phosphatase, and that overexpression of PSTPIP1 in 3T3 cells resulted in the morphological change of cells with evidence of the development of filopodia (Spencer et al., 1997). Lasky and colleagues investigated other potential binding partners with a yeast two-hybrid screen and, using PSTPIP1 as bait, isolated Wiskott-Aldrich Syndrome protein (WASp) (Y. Wu, Spencer, & Lasky, 1998). Wiskott-Aldrich Syndrome (WAS) is an X-linked immunodeficiency characterised by eczema and thrombocytopaenia, as well as morphological defects in lymphocytes (Molina, Kenney, Rosen, & Remold-O'Donnell, 1992; Ochs et al., 1980). Cote et al. also highlighted the link between PTP-PEST, WASp and PSTIPIP1 in 2002 and extended the model of cytoskeletal regulation (Cote et al., 2002). The authors showed that PTP-PEST dephosphorylates PSTPIP1 at p.Tyr344, and that PSTPIP1 provides the structural link between PTP-PEST and WASp, with PTP-PEST dephosphorylating WASp leading to reduced WASp activity. Shortly after this, Wise et al. noted that the binding and dephosphorylation of PSTPIP1 by PTP-PEST is reduced significantly when PSTPIP1 harboured either p.Ala230Thr or p.Glu250Gln mutations, the most common mutations reported in PAPA syndrome (Wise et al., 2002).

Using a yeast two-hybrid screen to determine proteins interacting with pyrin using pyrin as bait, Shoham et al. determined a cDNA clone matching PSTPIP1 (Shoham et al., 2003). To determine the significance, the authors used HeLa cells transfected with both pyrin and

PSTPIP1 to show colocalisation, and further that endogenous pyrin and PSTPIP1 interacted in THP1 cells. The pathophysiological implications of the interaction of pyrin and PSTPIP1 was documented by evaluating the binding of p.Ala230Thr and p.Glu250Gln PSTPIP1 to pyrin. PSTPIP1 harbouring these mutations are hyperphosphorylated, and binding with pyrin increased when compared with WT PSTPIP1 (Shoham et al., 2003).

Yu et al. used THP1 cells and a HEK-293T reconstitution system to look at how pyrin and PSTPIP1 interact in an attempt to elucidate the pathophysiology of PAPA syndrome (J. W. Yu et al., 2007). The authors showed that pyrin exists in an inactive homotrimer state, with the B-box domain responsible for the inactive state, potentially providing a structural barrier to PYD domain associating with ASC. In their hands, mutant PSTPIP1 bound more strongly to pyrin compared with WT PSTPIP1. They proposed that PSTPIP1 is absolutely required for release of pyrin from its baseline inhibitory state. This model was subsequently disputed in a paper by Waite et al. (Waite et al., 2009). Here, the authors used transfection/over expression models to show that pyrin and ASC always co-localise, even without PSTPIP1. PSTPIP1 and ASC did not co-localise without pyrin, and, even when pyrin is present, PSTPIP1 is only seen in approximately 70% of specks. This percentage increases to over 95% when mutations associated with PAPA syndrome are transfected. Despite the conflicting evidence, the idea that PSTPIP1 is involved in the regulation of pyrin in some way is more accepted.

A conditional knock-in murine model of PAPA syndrome failed to recapitulate the arthritis and skin phenotype seen in patients, but mice harbouring PAPA-associated mutant PSTPIP1 had partial embryonic lethality, growth retardation and raised inflammatory markers (D. Wang et al., 2013). It should be noted that the murine pyrin was not altered in this model. Interestingly, mice with mutant PSTPIP1 expressed in haematopoetic cells only did not have

elevated proinflammatory cytokines, suggesting that the phenotype seen was independent of these cells.

The clinical similarities between PAAND and PAPA syndrome are more marked than those between PAAND and FMF. The possible link between these condition is explored in the discussed in **Section 3.3**.

3.2 **Manuscript**

The citation of the manuscript included as the results for **Chapter 3** is as follows:

Moghaddas, F., et al., *A novel Pyrin-Associated Autoinflammation with Neutrophilic Dermatosis mutation further defines 14-3-3 binding of pyrin and distinction to Familial Mediterranean Fever.* Ann Rheum Dis, 2017

EXTENDED REPORT

A novel Pyrin-Associated Autoinflammation with Neutrophilic Dermatosis mutation further defines 14-3-3 binding of pyrin and distinction to Familial Mediterranean Fever

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ABSTRACT

Objective Pyrin-Associated Autoinflammation with Neutrophilic Dermatosis (PAAND) is a recently described monogenic autoinflammatory disease. The causal p.S242R *MEFV* mutation disrupts a binding motif of the regulatory 14-3-3 proteins within pyrin. Here, we investigate a family with clinical features consistent with PAAND in whom the novel p.E244K *MEFV* mutation, located in the +2 site of the 14-3-3 binding motif in pyrin, has been found.

Methods Multiplex cytokine analyses were performed on p.E244K patient and control serum. Peripheral blood mononuclear cells were stimulated ex vivo with lipopolysaccharide (LPS). In vitro, inflammasome complex formation was evaluated by flow cytometry of Apoptosis-associated Speck-like protein containing a Caspase recruitment domain (ASC) specks. Interleukin-1β (IL-1β) and IL-18 production was quantified by ELISA. The ability of the p.E244K pyrin mutation to interact with 14-3-3 was assessed by immunoprecipitation.

Results PAAND p.E244K patient serum displayed a different cytokine profile compared with patients with Familial Mediterranean Fever (FMF). In overexpression models, p.E244K pyrin was associated with decreased 14-3-3 binding and increased ASC speck formation. THP-1 monocytes expressing PAAND pyrin mutations demonstrated spontaneous caspase-1-dependent IL-1β and IL-18 secretion, as well as cell death, which were significantly greater than those of wild-type and the FMF-associated mutation p.M694V.

Conclusion In PAAND, disruption of the +2 position of a 14-3-3 binding motif in pyrin results in its constitutive activation, with spontaneous production of IL-1β and IL-18, associated with inflammatory cell death. The altered serum cytokine profile may explain the different clinical features exhibited by PAAND patients compared with those with FMF.

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INTRODUCTION

Pyrin-Associated Autoinflammation with Neutrophilic Dermatosis (PAAND) is a recently described monogenic autoinflammatory condition caused by a heterozygous mutation in the *MEFV* gene resulting in the p.S242R substitution in pyrin.[1] The dominant clinical phenotype of prolonged fever and neutrophilic dermatosis (eg, acne, pyoderma gangrenosum), and potentially the mechanism of disease, differs from the classical pyrin-associated disease, Familial Mediterranean Fever (FMF).

The p.S242 site of pyrin forms a 14-3-3 binding motif.[1 2] Although there are a number of variations of 14-3-3 recognition motifs reported, all contain a phosphorylated serine or threonine residue.[3 4] In its inactive state, pyrin is phosphorylated by serine-threonine kinases PKN1 and PKN2 at residues p.S208 and p.S242, and is bound to 14-3-3 proteins.[5] When triggered in response to RhoGTPase modifications, such as those induced by the pathogen *Clostridium difficile*, there is dephosphorylation of pyrin at p.S208 and p.S242 residues and loss of 14-3-3 binding.[1 5 6] In vitro models show that the p.S242R pyrin mutation is constitutively dephosphorylated, with reduced 14-3-3 binding.[1] The resulting increased pyrin inflammasome activation and enhanced IL-1β production appear to drive the pathology in PAAND.[1]

Here, we report a novel mutation in the *MEFV* gene in a family with clinical features of PAAND that results in an altered 14-3-3 binding motif and constitutive activation of pyrin. We also confirm phenotypic differences and identify cytokine differences between PAAND and FMF.

METHODS

Patients

We investigated three symptomatic patients in one family. We used patients with homozygous p.M694V FMF as disease controls, and blood donors as healthy controls. This study was approved by the Hospital Clinic-IDIBAPS Ethics Committee.

Patient cell stimulation and analysis

Fresh serum samples were collected from patients and controls, and cytokine quantification was performed by Luminex multiplex assay. PAAND patients had active clinical features at the time of collection. For human IL-18 and IL-18BP, serum was assayed in multiplex on a Luminex Magpix system (Bio-Rad, Hercules, California, United States). Bio-Rad group II cytokine standard was used for IL-18, whereas recombinant human IL-18BPa-Fc

chimeric protein (R&D Systems, Minneapolis, Minnesota, United States) was used as standard for IL-18BP.

Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque-1077 (Sigma-Aldrich, St Louis, Missouri, United States) and treated with *Escherichia coli* LPS serotype 055:B5 (Sigma-Aldrich; 1 µg/mL, 2 hours at 37°C) or left untreated. IL-1β was measured on cell supernatants by ELISA (eBioscience, San Diego, California, United States) while other cytokine quantification was performed by Luminex multiplex assay as described above. Cells were fixed with 2% paraformaldehyde and stained for the detection of Apoptosis-associated Speck-like protein containing a Caspase recruitment domain (ASC) specks by Time of Flight Inflammasome Evaluation using the rabbit polyclonal antibody anti-ASC (N-15)-R (Santa Cruz Biotechnology, Dallas, Texas, United States) as previously described.[7] Alternatively, for the detection of active caspase-1, PBMCs were incubated for 20 min with Fluorochrome Inhibitor of Caspases (FLICA)660 reagent (ImmunoChemistry Technologies, Bloomington, Minnesota, United States) and fixed following manufacturer's recommendations. Monocytes were detected with the APC-vio770 mouse anti-human CD33 antibody (Miltenyi Biotech, Bergisch Gladbach, Germany) and with the APC-Cy7-conjugated anti-human CD14 antibody (TONBO Biosciences, San Diego, California, United States). Stained cells were acquired on a BD FACSCanto cytometer.

Heat maps representing cytokine expression profiles were created using Morpheus software (Broad Institute, Cambridge, Massachusetts, United States).

Cell culture

HEK293T cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and transfected with mCherry-pyrin or GST-pyrin,[8] GFP-ASC,[9] or V5-Proline Serine Threonine Phosphatase-Interacting-Protein 1 (PSTPIP1) (HsCD00438559, DNASU Plasmid repository) constructs using Lipofectamine (Life Technologies) according to manufacturer's instructions. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 techniques were used for generation of *MEFV* KO and *CASP1* KO THP-1 cells, as has been described.[1 10] These cells were cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FCS.

Lentiviral infection of THP-1 cells

MEFV KO THP-1 cells were reconstituted with pyrin by lentiviral transduction. A lentiviral construct was generated through ligation of *MEFV* cDNA into *Bam*HI and *Age*I restriction sites on the pFUGW backbone after performing site directed mutagenesis of the *Bam*HI restriction site within *MEFV*. Lentivirus was produced as previously described.[10] One million THP-1 cells were seeded per well in six-well plates with 3.5 mL of virus and 24 µg of polybrene. A total of 6 million THP-1 cells were seeded per condition. Plates were centrifuged at 840 g for 3 hours and then incubated at 37°C overnight. Cells were collected the following day, washed in phosphate buffered saline (PBS), reseeded in fresh media and incubated at 37°C overnight. After a further 24 hours, live and dead cells were separated using Ficoll density gradient centrifugation (GE Healthcare, Chicago, Illinois, United States). Live cells were seeded for experiments. Supernatant was harvested after 24 hours for cytokine analysis by ELISA for IL-1β and IL-18 (DY201 and DY008, R&D Systems). Cytokines from cell culture supernatant were also quantified using Bio-Plex Pro Assay (Bio-Rad). Cell death was analysed by flow cytometry using propidium iodide (Sigma-Aldrich) staining at 1 µg/mL. Where indicated, priming of cells was performed with Pam3CSK4, a synthetic TLR1/2 agonist (InvivoGen, San Diego, California, United States). Cells were also lysed using radioimmunoprecipation assay buffer to assess expression of pyrin by western blotting.

Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuickChange Lightning Kit (210519–5, Agilent Technologies, Santa Clara, California, United States) according to manufacturer's instructions. Mutations were introduced to pyrin-expressing constructs using the following oligonucleotide primers:

p.E244K 5'-TAGAAATGGTGACCTTAAGGCTTCTAG GTCGCATC-3'
5'- GAT GCGA CCTA GAAG CCTT AAGG TCAC CATTTCTA-3'
p.M694V 5'-GGTACTCATTTTCCTTCACCATTATCA CCACCCAGTAG-3'
5'- CTA CTGG GTGG TGAT AATG GTGA AGGA AAAT GAGTACC-3'
p.S242R 5'-GAAATGGTGACCTCAAGCCTTCTAGGT CGCATCTT-3'
5'- AAG ATGC GACC TAGA AGGC TTGA GGTC ACCATTTC-3'
p.E244P 5'-GATGCGACCTAGAAGCCTTCCGGTCAC CATTTCTACAG-3'
5'- CTGTAGA AATG GTGA CCGG AAGG CTTC TAGG TCGCATC-3'
p.E244D 5'-CGACCTAGAAGCCTTGATGTCACCATT TCTACAGG-3'
5'- CCT GTAG AAAT GGTG ACAT CAAG GCTT CTAGGTCG-3'
p.E244R 5'AGATGCGACCTAGAAGCCTTAGGGTCA CCATTTCTACAGG-3'
5'- CCT GTAG AAAT GGTG ACCC TAAG GCTT CTAG GTCGCATCT-3'
R39R(Δ*Bam*HI) 5'-GGAGCACTCCAGAATCCCC CGGAGC-3'
5'-GCTCCGGGGGATTCTGGAGTGCTCC-3'
I666I(Δ*Bam*HI) 5'-CAGGCTCCCAGTATCCATGCTGT CTTGTCTCC-3'
5'-GGAGACAAGACAGCATGGATACTGGGAGCCTG-3'

Fluorescence microscopy and flow cytometry

HEK293T cells were transfected with 25 ng wild type (WT) or mutant mCherry-MEFV and 5 ng GFP-ASC, and ASC specks were quantified 16 hours later using flow cytometry, as previously described.[7] Colocalisation experiments were performed using mCherry-MEFV and GFP-ASC transfected into 1×10^5 HEK 293 T cells seeded in ibidi chamber slides (ibidi GmbH, Munich, Germany). Images were taken with a Zeiss LSM 780 Confocal microscope and were processed using FIJI software (National Institutes of Health, Bethesda, Maryland, United States).

Immunoprecipitation and western blotting

HEK293T cells (3×10^6 cells) were transfected with 5 µg of GST-tagged WT or mutant pyrin, with or without WT PSTPIP1. Where indicated, cells were treated with *Clostridium difficile* Toxin B protein (TcdB, 5 µg/mL, ab124001, Abcam, Cambridge, United Kingdom) 16 hours before harvesting. Cell lysates were generated 48 hours after transfection using 1% NP-40 lysis buffer

supplemented with protease inhibitors and sodium orthovanadate. Immunoprecipitation of pyrin was performed using glutathione sepharose 4B (GE Healthcare). After washing, bound proteins were eluted from beads using 2x sodium dodecyl sulphate (SDS) buffer and boiling at 90°C. Immunoblots were prepared using 4%–12% Novex SDS-Polyacrylamide gel electrophoresis (Invitrogen, Carlsbad, California, United States) gels in MES running buffer, followed by transfer on to nitrocellulose membranes. Membranes were blocked with tween/tris-buffered saline (TBST) +3% bovine serine albumin (BSA) at room temperature and subsequently probed overnight at 4°C with antibodies against pan-14-3-3 (1:500 Santa Cruz #sc-629-G), 14-3-3τ (1:500 Santa Cruz #sc-59414), 14-3-3ε (1:1000 Biorbyt #orb6357), pSer 14-3-3 binding motif (1:500 Cell signalling #9601), pyrin (1:500 AdipoGen #AL196), p10 Caspase-1 (1:200 Santa Cruz #sc-515), IL-1β (1:1000 R&D #AB-401-NA), GST (1:1000 in-house), PSTPIP1 (1:500 Abnova #H00009051) and actin (1:5000 Santa Cruz #sc-1616). All antibodies were prepared in TBST +1% BSA.

Statistical analysis

Mann-Whitney non-parametric test was used for the analysis of data in figure 2. Two-tailed t-tests were performed in other analysis using Prism software (GraphPad Software, La Jolla, California, United States). Data are represented as mean+/−SEM unless otherwise specified (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

RESULTS

PAAND family with a novel mutation in *MEFV*

The index patient is a 43-year-old woman of Spanish descent with a 30-year history of chronic and severe pustular acne, severe hidradenitis suppurativa, recurrent pyoderma gangrenosum, recurrent long-lasting febrile episodes, neutrophilic panniculitis as well as polyarthralgia and oligoarthritis of small and large joints (figure 1A). Raised C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and peripheral blood neutrophil count persisted despite treatment with corticosteroids and the IL-1 receptor antagonist (IL-1Ra), anakinra

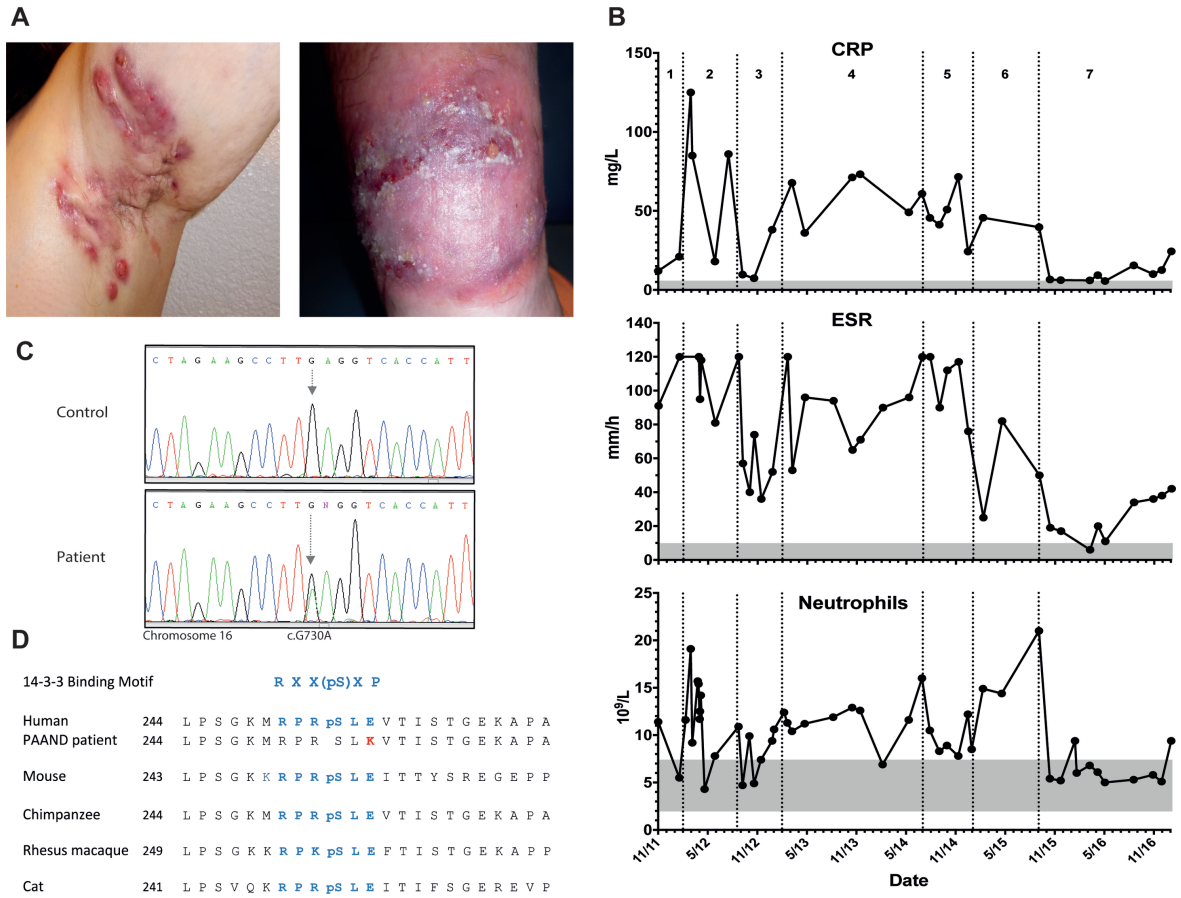

Figure 1 Clinical features of Pyrin-Associated Autoinflammation with Neutrophilic Dermatosis (PAAND). (A) Representative macroscopic images of dermatological manifestations in index case (left: hidradenitis suppurativa axillae; right: pyoderma gangrenosum lower leg). (B) Acute phase reactants and neutrophils over time, with treatment periods 1. infliximab+methotrexate; 2. prednisolone+ciclosporin A; 3. prednisolone+infliximab; 4. prednisolone+doxycycline; 5. prednisolone+anakinra+ clindamycin; 6. prednisolone+clindamycin+ moxifloxacin+dapsone; 7. prednisolone+clindamycin+ moxifloxacin+dapsone+ adalimumab. (top: C-reactive protein (CRP); middle: erythrocyte sedimentation rate (ESR); bottom: peripheral blood neutrophil count). (C) DNA chromatogram showing the heterozygous G-to-A transition at position corresponding to c.730 *MEFV*. (D) p.E244 pyrin is highly conserved across species. The glutamate is in position +2 of a 14-3-3 binding motif.

(figure 1B). Long-lasting (8 years) clinical benefit was seen with the chimeric anti-tumor necrosis factor (TNF)-α monoclonal antibody infliximab. However, loss of efficacy of infliximab was observed and necessitated switching to the human anti-TNF-α monoclonal antibody adalimumab when symptoms recurred. Although a clinical diagnosis of pyogenic arthritis, pyoderma gangrenosum and acne (PAPA) syndrome was suspected, genetic testing of *PSTPIP1* failed to reveal a pathogenic mutation. Pathogenic mutations in *NCSTN*, reported in familial cases of hidradenitis suppurativa, were absent.[11] The recent description of PAAND, a condition with significant clinical overlap with PAPA syndrome, prompted exon 2 *MEFV* sequencing in this patient, which revealed the heterozygous c.730G >A transition in the *MEFV* gene encoding for the p.E244K mutation (figure 1C). This mutation was absent from the 1000 Genomes Project, Exome Aggregatium Consortium, Exome Variant Server and 250 Spanish healthy controls. Furthermore, it had not been reported on the INFEVERS database.[12–14] The locus is highly conserved across species (figure 1D) and the amino acid substitution predicted to be damaging using MutationTaster,[15] Sorting Intolerant from Tolerant[16] and Polymorphism Phenotyping v2.[17] Evaluation of the patient's mother and brother, both of whom have had dermatitis and long-lasting (>30 years) severe nodulocystic acne affecting the face and trunk respectively, revealed the mutation of interest, suggesting an autosomal dominant disease with variable penetrance (see online supplementary figure S2).

PAAND family has a cytokine profile distinct from FMF patients

Serum cytokine analysis of the proband, mother and brother revealed a unique profile when compared with FMF patients (n=5) and healthy controls (n=7), highlighted on a heat map of relative values (figure 2A). The increased serum IL-18 was explored further with the measurement of IL-18 binding protein (IL-18BP). IL-18BP has a high affinity for IL-18, and renders it biologically inactive.[18] Free IL-18, rather than total, correlates better with disease activity in IL-18-driven conditions, such as haemophagocytic lymphohistiocytosis.[19] Interestingly, in our PAAND patients, IL-18BP was significantly elevated when compared with healthy controls, and the ratio of total IL-18 to IL-18BP was similar (figure 2B). Therefore, analysis of free IL-18 revealed no significant increase (data not shown).

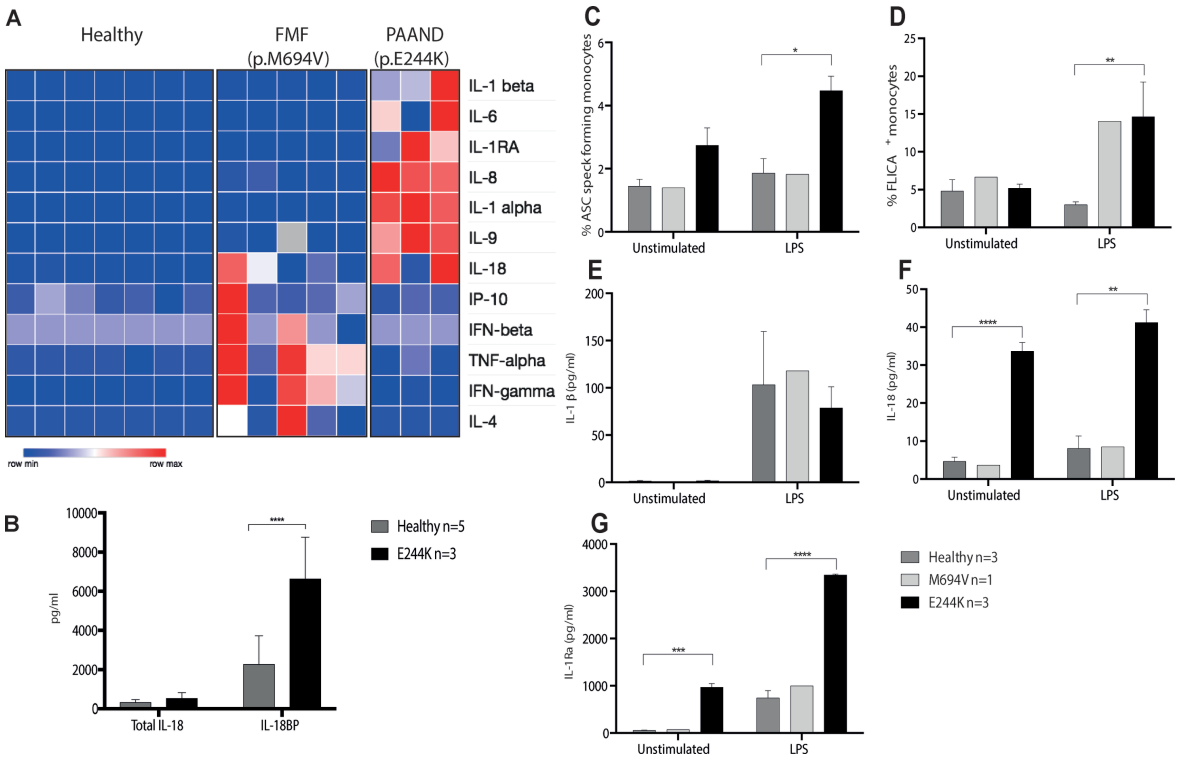

Figure 2 Pyrin-Associated Autoinflammation with Neutrophilic Dermatosis (PAAND) (p.E244K pyrin) has a distinct cytokine and inflammasome profile compared with Familial Mediterranean Fever (FMF). (A) Heat map of serum cytokine analysis of healthy controls, patients with FMF and genetically confirmed homozygous p.M694V *MEFV* mutation or patients with PAAND carrying the heterozygous p.E244K *MEFV* mutation. Representative of relative values of minimum and maximum concentrations measured per cytokine. (B) Serum total IL-18 analysis compared with IL-18BP. Assessment of (C) Apoptosis-associated Speck-like protein containing a Caspase recruitment domain (ASC) speck forming monocytes by flow cytometry and (D) active caspase-1 by YVAD-Fluorochrome Inhibitor of Caspases (FLICA) staining. Peripheral blood mononuclear cell IL-1β (E), IL-18 (F), and IL-1Ra (G) cytokine production at baseline and with Lipopolysaccharide (LPS) stimulation in healthy controls, FMF and PAAND patients. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

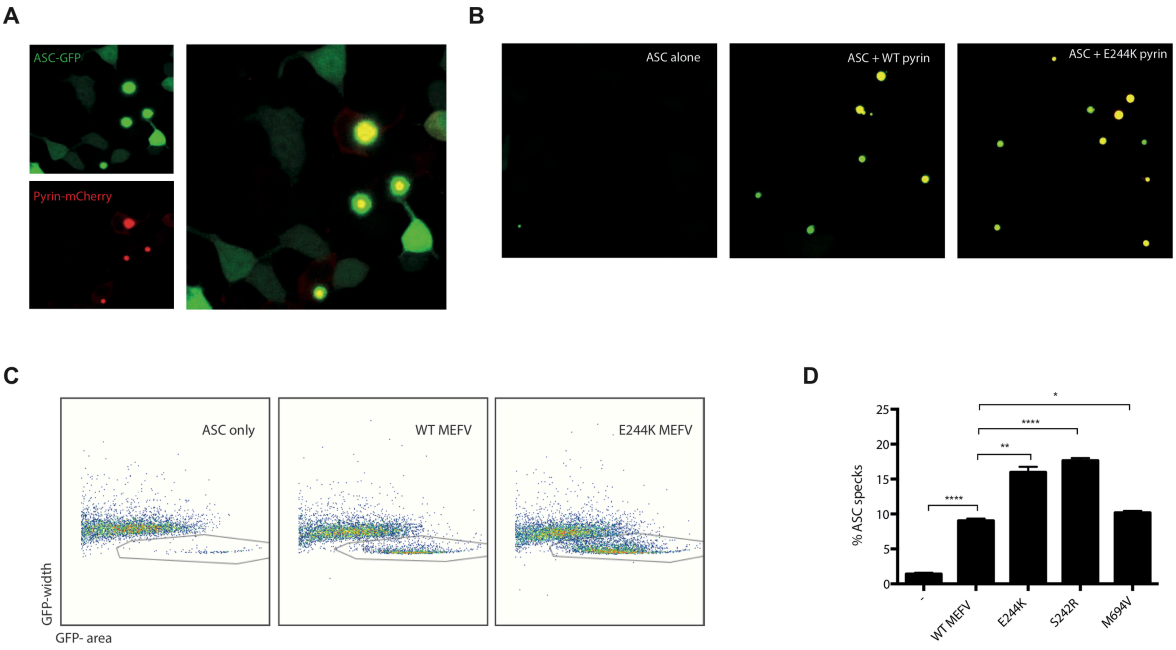

Figure 3 Increased inflammasome activation by p.E244K pyrin. (A) Confocal microscopy showing colocalisation of mCherry-tagged pyrin and GFP-tagged Apoptosis-associated Speck-like protein containing a Caspase recruitment domain (ASC) transfected into HEK293T cells. (B) Increase in spontaneous ASC speck formation in p.E244K pyrin compared with wild type (WT) pyrin or ASC alone control. (C) Fluorescence-activated cell sorting (FACS) analysis of HEK293T cells with mCherry-tagged pyrin and GFP-tagged ASC constructs. After 16 hours, cells were selected by forward scatter (FSC) and side scatter (SSC), expression of both constructs (mCherry and GFP) and finally GFP area versus width. (D) Flow cytometric quantification of ASC speck formation for WT and various pyrin mutants. Data pooled from three independent experiments. *p<0.05, **p<0.01, ****p<0.0001.

When activated, most inflammasome forming proteins, including pyrin, associate with the adaptor protein ASC to form a platform for procaspase-1 activation and cleavage of pro-IL-1β and pro-IL-18 to the mature forms.[20 21] Monocytes isolated from PAAND patients showed increased ASC speck formation with LPS exposure, and there was a trend toward an increase at baseline (figure 2C). Caspase-1 activity as measured by YVAD-FLICA staining was increased in PAAND monocytes when treated with LPS (figure 2D). Given these results, it was surprising that IL-1β production from PAAND patient PBMCs in response to LPS was unaltered (figure 2E). Nevertheless, the total IL-18 secreted by PBMCs was increased compared with healthy controls, both at baseline and following LPS stimulation, as were levels of IL-1Ra (figure 2F,G).

p.E244K pyrin is associated with increased ASC speck formation

To determine whether the above results were indeed caused by the novel p.E244K pyrin mutation, we assessed ASC speck formation in vitro as a surrogate marker for inflammasome formation. Colocalisation experiments were performed by expression of both mCherry-tagged pyrin and GFP-ASC in HEK293T cells (figure 3A). There was minimal spontaneous ASC speck formation. As expected, WT pyrin augmented this response, but p.E244K pyrin did so further (figure 3B). This was quantified using flow cytometry (see online supplementary Figure S3), with p.E244K pyrin resulting in a similar percentage of cells with ASC speck formation compared with the other known PAAND mutation p.S242R, both of which were greater than WT and p.M694V pyrin (figure 3C,D).

p.E244K pyrin is associated with increased IL-1β, IL-18 and pyroptosis

Further functional studies were performed using THP-1 monocytes. *MEFV* KO or *CASP1* KO THP-1 cells were reconstituted with *MEFV* using lentiviral transduction of WT or mutant cDNA. Even without stimulation, *MEFV* KO THP-1 cells expressing p.E244K pyrin displayed increased cell death (figure 4A), as well as IL-1β and IL-18 release (figure 4B,C). Surprisingly, this phenotype was present without 'priming' the inflammasome, which is usually required to induce pro-IL-1β expression.[22] Interestingly, IL-1β production in both p.E244K and p.S242R pyrin-expressing *MEFV* KO THP-1 cells was significantly higher than cells expressing FMF associated p.M694V pyrin (figure 4C). Genetic deletion of caspase-1 prevented p.E244K and p.S242R pyrin-induced cytokine production as well as cell death, suggesting the caspase-1 dependent inflammatory cell death (pyroptosis) (figure 4A–C). However, genetic deletion of caspase-1 did not affect Pam3CSK4-induced priming of pro-IL-1β (figure 4D). These in vitro data support the hypothesis that inflammasome activation in p.E244K pyrin patients is responsible for excessive cytokine release and pyroptosis.

p.E244K pyrin does not alter PSTPIP1 binding

In PAPA syndrome, mutant PSTPIP1 is hyperphosphorylated and binds more strongly to pyrin.[23] Given the clinical similarities between PAAND and PAPA syndrome, binding of PSTPIP1 to pyrin with and without PAAND mutations was assessed. Both GST-pyrin and PSTPIP1 were transfected into HEK293T cells and GST-immunoprecipitation performed. When comparing the

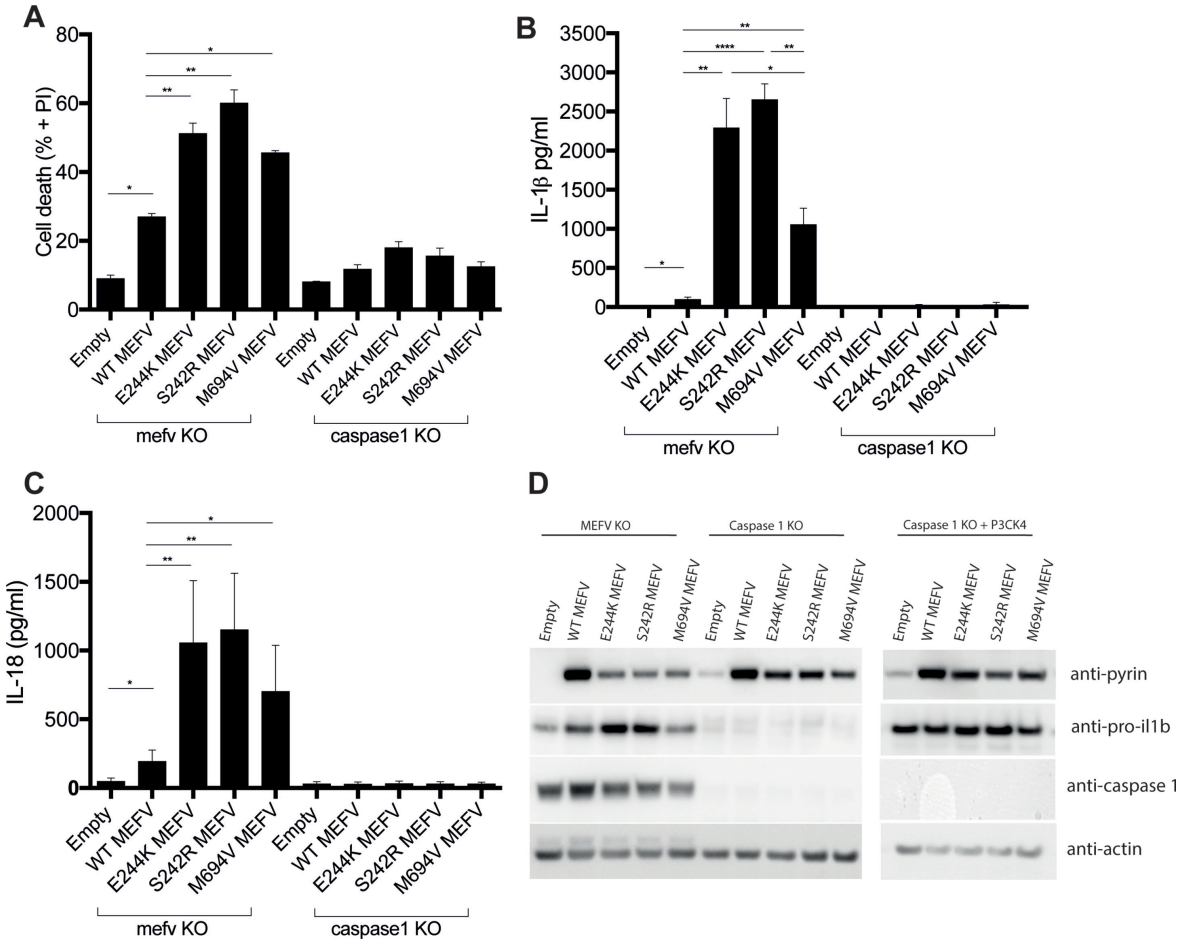

Figure 4 Pyroptosis and cytokine production by p.E244K pyrin. Monocytic THP-1 cells with pyrin or caspase-1 deleted by CRISPR were reconstituted with wild type (WT) and mutant pyrin using lentiviral vectors. (A) Cell death was measured by propidium iodide (PI) staining and flow cytometry, and (B) IL-1β and (C) IL-18 measured by ELISA 48 hours after lentiviral infection. The increased cell death (A), IL-1β (B) and IL-18 (C) seen in the pyrin mutants was abrogated in the caspase-1 KO THP1 cells. (D) Whole cell lysate was prepared from THP-1 cells and western blotting was performed, probing for pro-IL-1β to determine the mechanism of the IL-1β response. *CASP1* KO cells were further treated with Pam3CSK4 to look at a physiological priming response to ensure that pro-IL-1β could be generated in this cell line. Data pooled from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

binding of WT PSTPIP1 to WT, PAAND and FMF associated pyrin, no significant difference was observed. This suggests that the mechanism of this disease is not related to increased PSTPIP1 binding (see online supplementary figure S4).

p.E244K pyrin has reduced phosphorylation of 14-3-3 binding motif and reduced 14-3-3 binding

The initial report of PAAND showed that the mechanism of increased inflammasome activation was loss of 14-3-3 binding to pyrin and subsequent loss of autoinhibition.[1] Given that p.E244 is the +2 position of a 14-3-3 binding motif (figure 1D), preliminary experiments were conducted to examine 14-3-3 binding to p.E244K pyrin. Serine residues at positions p.S208 and p.S242 have previously been shown to interact with 14-3-3[1,2] and were used as comparators. Immunoprecipitation was performed using GST-tagged WT and mutant pyrin transfected into HEK293T cells. This revealed reduced binding of an antibody that recognises phosphorylated serine in the 14-3-3 binding motif in mutants p.E244K and p.S242R pyrin, but not in the FMF-associated p.M649V pyrin (figure 5A). Binding of 14-3-3 to pyrin was also affected, following the same pattern. Further evaluation of binding of the 14-3-3τ and 14-3-3ε isoforms to these mutants, as well as p.S208A and p.S208A/S242R pyrin, showed no differences, suggesting both isoforms behave similarly (figure 5B). These data suggest that PAAND pyrin mutations result in reduced phosphorylation of the 14-3-3 binding motif and reduced 14-3-3 binding to pyrin.

The p.E244 position is important in 14-3-3 binding to pyrin

Phosphorylated serine in specific motifs is important for 14-3-3 binding. Previous reports had suggested that proline was required at the +2 position of the motif for interaction between 14-3-3s and their target protein, documented as RXX(pS)XP (figure 1D). However, subsequent reports show that proline in +2 position is present in only 50% of 14-3-3 binding motifs.[24] To explore the importance of the +2 position in 14-3-3 binding and pyrin

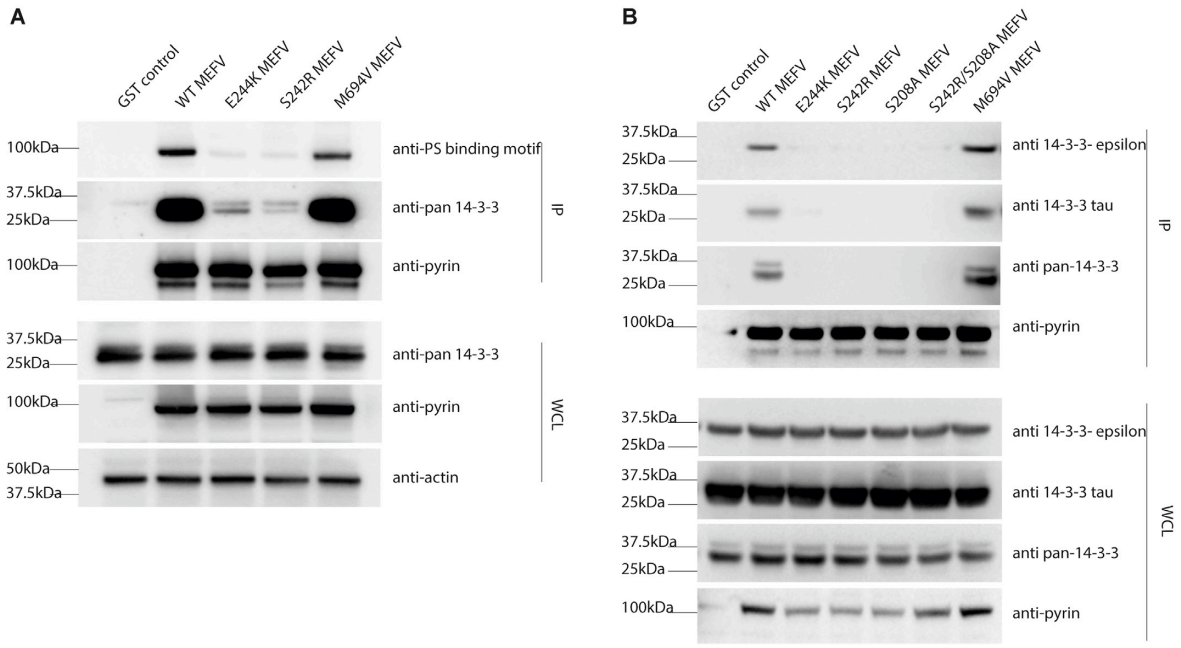

Figure 5 Reduced 14-3-3 binding by p.E244K pyrin. HEK293T cells were transfected with GST-tagged pyrin, with or without Pyrin-Associated Autoinflammation with Neutrophilic Dermatosis (PAAND) and Familial Mediterranean Fever (FMF) mutations, and immunoprecipitation performed. (A) Western blot was performed to compare phosphoserine (PS) 14-3-3 binding motif, pan-14-3-3 binding and pyrin expression in immunoprecipitate (IP) and whole cell lysate (WCL). Comparison was made to p.S242R and p.M694V pyrin. (B) Western blot was performed to compare pan-, τ or ε 14-3-3 binding and pyrin expression in IP and WCL. Comparison was made to p.S242R, p.S208A, p.S208A/S242R and p.M694V pyrin. Representative of three independent experiments.

regulation, p.E244 pyrin was mutated to various amino acids. Glutamate (E) was substituted by aspartate (D) or arginine (R) to explore charge and polarity, respectively, or proline (P) to explore the effect of the canonical 14-3-3 binding motif. Flow cytometric analysis of these mutants showed an increase in ASC speck formation in p.E244R pyrin mutant, while p.E244D and p.E244P pyrin mutations did not further activate pyrin in this assay (figure 6A). Immunoprecipitation showed that p.E244R pyrin had reduced binding to 14-3-3 when compared with WT, similar to p.E244K (figure 6B). Interestingly, p.E244P pyrin had increased 14-3-3 binding, suggesting that this mutation could potentially suppress pyrin activation. To test this hypothesis, cells were treated with the RhoGTPase inhibitor, TcdB, to activate pyrin. Although p.E244P increased binding of 14-3-3 to pyrin, this was insufficient to prevent activation by TcdB (figure 6C). Furthermore, the double mutant p.E244P/M694V had no effect on this, highlighting again a distinct pathophysiological mechanism of FMF and PAAND (figure 6C).

DISCUSSION

The initial clinical suspicion of PAPA syndrome in the index patient highlights the striking clinical overlap between PAAND and PAPA syndrome, as noted in the original description of PAAND.[1] Compared with the initial report, our family is distinct in suffering from polyarthritis as well as severe hidradenitis suppurativa, suggesting that even within the PAAND diagnosis, there is variability in clinical presentation, consistent with a spectrum of pyrin-associated features. Our results agree with the original description of PAAND, namely that excessive IL-1β is pyrin dependent. Although PAPA syndrome is also pyrin dependent,[23] the exact mechanisms underlying the similar clinical presentations of PAAND and PAPA syndrome have not been elucidated. We suggest that patients with clinically suspected PAPA syndrome who test negative for *PSTPIP1* mutations should undergo genetic evaluation of *MEFV*, with particular attention to the bases in exon 2 encoding 14-3-3 binding motifs.

The role of 14-3-3 in controlling the activation of pyrin is highlighted by this novel mutation causing PAAND. Reduced binding of 14-3-3 to pyrin was seen with both p.E244K and p.S242R pyrin, but not in the FMF-associated p.M694V mutation (figure 5). The loss of 14-3-3 binding following stimulation with TcdB suggests that 14-3-3 is required to maintain pyrin in an auto-inhibited state and reduced 14-3-3 binding to PAAND-associated pyrin leads to its auto-activation. We propose that with the same expression of pyrin across the mutants examined in our model, the PAAND pyrin is likely to be more active, with increased pyroptosis and availability of pro-IL-1 for cleavage. It is possible that PAAND is at one spectrum of pyrin-associated disorders in terms of severity, with PAAND pyrin being spontaneously active and FMF pyrin having a lower threshold for activation than WT pyrin.

The 14-3-3 binding motif of pyrin differs from the canonical RXX(pS)XP motif with a highly conserved glutamate at the +2 position. Substituting glutamate for proline or aspartate,

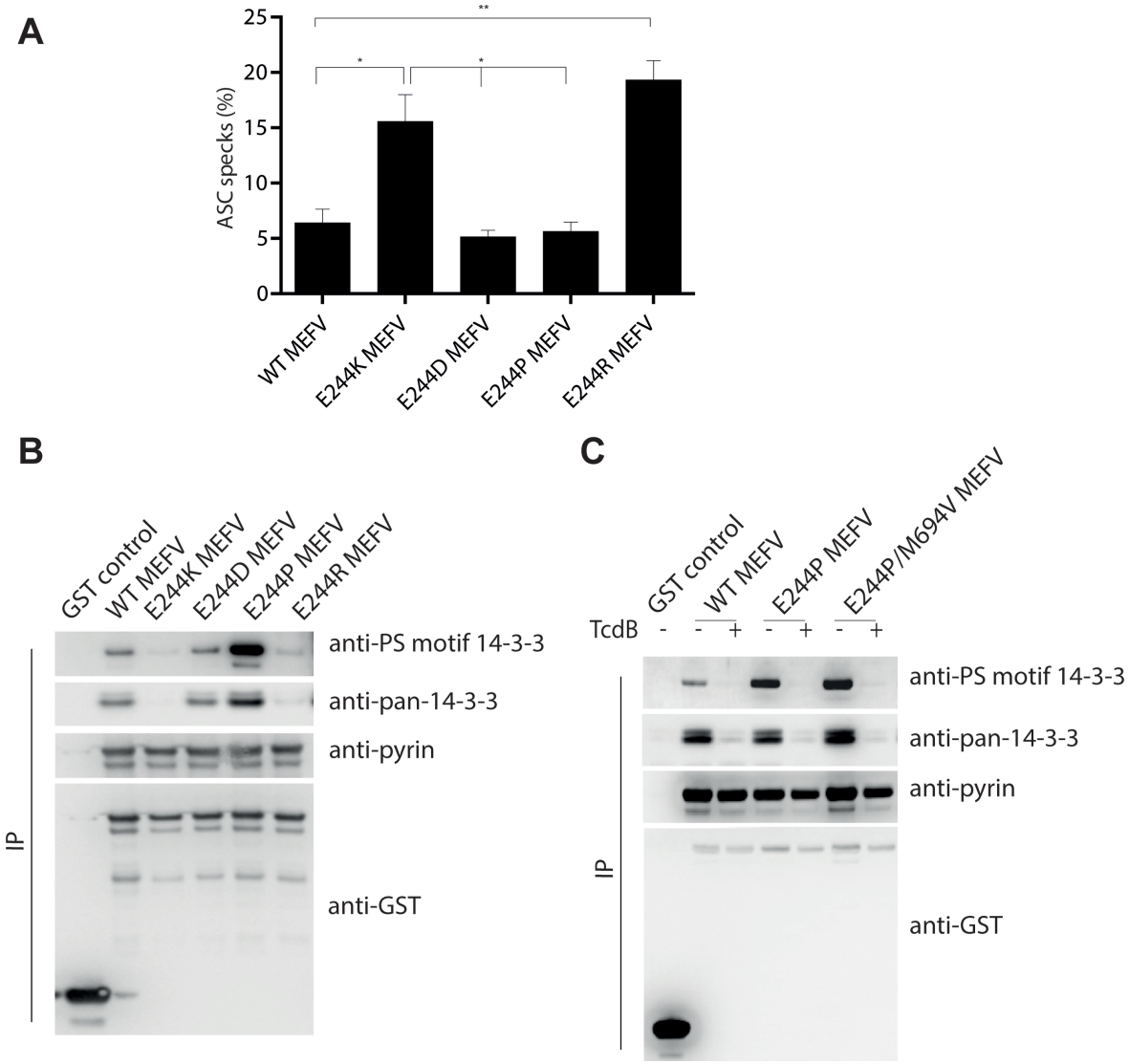

Figure 6 +2 position of 14-3-3 binding site is important in regulation of pyrin activation. (A) Flow cytometric analysis of Apoptosis-associated Speck-like protein containing a Caspase recruitment domain (ASC) speck formation performed on HEK293T cells transfected with mCherry-tagged pyrin with various mutations at position p.E244 and GFP-tagged ASC. Data pooled from three independent experiments. (B) HEK293T cells were transfected with GST-tagged pyrin with various mutations at position p.E244, and then immunoprecipitated and blotted with antibodies to detect the phosphorylated 14-3-3 binding motif, pan-14-3-3, pyrin or the GST tag. (C) Immunoprecipitation was performed as described above, but with *Clostridium difficile* Toxin B protein (TcdB) stimulation for 16 hours, to assess phosphorylation of 14-3-3 binding sites and 14-3-3 binding. Representative of three independent experiments. *p<0.05, **p<0.01. IP= immunoprecipitate.

non-polar and negatively charged amino acids, respectively, retained 14-3-3 binding to pyrin, whereas substitutions to lysine or arginine, both positively charged amino acids, do not appear to be tolerated. The structure of this region of pyrin has not been elucidated, making it difficult to predict the effect of amino acid substitutions. However, we demonstrate that the +2 position of the 14-3-3 binding motif is important, and that substitution at this site can alter the ability for 14-3-3 to bind to pyrin.

Although M694V pyrin results in increased inflammasome formation[25], the mechanism of auto-activation still remains to be elucidated. We saw no discernible difference between WT and p.M694V pyrin with regards to 14-3-3 binding, and Van Gorp *et al* documented unaltered phosphorylation at position p.S242 in p.M694V pyrin transfected HEK293T cells, which is required for 14-3-3 binding.[26] Interestingly, Park *et al* did see reduced 14-3-3ε binding in FMF-associated mutations.[5] It is possible that subtle differences in the experimental approach may influence this result, and given that PAAND is a more severe disease, we would expect FMF mutations to have a smaller mechanistic effect on 14-3-3 binding.

In addition to 14-3-3 binding, the clinical presentation, mode of inheritance and biochemical status of PAAND differ from

FMF. Although this study focuses on only two generations of one family, the heterozygous mutation and variable phenotype suggest a dominant disorder with variable penetrance, compared with the typically autosomal recessive inheritance of FMF. All members of the PAAND family have marked dermatological manifestations, further differentiating this condition from FMF.

Another distinction between these pyrin-associated conditions is evident from the serum cytokine profile, and cytokine production by PBMCs, both at baseline and after LPS-priming. Although the FMF patients were asymptomatic, there was evidence of systemic inflammation with raised CRP in four of the five controls (see online supplementary table S1). Furthermore, their serum cytokine profile was distinct from healthy controls as well as PAAND patients, suggesting that there are indeed differences that are not accounted for by symptom control.

Despite elevated IL-1β in these analyses, one patient with the p.E244K mutation did not improve with a trial of anakinra. Interestingly, the elevated IL-1Ra levels in this individual may explain why a recombinant IL-1Ra did not provide further benefit. Our FMF patients did not have elevated IL-1Ra levels, and a number of recent publications suggest that colchicine-resistant FMF can be adequately treated with IL-1 antagonism.[27–29] Despite elevated IL-18 levels in PAAND PBMC secreted at baseline and in response to LPS (figure 2F), an increase in IL-18BP levels (figure 2B) suggests that targeting this pathway may not be as effective as shown for patients with activation of the Nod-Like Receptor CARD containing protein 4 (NLRC4) inflammasome.[30] The clinical response to TNF inhibition in our patient suggests that this is an important cytokine in PAAND, even though TNF was not elevated in the serum of these patients. This may be because at the time of the study, the patient was receiving treatment with immunomodulatory drugs including adalimumab. Alternatively, increased cell death in PAAND (figure 4A) could release damage-associated molecular patterns that trigger local cytokine production in tissues such as the skin. Furthermore, it would be interesting to assess tissue specific cytokines and cell responses as these may reveal pathogenic factors not present in peripheral blood. Regardless, given the difficulty controlling disease activity and the need for multiple therapeutic agents, PAAND is likely to be driven by more than a single cytokine.

The p.E244K pyrin mutation in PAAND patients highlights the importance of the 14-3-3 binding motif in pyrin activation, in addition to the p.S242R mutation described originally. Our study suggests that although PAAND and FMF mutations are located in the same gene, they are distinct diseases clinically, with unique cytokine profiles, cellular responses and 14-3-3 binding.

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Contributors FM, JIA, SLM: Conception and design of the work. FM, RL, DDN, HMB, JJMG, PMdeC, PJB, VG, AMV, SC, IPW, PP, JIA, SLM: Performed experiments, data collection, analysis and interpretation. All authors were involved in drafting and approval of the manuscript.

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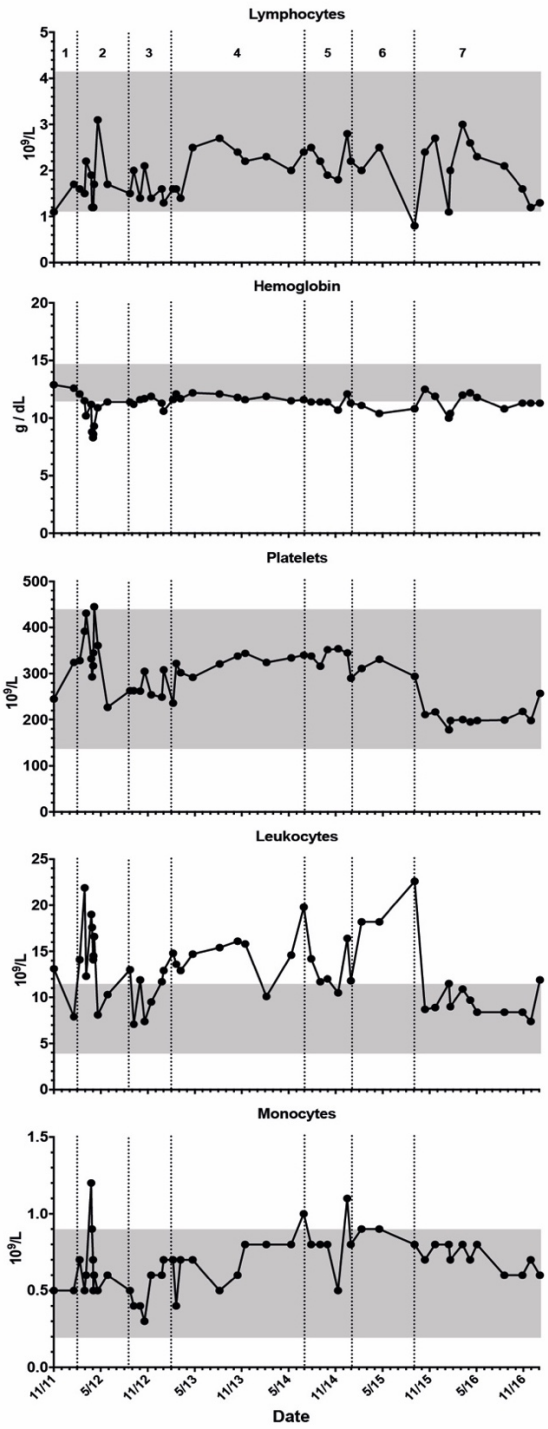
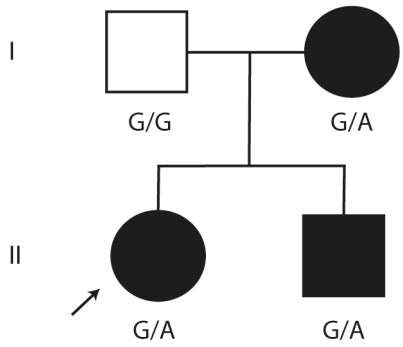
Supplementary Figure 1



Supplementary Figure 1: Further clinical information. Haematological and biochemical data of index case charted across time, with treatment periods 1. infliximab + methotrexate; 2. prednisolone + cyclosporin A; 3. prednisolone + infliximab; 4. prednisolone + doxycycline; 5. prednisolone + anakinra + clindamycin; 6. prednisolone + clindamycin + moxifloxacin + dapsone; 7. prednisolone + clindamycin + moxifloxacin + dapsone + adalimumab.

Supplementary Figure 2



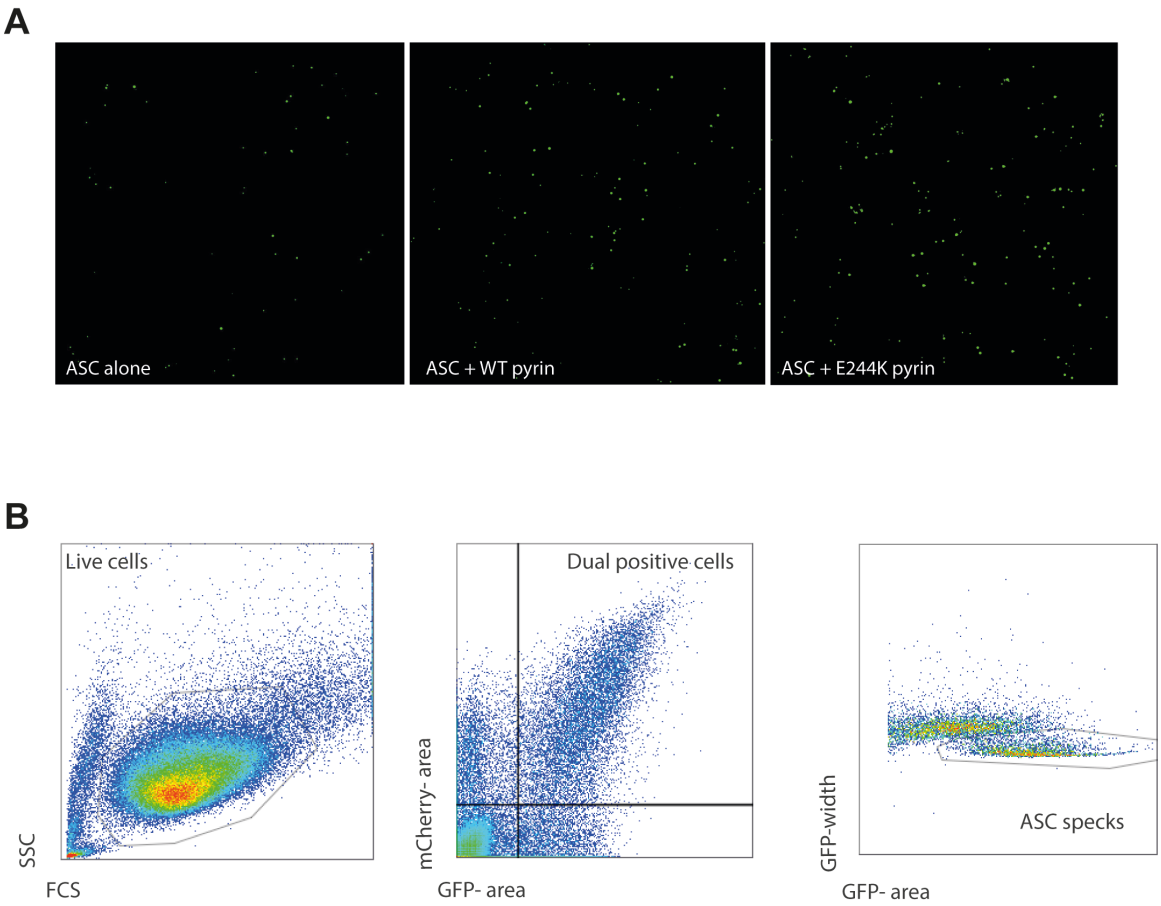
Supplementary Figure 2: Pedigree of the family. Solid symbols (squares=male, circles=females) denote affected individuals. Open symbol denotes unaffected individuals. The arrow indicates the proband of the family.

Supplementary Table 1

Control number	Genotype	Symptomatic	Treatment	CRP (<10mg/L)
1	M694V/M694V	No	Colchicine	12.7
2	M694V/M694V	No	Colchicine	20.3
3	M694V/M694V	No	Unknown	3.2
4	M694V/M694V	No	Unknown	70.9
5	M694V/M694V	No	Unknown	45.2

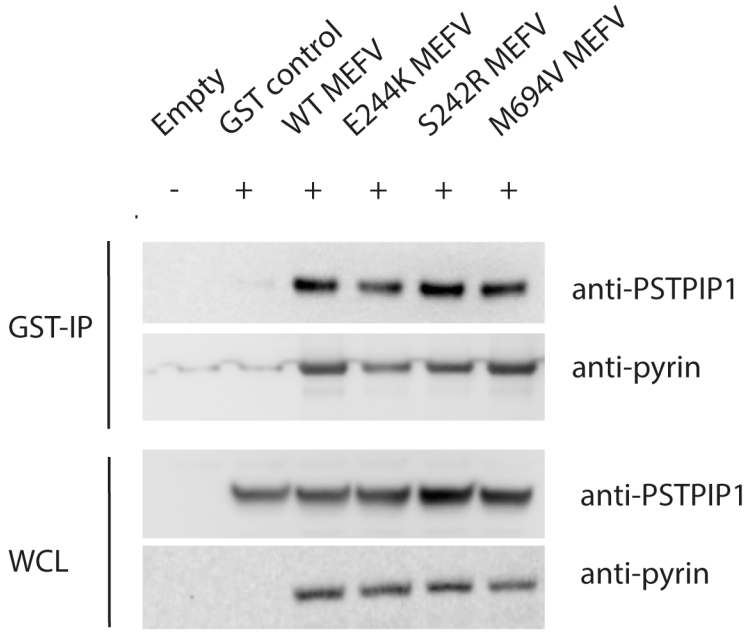
Supplementary Table 1: Summary of FMF controls. Table of FMF controls on whom serum cytokines assessed. All patients were asymptomatic but four of five had evidence of subclinical inflammation.

Supplementary Figure 3



Supplementary Figure 3: ASC Speck analysis. (A) Larger field of view of immunofluorescence showing background ASC speck formation, and an increase with WT and pyrin p.E244K transfection. (B) Gating strategy for quantification of ASC speck formation. HEK293T cells were gated on forward and side scatter, selecting live cells. Dual GFP and mCherry positive cells were selected. ASC specks were determined by reduced width and increased area of GFP signal.

Supplementary Figure 4



Supplementary Figure 4: Binding of PSTPIP1 to PAAND pyrin is unaltered. HEK293T cells were transfected with GST-tagged pyrin, with or without PAAND and FMF mutations, in addition to WT PSTPIP1. GST-immunoprecipitation performed and comparison of PSTPIP1 binding to various MEFV constructs made. Representative of three independent experiments.

3.3 Discussion

Evidence of pathogenicity of p.Glu244Lys *MEFV* found in a family with PAAND is provided here using ASC speck formation as a surrogate marker for inflammasome activation, as well as a THP1 monocyte-like cell reconstitution system documenting Caspase-1-dependent IL-1β, IL-18 and cell death. Based on work presented in **Figure 6**, p.Glu244Arg pyrin is also predicted to be pathogenic and may result in a similar presentation to PAAND depending on the penetrance of the genotype. Supporting evidence for the importance of the 14-3-3 binding motif is the infrequency of SNPs in conserved positions -2, 0 or +2 on databases of healthy controls (Lek et al., 2016).

Despite both being caused by mutations in *MEFV*, the clinical features of FMF and PAAND are markedly disparate. One hypothesis is that FMF and PAAND represent disorders on a continuum of pyrin-associated autoinflammatory diseases. The reduced binding of 14-3-3 to pyrin seen in FMF patient PBMCs compared with healthy control PBMCs when stimulated with IFN-γ to increase pyrin expression and LPS suggests that reduced binding of 14-3-3 may be common to both conditions. This is in contrast to data presented in **Figure 5** of the manuscript (Moghaddas et al., 2017). An important experiment to clarify this would be the comparison of 14-3-3 binding to endogenous pyrin of unstimulated PBMCs from patients with FMF and PAAND as well as healthy controls. This may be technically difficult due to the low expression of endogenous pyrin in PBMCs without stimulation (Centola et al., 2000).

Importantly, if PAAND and FMF are indeed on a continuum of pyrin-associated disorders, this spectrum does not necessarily equate to severity. Although the dermatological features of PAAND are indeed striking, these patients have, thus far, not developed consequences of amyloidosis despite long periods of uncontrolled inflammation. Furthermore, evidence is

provided in **Figure 2** of the manuscript that the cytokine profile is also distinct suggesting that these two conditions may have separate pathophysiological mechanisms (Moghaddas et al., 2017).

It is possible that mutations in the 14-3-3 binding domain of pyrin may alter the binding or function of pyrin interacting proteins that mutations in the B30.2 domain do not. These interacting proteins may have differential tissue expression leading to different presentations of PAAND and FMF. A key candidate for this is PSTPIP1, especially given the clinical similarities between PAAND and PAPA syndrome. As discussed in **Section 3.1.4.4**, the literature suggests that PSTPIP1 interacts with pyrin, and PAPA-associated mutant PSTPIP1, in a hyperphosphorylated state, binds more strongly to pyrin. Through an siRNA pyrin knock down model in THP1 cells with mutant PSTPIP1 overexpressed, the IL-1β and cell death seen was pyrin-dependent (J. W. Yu et al., 2007). The mechanism by which the increased binding of PSTPIP1 leads to increased pyrin activity is uncertain. It is possible that PSTPIP1 is required for the activation of pyrin, and that an increased binding affinity of PSTPIP1 to pyrin leads to increased inflammasome activity. PSTPIP1 could mediate this itself through mechanisms that are currently unknown, or alternatively it could be acting as a binding partner between pyrin and another protein such as PTP-PEST or WASp. For example, PSTPIP1 could compete with PKN1 binding to pyrin resulting in reduced phosphorylation of p.Ser242.

Experimental evidence provided in **Figure S4** of the manuscript suggests that the binding of PSTPIP1 to pyrin is not affected by the presence of PAAND mutations. Further evaluation of the role of PSTPIP1 in PAAND could include the creation of *PSTPIP1* KO THP1 cells through CRISPR/Cas9 gene editing techniques**.** By stimulating these cells with TcdB, it could be determined whether activation of the pyrin inflammasome pathway is possible in the absence

of PSTPIP1. Furthermore, comparison of cytokine release and cell death between WT and PSTPIP1 KO cells after transduction of with a lentiviral cDNA pyrin construct harbouring the p.Glu244Lys mutation would address whether the increased inflammation seen in PAAND is dependent on PSTPIP1.

Another approach to clarifying the possible link between these two conditions is the assessment of the phosphorylation of p.Ser242 of pyrin and 14-3-3 binding in the presence of either WT or PAPA-associated PSTPIP1. This line of enquiry would determine whether the presence of PSTPIP1 alters the phosphorylation status of pyrin. The HEK293T overexpression system optimized in **Figure 5** and **Figure 6** of the manuscript could be used with WT or mutant PSTPIP1 transfected with WT pyrin with subsequent immunoprecipitation of pyrin. Western blot for phosphoserine binding motif and total 14-3-3 binding to pyrin would be informative. An important caveat to this experiment is that the binding partners of PSTPIP1 and pyrin, such as PTP-PEST or PKN1 respectively, are not transfected and may not be adequately expressed in HEK293T cells.

An alternative consideration is the possible presence of a variant in another gene that leads to a distinct clinical presentation in patients with PAAND compared with patients with FMF, potentially defining it as a digenic rather than monogenic disorder (**Chapter 6**). The family presented here had exon 2 of *MEFV* sequenced via Sanger technique as the clinical suspicion of PAAND was high. Assessment for the presence of another potentially relevant genetic variant is not possible. The original PAAND publication by Masters et al. described 17 cases with p.Ser242Arg pyrin mutation, but noted that one of these patients did not have features consistent with PAAND and another was asymptomatic at the time of the report (Masters et al., 2016). The large pedigree presented had limited WES performed and then Sanger

sequencing to determine segregation with disease in the remaining members of the family. Is it possible that the two cases that are inconsistent with the presentation lack another variant of importance? An approach that could be taken is to perform WES of all patients with PAAND and their family and analyse for linkage through two-locus linkage analysis.

Highlighted in this study of one family with a dominantly inherited autoinflammatory condition is the phenotypic differences between two conditions, FMF and PAAND, that are caused by different mutations in one gene, *MEFV*, encoding the protein pyrin. Given that there have only been two publications of PAAND, the spectrum of clinical features is likely to broaden with description of more cases. The description presented opens up the possible link between pyrin, PSTPIP1 and their respective binding partners that would be interesting to explore further. Additionally, it prompts consideration of alternative explanations for such phenotypic variability and whether another contributing variant is yet to be discovered.

4 AUTOINFLAMMATION AND MACROPHAGE ACTIVATION SYNDROME CAUSED BY MUTATION IN THE LEUCINE RICH REPEAT DOMAIN OF NLRC4 DELINEATES MECHANISMS OF INFLAMMASOME ASSEMBLY.

4.1 Introduction

Gain of function mutations in NLRs are the cause of a number of monogenic AIDs including CAPS (**Section 1.1.1.1.2.2)**, autoinflammation with arthritis and dyskeratosis (**Section 1.1.1.1.2.1**), and NLRC4-associated autoinflammatory disorders (NLRC4-AID) (**Section 4.1.4**). Although increased inflammasome formation has been documented in most cases, the exact mechanism of auto-activation in the context of disease is unclear. Setting NLRC4 apart from other NLRs is that a crystal structure of the murine homologue has been elucidated. This has led to the ability to model disease causing mutations and predict the impact of amino acid substitutions on the structure and function of NLRC4.

Here, the current understanding of NLRC4, as well as inhibited and active structural conformation information, are used to investigate the pathogenicity as well as potential mechanisms of increased activation of a novel variant in the LRR domain of NLRC4 in two unrelated children with autoinflammation and MAS. Provided in this introduction is a detailed review of the current understanding of the structure, function, and effector mechanisms of NLRC4, summarized in **Figure 4.4**. This background information is vital for understanding the choice of experiments performed, the interpretation of results presented throughout the chapter as well as the discussion that follows.

4.1.1 NLRC4 structure

NLRC4, also known as Ipaf, CARD12 or CLAN, was identified by three groups independently in 2001 through a search of CARD encoding cDNA from a human peripheral blood leukocyte library (Damiano, Stehlik, Pio, Godzik, & Reed, 2001; Geddes et al., 2001; Poyet et al., 2001). Similar to NOD1 and NOD2, homologues of the *C. Elegans* apoptosis initiator caspase adaptor protein CED-4, NLRC4 contains a NOD and a C-terminal LRR domain in addition to an N-terminal CARD (Damiano et al., 2001; Geddes et al., 2001; Poyet et al., 2001) (**Figure 4.1**). The crystal structure of the closed murine NLRC4, sharing 75% sequence identity with human NLRC4, has been defined, with ADP mediated winged helix domain (WHD) and NOD interaction important for autoinhibition (Hu et al., 2013).

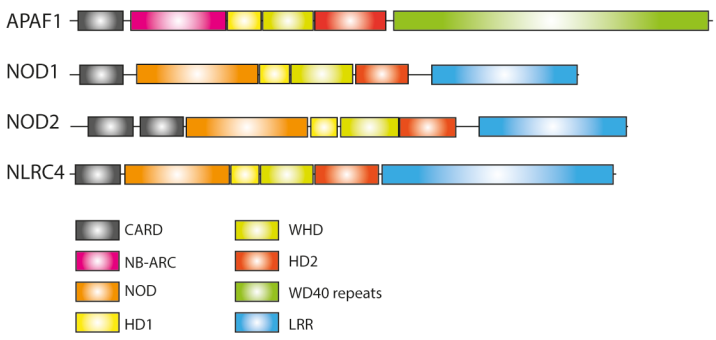


Figure 4-1 Human homologues of the C. Elegans apoptosis initiator caspase adaptor protein.

All members of this family contain a caspase activation and recruitment domain (CARD), and NOD1 and 2 have a nucleotide oligomerisation binding domain (NOD), hinge domain 1 and 2 (HD1, HD2), winged helix domain (WHD) and leucine rich repeat domain (LRR) in common with NLRC4. NB-ARC = nucleotide-binding adaptor shared by APAF-1, certain R gene products and CED-4.

4.1.2 NLRC4 function

Immunoprecipitation of NLRC4 from co-transfection experiments performed in HEK293T cells suggested early on that NLRC4 associates with Caspase-1 (Damiano et al., 2001; Geddes et al., 2001), and possibly with ASC as determined by a mammalian two-hybrid analysis (Geddes et al., 2001). Interestingly, Poyet et al. predicted that NLRC4 was engaged in CARD-

mediated oligomerization and documented CARD-dependent activation of pro-Caspase-1, as well as the inhibitory role of the LRR domain (Poyet et al., 2001). Additionally, luciferase assay experiments suggested that, unlike NOD1 and NOD2, there was little induction of NF-κB with transfection of NLRC4 in HEK293T cells (Poyet et al., 2001).

Since the work by Poyet et al., the inflammasomes have been defined (**Section 1.1.1.1**), followed by a plethora of work elucidating the role of NLRC4 in response to pathogens. Dixit and colleagues challenged LPS stimulated WT and *Nlrc4*$^{-/-}$ macrophages with *Salmonella typhimurium* and determined that the cell death and IL-1β secretion seen were dependent on NLRC4 (Mariathasan et al., 2004). Cytosolic flagellin was subsequently identified as the component of *Salmonella* required for this response (Franchi et al., 2006; Miao et al., 2006). Miao et al. compared the response of BMDMs to purified flagellin and determined that cells responded with high cell death and IL-1β secretion only when flagellin was transfected, and that this response was completely lost in *Nlrc4*$^{-/-}$ BMDMs (Miao et al., 2006). Furthermore, using murine macrophages that lack expression of TLR5 or MyD88, the authors also confirmed that the response to cytosolic flagellin was independent of the TLR5 pathway. Nunez and colleagues adopted a similar approach, infecting WT, *Nlrc4*$^{-/-}$, and *Tlr5*$^{-/-}$ BMDMs with either WT *Salmonella* or the *fliC-fljB* mutant strain lacking flagellin (Franchi et al., 2006). Both NLRC4 and flagellin were required for cleavage of pro-Caspase-1 to its active form and for the secretion of IL-1β.

4.1.2.1 NAIP as a sensor of flagellin and Type III secretion system components.

Given these findings, the working hypothesis at the time was that NLRC4 was a direct sensor of flagellin. The complexity of activation was highlighted, however, when Miao et al. provided evidence that rod proteins of a number of bacteria, sharing a similar sequence motif with

flagellin, could also activate the NLRC4 inflammasome (Miao, Mao, et al., 2010). The rod protein, a component of the T3SS found in a number of gram-negative pathogens, enables bacterial effector proteins to be delivered into the cytoplasm of cells. A schematic representation of flagellin and T3SS is presented in **Figure 4.2.**

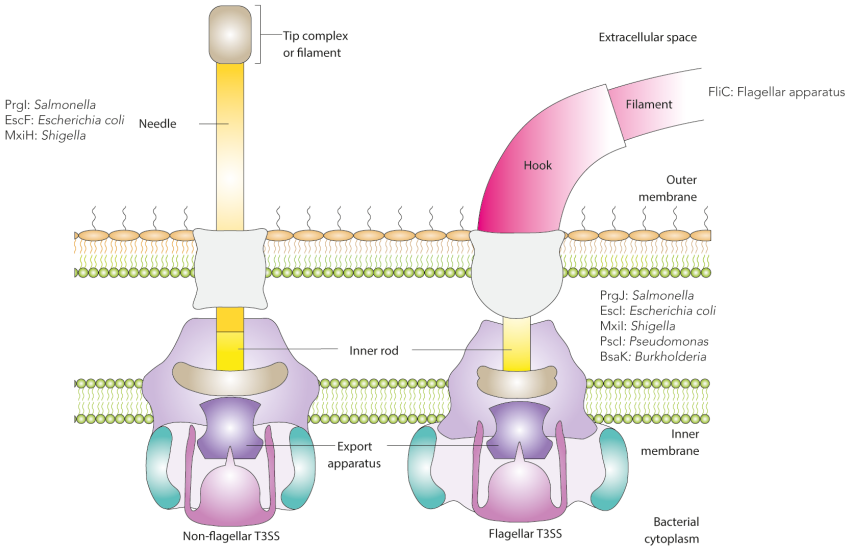


Figure 4-2 Schematic representation of T3SS and flagellin.

Type three secretion system (T3SS) apparatus (left) and flagelar apparatus (right) shown highlighting key features enabling an NLRC4 response such as needle protein, inner rod protein and the filament. Included in the figure are T3SS components used in experiments performed in this thesis, including PrgI, the needle protein of *Salmonella typhimurium*. Adapted from (W. Deng et al., 2017).

An important observation made in the context of *Legionella pneumophila* infection was the association of neuronal apoptosis inhibitory protein (NAIP) 5 to NLRC4 (Zamboni et al., 2006). NAIP, also known as baculovirus inhibitor of apoptosis repeat-containing 1 (BIRC1) protein, is a homologue of NLRC4 and a member of a family of IAP proteins. Well studied members of the IAP family, XIAP and cIAP1 and 2, interact with and regulate TNF, TLR and NOD receptor mediated signalling pathways. The IAPs are related by the presence of one to three BIR domains which are essential for mediating protein-protein interactions. Unlike other

IAPs, NAIP lacks E3 ligase activity and contains a NOD and LRR domain (**Figure 4.3**) (Miller, 1999).

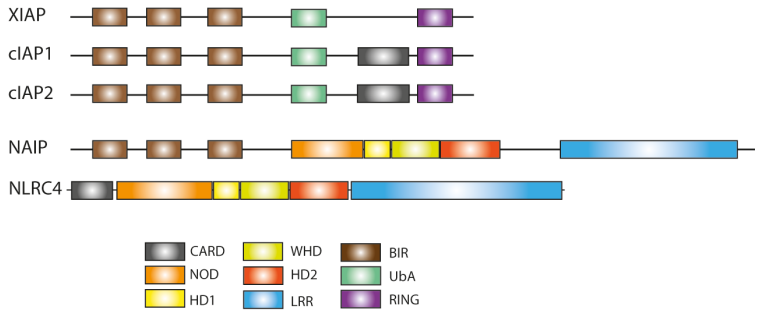


Figure 4-3 Human IAP family of proteins.

The inhibitor of apoptosis proteins (IAPs) are unified by their baculovirus inhibitor of apoptosis repeat (BIR) domains. Besides NAIP, IAPs harbour RING domains which mediate interactions with E2 ligases, and ubiquitin associated (UbA) domains. CARD = caspase associated recruitment domain, NOD = nucleotide oligomerisation binding domain, HD1 = hinge domain 1, WHD = winged helix domain, HD2 = hinge domain 2, LRR = leucine rich repeat domain.

A murine *L. pneumophila* susceptibility locus was identified containing at least six homologous copies of *Naip* (Diez, Yaraghi, MacKenzie, & Gros, 2000). Subsequent work by Zamboni et al. highlighted that in HEK293T cells infected with *L. pneumophila,* transfection of plasmids expressing NAIP5 and Caspase-1 was required for cell death (Zamboni et al., 2006). Both the BIR and NOD domains of NAIP5 were required, and the bacterial products needed to be present in the host cytosol, for activation of Caspase-1 to occur. To investigate the possible adaptor protein between NAIP5 and Caspase-1, the authors infected *Asc*$^{-/-}$ and *Nlrc4*$^{-/-}$ macrophages with *L. pneumophila*. NLRC4, but not ASC, was required for restriction of bacterial replication.

This finding prompted a number of groups to explore in more detail the role of NAIP in the activation of NLRC4. In an elegant series of experiments, Kofoed and Vance explored the

function of the four known murine NAIP proteins (Kofoed & Vance, 2011). Primary BMDMs with *Naip2* knocked down using shRNAs had reduced cell death and Caspase-1 cleavage in response to infection with *Listeria monocytogenes* expressing PrgJ when compared with control cells. Using HEK293T cells transfected with NLRC4, Caspase-1 and NAIP5 cDNA containing plasmids, the authors determined that the cell death seen in response to flagellin required all components to be present, suggesting that NAIP5 could not activate Caspase-1 directly. A similar cotransfection experiment was used with NLRC4, Caspase-1 and either murine NAIPS 1, 2, 5 or 6. Cell death was increased when NAIP2 expressing cells were transfected with PrgJ, whereas NAIP5 or NAIP6 expressing cells died in response to flagellin. The specificity of response was further supported by blue native page analysis of NLRC4, showing formation of an oligomer in response to flagellin when cotransfected with NAIP5 or NAIP6, or in response to PrgJ when cotransfected with NAIP2. These findings were independently confirmed by Shao and colleagues, who then extended the work by providing evidence in coimmunoprecipitation assays that NAIP2 can interact with PrgJ (Zhao et al., 2011). Furthermore, they explored the role of human NAIP. Unlike its murine orthologue, human NAIP is encoded by a single gene. Human U937 monocyte-like cells demonstrated NLRC4- and ASC-dependent Caspase-1 cleavage in response to a needle subunit of *Chromobacterium violaceum*, but not rod proteins or flagellin. Since this finding, two groups have reproduced this result, and clarified the role of NAIP1 and human NAIP in the response to needle proteins (Rayamajhi, Zak, Chavarria-Smith, Vance, & Miao, 2013; J. Yang, Zhao, Shi, & Shao, 2013). The limitations of performing experiments on, and drawing conclusions from, tumour derived cells such as U937 and THP1 cells were underlined when investigation of primary macrophages from healthy donors demonstrated a robust NAIP-dependent cell death and IL-1β response to flagellin (Kortmann, Brubaker, & Monack, 2015). Two isoforms of NAIP were identified in the primary cells, whereas tumour-derived cells only expressed one.

The ability of U937 cells to respond to flagellin was restored when the full length NAIP isoform was expressed. More recently, full length human NAIP has been shown to recognize rod and needle proteins, as well as flagellin (Reyes Ruiz et al., 2017). The mechanism by which a single protein is able to recognize such a range of ligands is, thus far, unclear. Altogether, the evidence to date demonstrates that NAIP recognizes certain bacterial effector proteins in the cytosol and induces an NLRC4-dependent inflammasome response.

Structural modelling of murine NLRC4 in its active form with NAIP2 and PrgJ using cryo-electron microscopy revealed a wheel like structure, with a single NAIP2 and PrgJ in a complex with 10 or 11 NLRC4 monomers (Hu et al., 2015; Zhang et al., 2015). In response to PrgJ, NAIP2 undergoes a conformational change, initially proposed by Vance and colleagues (Tenthorey, Kofoed, Daugherty, Malik, & Vance, 2014), and exposes a basic catalytic surface which is complementary to an acidic receptor surface on NLRC4 (Hu et al., 2015; Zhang et al., 2015). This interaction creates a conformational change, exposing a catalytic surface on NLRC4 that can initiate the same conformational change in NLRC4 monomers. This complex forms a suitable platform for Caspase-1 activation. Recently, a similar model was published of a flagellin-NAIP5-NLRC4 complex, again highlighting the ability of NLRC4 to self-propagate in its active conformation (Tenthorey et al., 2017).

4.1.2.2 Role of ASC in Caspase-1 activation and IL-1β release

Classically, as in the case of pyrin and NLRP3, an inflammasome-forming immune sensor associates with the adaptor protein ASC, with subsequent ASC and Caspase-1 coupling through their respective CARDs. As NLRC4 has a C-terminal CARD, the role of ASC in NAIP-NLRC4 inflammasome is less clear. Dixit and colleagues examined the role of both ASC and NLRC4 in *S. typhimurium* infection of murine macrophages (Mariathasan et al., 2004)

(**Section 4.1.2**). *Asc*$^{-/-}$ macrophages failed to cleave Caspase-1 as determined by western blot analysis of WCL after infection at time points 0, 10, 20 and 70 minutes. Furthermore, mature IL-1β release was also completely abrogated, a result since independently reproduced (Proell, Gerlic, Mace, Reed, & Riedl, 2013). This abrogation was also seen with *Nlrc4*$^{-/-}$ macrophages, suggesting that ASC or NLRC4 are necessary for the cell death and IL-1β cytokine response to *S. typhimurium*, and raising the possibility that ASC acts downstream of NLRC4. Interestingly, at later time points, the cell death of the *Asc*$^{-/-}$ macrophages was increased compared with that of WT macrophages in response to infection with *S. typhimurium.* No Caspase-1 cleavage was detected, suggesting a Caspase-1-independent cell death process. Importantly, cell death in the *Nlrc4*$^{-/-}$ macrophages remained low, even at this extended time point. A possible explanation is that ASC and NLRC4 are required for the initial Caspase-1 mediated cytokine response, and that NLRC4 may have a distinct (non-Caspase-1) pathway for mediating later cell death. In an attempt to explore this further, Miao et al. transfected flagellin into LPS primed *Asc*$^{-/-}$ and *Nlrc4*$^{-/-}$ BMDMs and, using a time point of between 30-60 min, determined that IL-1β release was completely reduced by the deletion of *Nlrc4* and partially by the deletion of *Asc* (Miao et al., 2006) suggesting a non-essential role for ASC. In another model, ASC was dispensable for the control of *L. pneumophila* bacterial replication, but was absolutely required for IL-1β secretion seen in response to infection (Zamboni et al., 2006), highlighting the complexity of the role of ASC and NLRC4 in the response to infection. Furthermore, bone marrow-derived murine neutrophils exposed to *S. typhimurium* demonstrated a multiplicity of infection (MOI)-dependent release of IL-1β that was entirely Caspase-1- dependent and partially ASC-dependent (K. W. Chen et al., 2014). Importantly, unlike their BMDM counterparts, neutrophils did not undergo pyroptosis, and the pyroptosis seen in the BMDMs was ASC independent. Although the exact role or possible interaction between the two CARD containing proteins remains to be elucidated, this finding provides

further evidence that, at least in mice, distinct pathways may exist for ASC and NLRC4 in the response to certain gram-negative organisms and that this may be cell type specific.

4.1.2.3 Role of NLRP3

The observation that both NLRP3 and NLRC4 played a role in the host response to *S. typhimurium* led Bryant and colleagues to investigate whether these two NLRs were both recruited to an individual ASC speck within a cell (Man et al., 2014). Using super-resolution microscopy, the authors imaged endogenous NLRP3 and NLRC4 in THP1 cells after infection with *S. typhimurium*. Remarkably, each speck imaged contained both NLRP3 and NLRC4, with a distinct ring structure observed for each NLR. This relationship was further explored by Dixit and colleagues (Qu et al., 2016). Although they failed to detect NLRP3 in a chromatography fraction of macromolecular complexes of NLRC4 formed in response to *S. typhimurium*, they noted that NLRC4 and NLRP3 coimmunoprecipitated when BMDMs were primed with LPS and transfected with flagellin. This interaction was dependent on the NOD of NLRC4 but the exact mechanism, its relevance in other infection models or in human cells, remain to be explained.

4.1.2.4 Caspase-8 as an effector of NLRC4

The first examination of the role of Caspase-8 in NLRC4-induced cell death was made in the context of A549 human lung epithelial cells and a search for interacting partners of NLRC4. A yeast two-hybrid screen with NLRC4 as bait yielded a cDNA fragment encoding Sug1, with interaction assessed by coexpression in HEK293T cells and immunoprecipitation (Y. Kumar, Radha, & Swarup, 2010). The authors transfected Sug1, a component of the 26S proteasome, with NLRC4 lacking the LRR domain and noted cell death in A549 cells. This cell death was abrogated with the co-transfection of a construct expressing a dominant negative Caspase-8,

but not when a dominant negative Caspase-1 was expressed. NLRC4, Sug1 and Caspase-8 were shown to co-localise in cytoplasmic aggregates, with each cell containing multiple foci. The authors also explored the role of ubiquitination of NLRC4 in cell death and noted that a ubiquitinated NLRC4 interacts more strongly with Caspase-8 leading to increased cell death. Interpretation of these results requires caution, however, as the interaction between Sug1 and NLRC4 has not been independently verified, nor has the requirement for ubiquitination in NLRC4 induced cell death. Furthermore, all experiments were performed using overexpressed, rather than endogenous, proteins.

A number of groups have since provided evidence that Caspase-8 is a down-stream effector of NLRC4. Bryant and colleagues infected unprimed BMDMs with *S. typhimurium* and visualized cells shortly after to show colocalisation of ASC, Caspase-1 and Caspase-8 in a single focus per cell (Man et al., 2013). The authors subsequently showed this colocalisation in THP1 cells (Man et al., 2014). The Caspase-8 cleavage seen in response to *S. typhimurium* in WT BMDMs was absent in *Nlrc4*$^{-/-}$ and *Asc*$^{-/-}$ BMDMs, suggesting an NLRC4- and ASC-dependent pathway (Man et al., 2013). The effects of Caspase-8 deletion on cytokine responses is less clear. *Casp8*$^{-/-}$*/Ripk3*$^{-/-}$ BMDMs (used due to the embryonic lethality of Caspase-8 deletion) had reduced IL-1β release in response to *S. typhimurium* as noted by ELISA. Although western blot revealed reduced IL-1β in the supernatant, pro-IL-1β in WCL was also markedly reduced indicating abnormal priming. This is in contrast *Casp1*$^{-/-}$ BMDMs, which displayed normal pro-IL-1β, but reduced mature IL-1β secreted in supernatant.

Two recent publications have contributed to the understanding of the interplay between Caspase-8 and NLRC4. Whilst previous publications had used haematopoetic cells in *S. typhimurium* infection models, Vance and colleagues explored the role of the intestinal

epithelial cell (IEC) NLRC4 inflammasome (Rauch et al., 2017). This cell type has clinical relevance as gastrointestinal symptoms are a prominent feature of NLRC4-AID, discussed further in **Section 4.1.4**. Mice generated to express NLRC4 only in IECs and treated with a reagent that delivers flagellin into the cytosol, succumbed to hypothermia that was not seen in the *Nlrc4*$^{-/-}$ mice, but was similar to WT mice. Interestingly, the IEC NLRC4 expressing mice experienced gastrointestinal symptoms that were not seen in mice expressing NLRC4 only in myeloid cells, suggesting a tissue specific effect. An intestinal epithelial stem cell-derived organoid model was used to show that the expulsion of IECs into the lumen and lytic cell death in response to flagellin was cell intrinsic. The lytic cell death, but not expulsion of IECs, was dependent on Caspase-1 and GSDMD. The expulsion of IECs in the absence of Caspase-1 or GSDMD was hypothesized to be due to Caspase-8 activation. Immunohistochemical staining for cleaved Caspase-8 was performed on intestinal cells from *Casp1*$^{-/-}$ mice treated with flagellin. These cells contained an ASC speck that stained positive for cleaved Caspase-8, a feature not seen in intestinal cells from *Asc*$^{-/-}$ and *Nlrc4*$^{-/-}$ mice, suggesting that ASC is required for the activation of Caspase-8 in this context.

Similar results were found in the context of *L. pneumophila* infection of BMDMs (Mascarenhas et al., 2017). In the presence of Caspase-1, ASC was not required for control of *L. pneumophila*, suggesting that NLRC4 and Caspase-1 interaction is not entirely dependent on ASC. Similar to *S. typhimurium*, *Casp1*$^{-/-}$ BMDMs infected with *L. pneumophila* still formed a single ASC speck per cell, which stained positive for ASC and Caspase-8 on immunofluorescence. Quantification of colocalisation was close to 100%. The Caspase-1 independent, Caspase-8-dependent cell death seen was independent of GSDMD, suggesting possible engagement of alternative gasdermins. As pathogens may have mechanisms that inhibit Caspase-1 to promote

their own replication, it is possible that the Caspase-8 pathway can control infection in the absence of Caspase-1.

4.1.3 NLRC4 regulation

As with most inflammasome forming proteins, the consequences of inappropriate activation of NLRC4 are profound (**Section 4.1.4**), hence strong regulatory mechanisms for these pathways are likely. Besides the possibility of ubiquitination (touched on in **Section 4.1.2.4**), the phosphorylation of NLRC4 has been the key post-translational modification considered to date. Dixit and colleagues created a knock-in mouse expressing a FLAG-tagged NLRC4 and performed immunoprecipitation of NLRC4 of BMDMs after transfection with *S. typhimurium* flagellin (Qu et al., 2012). Compared with NLRC4 that was not exposed to transfected flagellin, mass spectroscopy of NLRC4 revealed a phosphorylated p.Ser533 residue, with phosphorylation present in approximately one quarter of all NLRC4 peptides. Cleavage of Caspase-1 and IL-1β secretion was seen in WT NLRC4 expressing macrophages stimulated with *S. typhimurium.* Both cell death and IL-1β secretion were significantly reduced in p.Ser533Ala NLRC4 (phosho-dead NLRC4) expressing macrophages. Using mass spectrometry, the phosphokinase was identified as PKCδ, and functional evidence of the role of PKCδ in NLRC4 activation was provided using *Pkcδ*$^{-/-}$ BMDMs. Subsequent work determined that the phosphorylation of NLRC4 was not an absolute requirement for activation, but that p.Ser533Ala NLRC4 expressing BMDMs had a reduced and delayed cell death and IL-1β release compared with WT BMDMs, albeit greater than *Nlrc4*$^{-/-}$ BMDMs. This finding suggested that phosphorylation enhances NLRC4 activation in mice with *S. typhimurium* infection. Lamkanfi and colleagues highlighted that phosphorylation of NLRC4 in response to flagellin occurs prior to engagement with NAIP5 (Matusiak et al., 2015). Collectively, these data suggest that NLRC4 is phosphorylated as a post translational 'priming' step, and engages

more readily with NAIP5. This is in contrast to work by Nunez and colleagues on *Shigella* inner rod effector protein MxiI (S. Suzuki et al., 2014). MxiI coimmunoprecipitated with NAIP2 and NLRC4, forming a Caspase-1 cleavage platform with ASC. Stimulation of *Pkcδ*$^{-/-}$ BMDMs with MxiI showed that PKCδ was completely dispensable for the activation of NLRC4, with increased rather than decreased IL-1β production. This finding raises the possibility that phosphorylation is important for NAIP5-NLRC4 but not NAIP2-NLRC4 activation.

The potential contribution of an alternative phosphokinase was raised by Liu et al. (W. Liu et al., 2017). Peritoneal macrophages from mice deficient in LRRK2, an IFN-γ inducible protein with a documented role in control of *L. monocytogenes,* had reduced Caspase-1 cleavage and IL-1β secretion when challenged with *S. typhimurium*, rod protein PrgJ or flagellin. Furthermore, survival of *Lrrk2*$^{-/-}$ mice when challenged with *S. typhimurium* was reduced when compared with WT mice. Through immunoprecipitation assays using endogenous LRRK2 as bait, the authors documented that LRRK2 interacts with NLRC4 when peritoneal macrophages are infected with *S. typhimurium*. The kinase activity of LRRK2 was important for phosphorylation and optimal function of NLRC4. This recent finding has yet to be reproduced but suggests that there are potentially multiple kinases involved in NLRC4 phosphorylation, and that future studies should consider the T3SS, NAIP, cell and species specificity.

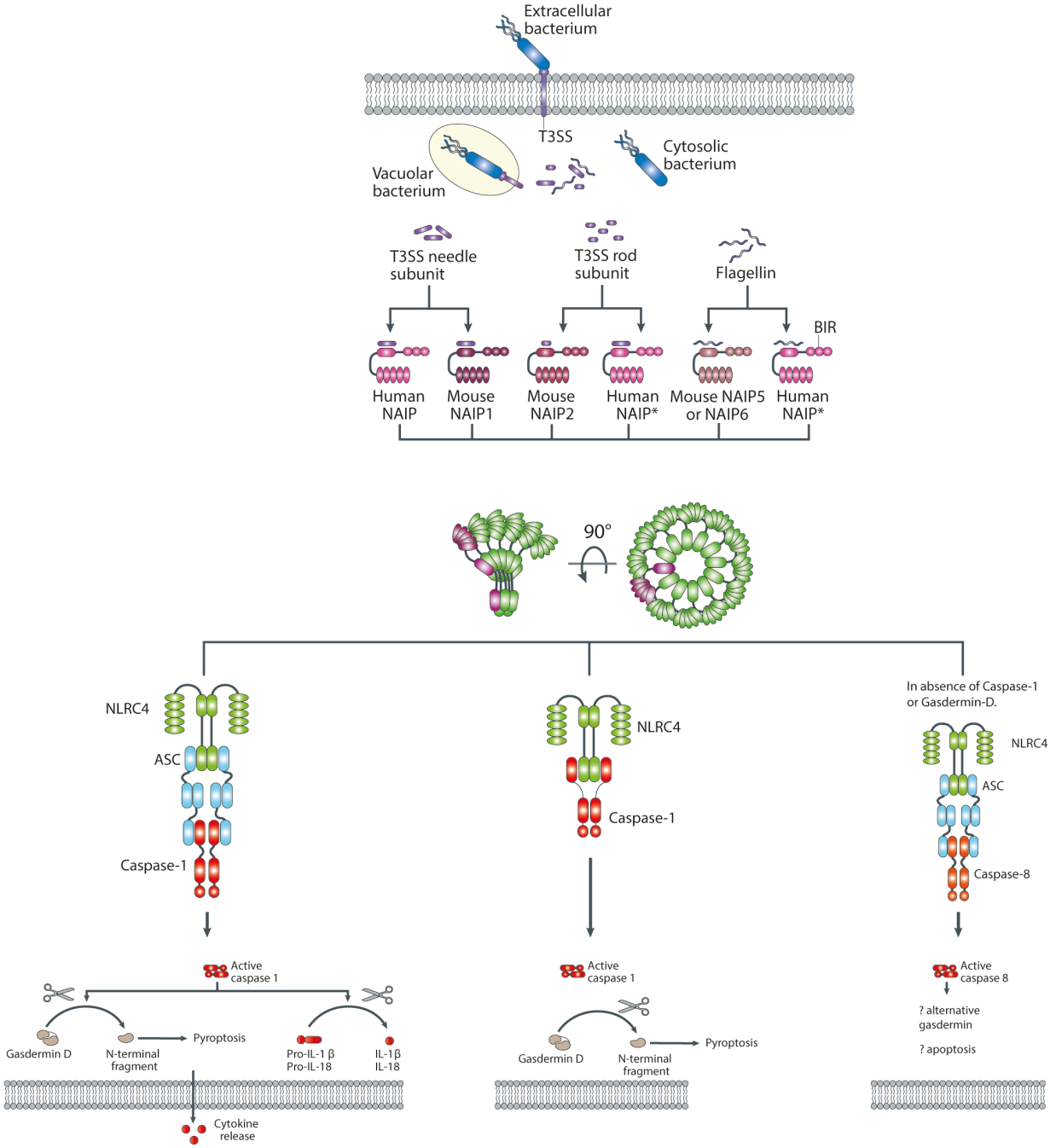


Figure 4-4 Possible NLRC4 activation pathways.

To date, evidence suggests that NLRC4 is activated through its association with NAIP, where NAIP is a sensor of a number components of the type three secretion system (T3SS). Once activated, NLRC4 oligomerises and may associate with the apoptosis-associated speck-like protein containing a CARD (ASC, left panel) and together provide a platform to activate Caspase-1, resulting in cleavage of both gasdermin-D and cytokines pro-IL-1β and pro-IL-18 to their active form. Alternatively, NLRC4 may associate with Capsase-1 directly through its CARD domain (middle panel), resulting in cell death without significant cytokine response. In the absence of Caspase-1 or gasdermin-D, NLRC4 engages with Caspase-8 and results in cell death through mechanisms that are unclear (right panel). Adapted from (Broz & Dixit, 2016). * various isoforms

4.1.4 NLRC4-associated autoinflammatory diseases

NLRC4-AID, also described as syndrome of enterocolitis and autoinflammation associated with mutation in *NLRC4* (SCAN4) and autoinflammation with infantile enterocolitis (AIFEC), is caused by GoF mutations in *NLRC4.* Key features are of MAS and high serum IL-18 levels. The first two reports (*Nature Genetics,* 2014) detailed patients with MAS and histological evidence of enterocolitis, who each harboured heterozygous mutations in the region of *NLRC4* encoding the WHD (Canna et al., 2014; Romberg et al., 2014). The first pedigree detailed by Romberg et al. documented three generations, suggesting a *de novo* p.Val341Ala NLRC4 mutation in an individual and a subsequent autosomal dominant inheritance pattern in two individuals, one of whom succumbed to disease (Romberg et al., 2014). Both surviving patients were symptomatic and had markedly elevated serum total IL-18 levels. Patient-derived monocytes differentiated into macrophages *in vitro* had higher cell death and increased IL-1β and IL-18 in response to low dose LPS stimulation compared with healthy controls. Using a HEK293T cell reconstitution model, transfection of constructs expressing mutant NLRC4 and pro-Caspase-1 resulted in increased Caspase-1 cleavage when compared with WT NLRC4. Furthermore, co-transfection of mutant NLRC4 with ASC resulted in a higher percentage of cells with an ASC speck compared with WT NLRC4. The second report by Canna et al. followed a child with a *de novo* p.Thr337Ser NLRC4 mutation, who was symptomatic of an urticarial rash, intestinal inflammation as well as MAS (Canna et al., 2014). Serum multiplex cytokine profiling revealed a distinct pattern when compared with healthy controls and patients with CAPS that included elevated IL-18 and macrophage-stimulating cytokines. Unstimulated patient-derived monocytes had elevated IL-18 secretion compared with healthy controls and CAPS patients, and when differentiated into macrophages *in vitro*, had an increase in ASC speck formation, as determined by immunofluorescence. IL-1R antagonist therapy in the NLRC4-AID patient caused reduction in clinical symptoms but serum inflammatory cytokine

levels remained elevated. These authors therefore suggested that targeting IL-18 may warrant consideration and subsequently reported the successful use of recombinant human IL-18 binding protein (rhIL18BP) in a patient with NLRC4-MAS (Canna et al., 2017). Since this time, there have been a number of publications documenting GoF mutations in *NLRC4* in patients with a spectrum of clinical features, ranging from urticaria (Volker-Touw et al., 2017), to thrombotic vasculopathy (Liang et al., 2017), and NOMID-like manifestations (Kawasaki et al., 2017). These reports are summarized in **Table 4.1**.

4.1.4.1 Role of IL-18 in macrophage activation syndrome

Haemopagocytoic lymphohistiocytosis (HLH), also known as MAS when associated with an underlying autoinflammatory or rheumatological condition, is characterised by dysregulation of macrophages, NK cells and cytotoxic T lymphocytes culminating in markedly elevated proinflammatory cytokines and haemophagocytosis (Al-Samkari & Berliner, 2017). Classically, primary HLH, also known as familial HLH, occurs in patients who have a genetic mutation affecting the normal packaging and trafficking of cytolytic granules containing perforin and granzymes (Perez, Virelizier, Arenzana-Seisdedos, Fischer, & Griscelli, 1984). Rather than inducing death of cells infected with an organism as a control mechanism, a defective cytolytic granule response leads to persistence of the infection and the immune activating stimulus (Grom, Horne, & De Benedetti, 2016; Morimoto, Nakazawa, & Ishii, 2016). IFN-γ has been implicated in the pathogenesis of HLH, with neutralization of IFN-γ improving disease in murine models (M. B. Jordan, Hildeman, Kappler, & Marrack, 2004; Pachlopnik Schmid et al., 2009). Clinical trials are currently underway to examine the safety and efficacy of neutralizing IFN-γ in patients with primary HLH, as well as assessing the use of a monoclonal anti-IFN-γ antibody for the prevention of MAS in patients with sJIA (clinical trial #NCT01818492 and #NCT03312751).

Initially considered to be an IFN-γ inducing factor (Nakamura, Okamura, Wada, Nagata, & Tamura, 1989), IL-18 has since been implicated in the induction of a number of pathways through the coupling of its receptor with MyD88 and subsequent NF-κB translocation (Dinarello, Novick, Kim, & Kaplanski, 2013). This IL-1 family member is similar to IL-1β in requiring cleavage by Caspase-1 for biological activity, but, distinctively, it exists in its inactive precursor form constitutively in monocytes, macrophages, antigen presenting and epithelial cells (Puren, Fantuzzi, & Dinarello, 1999). Early studies of IL-18 explored its role in the T helper type (Th) 1 response in concert with either IL-12 or IL-15. Subsequent work has shown that IL-18 can promote a Th2 response when acting with IL-2, and, significantly, has pro-inflammatory effects when acting alone. The systemic effects of IL-18 are regulated through interactions with its binding partner, IL-18 binding protein (IL-18BP) and only free IL-18 has biological activity (Novick et al., 1999).

The relationship between IL-18 and IFN-γ, and the role of IFN-γ in MAS, prompted studies exploring the role of IL-18 in MAS. Takada et al. measured serum IL-18 levels in patients with primary HLH and determined that total IL-18 levels were elevated when compared with healthy controls, and that this correlated with disease activity (Takada, Nomura, Ohga, & Hara, 2001). A method for detecting IL-18BP allowed for calculation of free IL-18 in the serum of patients with secondary HLH (Mazodier et al., 2005; Novick et al., 2001). Free IL-18 levels correlated with biologic markers of disease activity in HLH and inversely with NK cell number and function (Novick et al., 2001). A subsequent comparison of sJIA patients with and without MAS revealed that, as expected, IFN-γ was significantly increased in patients with MAS. However free serum IL-18 levels did not discriminate between sJIA patients with or without MAS in this study (Put et al., 2015). Although this finding could be sJIA-associated MAS

specific, it underlines that more work is required to establish the clinical utility of free IL-18 as a predictive marker of MAS.

Table 4-1 Summary of NLRC4-AID literature.

Six mutations in *NLRC4* have been reported to cause NLRC4-AID. The majority of patients described had features of macrophage activation syndrome and gastrointestinal symptoms as well as raised IL-18 serum cytokine levels. In vitro and in vivo modelling are also summarised.

Genetic change	Amino acid change	Domain	Clinical features	Serum cytokine analysis	*In vitro* model	*In vivo* model	Ref
c.512 C>T	Ser171Phe	NBD	Congenital anaemia HLH Fetal thrombotic vasculopathy Ascites Necrotizing enterocolitis	NA	NA	NA	(Liang et al., 2017)
c.529 A>G *somatic	Thr177Ala	NBD	NOMID-like	↑ IL-18.	iPSC: clonal sequencing. CRISPR/Cas9 deletion of *NLRC4*		(Kawasaki et al., 2017)
c.1009 A>T	Thr337Ser	HD1	Fever Urticarial-like rash Splenomegaly Enterocolitis MAS	↑ IL-18 levels. Distinct profile on multiplex analysis	Patient derived macrophages: ↑ cell death, ↑ IL-1β and IL-18 with LPS priming + flagellin. ↑ ASC speck formation in unstimulated cells. THP1 cells transduced with mutant: ↑ IL-1β and IL-18 levels.		(Canna et al., 2017)
c.1022 T>C	Val341Ala	HD1	Fever Enterocolitis Splenomegaly MAS	↑ IL-18	Monocyte derived macrophages: LPS→ ↑ cell death, ↑ IL-1β and IL-18 levels HEK293T inflammasome reconstitution: ↑ ASC speck + Caspase-1 cleavage.		(Romberg et al., 2014)
c.1328 A>	His443Pro	WHD	Cold-induced urticaria Arthritis Large pedigree	IL-1β (N) IL-18 not presented	Patient monocytes: PrgI produced ↑ IL-1β compared with healthy controls. HEK293T transfection: Spontaneous oligomerization of mutant NLRC4 HEK293T inflammasome reconstitution: ↑ IL-1β release.	Mutant mice: Autoinflammation. ↑ with exposure to cold. Splenomegaly. Neutrophilia.	(Kitamura, Sasaki, Abe, Kano, & Yasutomo, 2014)
c.1333 T>C	Ser445Pro	WHD	Painful erythematous nodes Urticarial rash Arthralgias +/- colitis Large pedigree	↑ IL-18	NA	NA	(Volker-Touw et al., 2017)

4.2 **Manuscript**

The following manuscript was accepted for publication in the Journal of Allergy and Clinical Immunology on the 4th April 2018.

Autoinflammatory mutation in NLRC4 reveals an LRR-LRR oligomerization interface

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Abstract

Background: Monogenic autoinflammatory disorders are characterised by dysregulation of the innate immune system. A significant number of this broadening group of disorders are caused by gain-of-function mutations in inflammasome forming proteins, such as NLRC4. A number of mutations in *NLRC4* have been described, leading to a spectrum of NLRC4-associated autoinflammatory disorders (NLRC4-AID).

Objective: We studied two unrelated patients with early onset macrophage activation syndrome caused by *de novo* mutations in *NLRC4* (c.G1965C, p.W655C). Unlike other mutations in *NLRC4* described to date, p.W655 is located within the leucine rich repeat (LRR) domain. For this reason, we investigated mechanisms by which this mutation contributes to the pathogenesis of autoinflammatory disease.

Methods: Next generation and/or Sanger sequencing techniques were used for genetic analysis. Enzyme linked immunosorbent assay (ELISA) was performed to quantify serum cytokine levels. *In vitro*, inflammasome complex formation was quantified using flow cytometric analysis of Apoptosis-associated Speck-like protein containing a Caspase recruitment domain (ASC) specks. Monocytic cell lines were generated by genetic deletion of *NLRC4* from THP-1 cells using CRISPR/Cas9 techniques followed by lentiviral transduction of wild type (WT) or mutant *NLRC4* cDNA. Cell death and release of IL-1β/IL-18 were quantified using flow cytometry and ELISA respectively.

Results: Both reported patients succumbed to macrophage activation syndrome early in life, associated with increased IL-18 serum levels. The *NLRC4* mutation identified, c.G1965C/p.W655C, caused increased ASC speck formation *in vitro*, similar to other

pathogenic NLRC4-AID mutations. In human monocyte-like cells (THP-1), introduction of c.G1965C/p.W655C NLRC4 results in increased caspase-1-dependent cell death, with increased IL-1β and IL-18 production. The enhanced response was independent of NLRP3 and caspase-8. ASC contributed to NLRC4 p.W655C mediated cytokine release, but not cell death. W655 is located at the interface between adjacent LRR domains in the oligomeric inflammasome structure. Mutation of W655 activates the NLRC4 inflammasome complex by engaging with two interfaces on the opposing LRR domain. One key set of residues (p.D1010, p.D1011, p.L1012 and p.I1015) participates in LRR-LRR oligomerization when it is triggered by NLRC4-AID mutations and T3SS effector (PrgI) stimulation of the NLRC4 inflammasome complex.

Conclusion: This is the first report of a mutation in the LRR domain of NLRC4 causing NLRC4-AID. c.G1965C/p.W655C NLRC4 increases inflammasome activation, leading to constitutive IL-18 production and increased IL-1β release upon priming, where ASC contributes to the cytokine response, but not to cell death. Data generated from various *NLRC4* mutations suggests that the tryptophan at p.W655 does not tolerate substitution, and provides evidence that the LRR-LRR interface has an important, previously unrecognized role in oligomerization of the NLRC4 inflammasome complex.

Key messages:

- Two patients with p.W655C NLRC4 had fatal macrophage activation syndrome and significantly elevated serum IL-18 levels.
- *De novo* c.G1965C *NLRC4* mutation encoding p.W655C NLRC4 is the first mutation reported in the LRR domain of NLRC4 to cause disease.

- An LRR-LRR interface is important for NLRC4 inflammasome activation by NLRC4-AID mutations and the T3SS effector PrgI.

Capsule summary: Two patients with macrophage activation syndrome and elevated serum IL-18 levels were found to have a novel gain of function mutation in the leucine rich repeat domain of NLRC4.

Key words: Autoinflammatory disease, periodic fever syndrome, NLRC4, macrophage activation syndrome, inflammasome, Nod-like receptor, IPAF, IL-18

Abbreviations:

AIFEC	Autoinflammation with infantile enterocolitis
ASC	Apoptosis-associated Speck-like protein containing a Caspase recruitment domain
CARD	Caspase activation and recruitment domain
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRP	C-reactive protein
CSF	Cerebrospinal fluid
DAMP	Damage associated molecular patterns
ELISA	Enzyme linked immunosorbent assay
HD	Hinge domain
LRR	Leucine rich repeat domain
MAS	Macrophage activation syndrome
NAIP	NLR family Apoptosis Inhibitor Proteins
NOD	Nucleotide-binding oligomerization domain

116	NLRC4	Nod-like receptor family CARD containing 4 protein
117	NLRC4-AID	NLRC4-associated autoinflammatory disorders
118	NLRP3	NOD-like receptor family, pyrin domain containing 3
119	PAMP	Pathogen associated molecular pattern
120	T3SS	Type 3 secretion system
121	TOFIE	Time of Flight Inflammasome Evaluation

122

INTRODUCTION

Inflammasomes are large, multimeric complexes formed in response to pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs). Some innate immune sensors oligomerise with the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and caspase-1 to form a platform for the cleavage of pro-IL-1β and pro-IL-18 to their active forms. Gain-of-function mutations in inflammasome forming proteins are a major cause of monogenic autoinflammatory disorders, a heterogeneous group of conditions characterised by innate immune dysregulation.

Nod-like receptor family CARD containing 4 protein (NLRC4) forms an inflammasome in response to Type III secretion system (T3SS) proteins from invading gram-negative bacteria such as *Salmonella*. Components of the T3SS are recognized by cytosolic sensors known as NLR family Apoptosis Inhibitor Proteins (NAIPs) (1-3). NAIP proteins associate with NLRC4, initiating a conformational change that allows for NLRC4 oligomerization through self-propagation of the nucleotide-binding oligomerization domain (NOD) (4, 5).

Mutations in the NOD of NLRC4 result in autoinflammation, with a spectrum of clinical manifestations ranging from cold-induced urticaria to life-threatening macrophage activation syndrome with severe enterocolitis (6-10). NLRC4-associated autoinflammatory disorders (NLRC4-AID) are characterised by high levels of free IL-18 in the serum of patients, distinguishing it from other monogenic inflammasomopathies such as Familial Mediterranean Fever or Cryopyrin Associated Periodic Syndrome. Importantly, successful treatment using a recombinant IL-18 binding protein (IL-18BP) has been reported in one patient with autoinflammation with infantile enterocolitis (AIFEC, OMIM 616050), an NLRC4-AID (11).

148 Here, we identify a previously unknown mutation in the leucine rich repeat (LRR) domain of
149 NLRC4 in two unrelated patients with macrophage activation syndrome. This is the first report
150 of such a mutation in *NLRC4*, and we provide *in vitro* evidence of the importance of LRR-LRR
151 interactions in the disease pathophysiology in these patients.

METHODS

Patient and study approval

Informed consent for genetic sequencing was obtained from the patients' guardians. Patient one (P1) was recruited through routine care. Patient two (P2) and age and sex matched control subjects were recruited through the Guangzhou Women and Children's Medical Center ethics committee (2016021602). Further informed consent was obtained for publication of case descriptions and clinical images.

Genetic analysis

Genomic DNA was extracted from whole blood using the QIAamp DNA Micro Kit (Qiagen, 56304). Targeted sequencing was performed on P1. *NLRC4* was amplified by polymerase-chain-reaction (PCR) and sequenced using the Sanger method and primers listed in **Table S1**. Whole exome sequencing was performed on P2 and P2's family members using the Agilent SureSelect Human All Exon V6 kit, sequenced on an Illumina platform. Bioinformatics analysis with read mapping and variant calling was performed using Genome Analysis Toolkit (GATK) Haplotype Caller. The variant of interest was confirmed with Sanger sequencing.

Serum cytokine analysis

For Patient 1, serum was diluted in sample buffer and assayed in multiplex on a Luminex Magpix system (Bio-Rad). Human IL-18BPa beads were generated with magnetic beads (Bio-Rad) conjugated to clone MAB1192, and detected with clone BAF119 (both R&D systems). Bioplex Pro group II cytokine standard was used for IL-18 and CXCL9, whereas recombinant human IL-18BPa-Fc (R&D system) was used for IL-18BPa. Patient 2 serum cytokine levels were quantified by enzyme linked immunosorbent assay (ELISA) for IL-1β (Biotech, CHE001) and IL-18 (Biotech, CHE007), as per manufacturer's guidelines.

177

178 ***Generation of NLRC4-deficient cells***

179 The method of generating knock out cells using CRISPR/Cas9 techniques, as well as lentiviral
180 production, has been previously described (12, 13). The sgRNA constructs used to make
181 *CASPASE-1* KO, *CASPASE-8* KO and *ASC* KO cells have been previously described (14-16).

182

183 ***Generation of lentiviral construct***

184 Lentiviral constructs were generated by amplification of *NLRC4* cDNA with Phusion ® DNA
185 polymerase (M0530S, New England BioLabs), using primers flanked with restriction enzyme
186 sequences allowing for cloning into the pFUGW backbone(17) (see **Table S1**). Both pFUGW
187 and amplified cDNA were digested using *Age*I-HF (R3552) and *BamH1*-HF restriction
188 enzymes (R3136, New England BioLabs), followed by agarose gel electrophoresis and
189 extraction of DNA. The vector and insert were ligated using T4 DNA Ligase (B0202S, New
190 England BioLabs).

191

192 ***Site directed mutagenesis***

193 Site directed mutagenesis was performed using the QuickChange Lightning Kit (210519-5,
194 Agilent Technologies), according to manufacturer's instructions. Mutations were introduced
195 to constructs using the oligonucleotides listed in supplementary **Table S1**.

196

197 ***Cell culture procedures***

198 Human THP-1 and HEK 293T cells were grown at 37°C in humidified atmosphere of air with
199 10% CO_2. THP-1 cells were maintained in HT RPMI (1% (w/v) RPMI-1640, 0.2% (w/v)
200 $NaHCO_3$, 0.011% (w/v) $C_3H_3NaO_3$, 0.1% (w/v) streptomycin, 100U/mL penicillin)
201 supplemented with 10% (v/v) Foetal Bovine Serum (FBS, Sigma-Alrich). HEK293T cells were

maintained in DMEM (1% (w/v) D-glucose, 0.11% (w/v) sodium pyruvate, 0.1% (w/v) streptomycin, 100U/mL penicillin) supplemented with 10% (v/v) FBS (Sigma-Alrich).

Transduction of knock out cell lines

NLRC4 KO cells were reconstituted using 3rd generation lentiviral vector transduction. We were not able to generate stable cell lines carrying pathogenic *NLRC4* mutations due to high levels of spontaneous cell death. As a result, lentiviral transduction was undertaken prior to each experiment as previously described (18). $2x10^6$ THP-1 cells were infected per condition, with $1x10^6$ cells per well in a 6-well plate. 1 mL of viral supernatant was added to each well, supplemented with 2.5mL RPMI and 8µg/mL polybrene, followed by centrifugation for 3 h at 840 x g at 32ºC. Cells were incubated overnight at 37ºC and the following day, washed in PBS and reseeded in fresh complete RPMI. After a further 24h, cells were seeded for experiments and protein expression was determined on whole cell lysates.

Cell stimulation

THP-1 monocytes underwent retroviral transduction with pMXsIG_PrgI_GFP (19). Briefly, $3x10^6$ HEK293T cells were seeded in 10cm Petri dishes. After adherence, cells were transfected using Lipofectamine 2000 (Life Technologies) with prgI-IRES-GFP (10µg) along with pGag-pol (5 µg) and pVSV-G (500 ng). Following a media change to complete RPMI + 10% FBS at 24h, viral supernatant was collected at 48h and stored at -80°C until required. THP-1 cells were plated in 96-well plates to a final density of $5x10^4$ per well. Priming was performed with synthetic TLR2/1 agonist Pam3CSK4 (Invivogen) at a final concentration of 100 ng/mL for 3 h. A titration of viral supernatant was used, with RPMI added to bring the volume of each well to 100µL. Polybrene was added to a final concentration of 7 µg/mL. Supernatant was collected at 24h for cytokine quantification by ELISA and cells were stained

with PI (1μg/ml, Sigma-Aldrich) to quantify cell death by flow cytometry. NLRP3 was activated by treating cells with nigericin (10 μM, Invivogen) for 1 h prior to collection of supernatant and cell death analysis. Where indicated, the NLRP3 inhibitor, MCC950 (20 ng/ml) was used 30 min prior to stimulation with PrgI or nigericin, or in the case of mutant cell lines, after priming.

Cytokine quantification from cell culture supernatants

Presence of cytokines in supernatant was assessed by ELISA for IL-1β and IL-18 using DY201 and DY008 kits, respectively (R&D Systems), as per manufacturers guidelines.

Western Blot analysis

THP-1 cells and HEK293T cells were lysed using RadioImmunoprecipitation Assay (RIPA) buffer supplemented with *cOmplete* protease inhibitors (11697498001, Roche Biochemicals). Whole cell lysates were incubated on ice for 30 min and clarified by centrifugation. Whole cell lysates were eluted with SDS-PAGE sample buffer, resolved on Novex 4-12% SDS-PAGE gels with MES running buffer and subsequently transferred on to nitrocellulose membranes. Membranes were blocked overnight in 3% BSA + 0.1% Tris Buffered Saline Tween 20 at room temperature for 1 h and then probed overnight at 4°C with primary antibodies including: α-NLRC4 (rabbit α-NLRC4, Cell Signaling Technology D5Y8E), α-caspase-1 (mouse α-caspase-1 p20, Adipogen), α-ASC (rabbit α-ASC, Santa Cruz sc-22514), α-caspase-8 (mouse α-caspase-8, Cell Signaling Technology #9746), and α-Actin (goat α-actin, Santa Cruz sc-1616).

Time of Flight Inflammasome Evaluation (TOFIE)

Flow cytometry for quantification of ASC speck formation by TOFIE was used as a surrogate marker of inflammasome activation (20). HEK293T cells were transfected with 5 ng of GFP-ASC and 10 ng of pCR3_NLRC4_VSV. ASC speck formation was quantified using flow cytometry 16 h post transfection. For examination of the response to type III secretion system proteins (T3SS), TOFIE were conducted in HEK293T cells stably expressing ASC-RFP via a published retroviral transduction (21). These cells were transfected with 10 ng pCR3_NLRC4_VSV as well as variable amounts of pMXsIG_PrgI_GFP and pCS2_hNAIP_myc, as described in the relevant figure legends (22).

Structural analysis

Previously published Protein Data Bank files 4KXF (23), 3JBL (4), and 6B5B (24) were used to generate ribbon figures of NLRC4's structure in PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC). The NLRC4 active and inactive conformations were compared by aligning the LRR domains.

Statistical analysis

Prism software (GraphPad) was used to perform two-tailed *t* tests. Data is pooled from at least three independent experiments and represented as mean + SEM, unless otherwise specified. *$P < 0.05$, **$P<0.01$, ***$P < 0.001$, ****$P < 0.0001$ were considered statistically significant.

RESULTS

Case One

Patient 1 (P1) presented at age 11 days with high grade fever, urticarial-like rash (**Figure 1A**), and elevated acute phase reactant C-reactive protein (CRP, **Figure 1E**). Broad spectrum antibiotic therapy was initiated for suspected neonatal sepsis. Multiple blood and cerebrospinal fluid (CSF) cultures remained sterile. Despite antimicrobial therapy, the patient continued to deteriorate, with development of thrombocytopaenia and acute renal injury (**Figures 1F and 1H**) necessitating intermittent peritoneal dialysis. The patient developed hepatosplenomegaly and the urticarial-like rash evolved to a combination of petechiae and ecchymosis (**Figures 1B-D**). Progressive pancytopaenia as well as elevation in soluble IL-2 receptor (sIL2R), ferritin, transaminases, and triglycerides (**Figure 1G, S1**) provided biochemical evidence of macrophage activation syndrome (MAS), which was confirmed on bone marrow biopsy. Multiple anti-inflammatory and immunomodulatory agents were trialed, including high dose corticosteroids and the terminal complement component inhibitor eculizumab (300mg single dose) without significant effect (**Figure 1I**). The patient developed severe secretory diarrhea despite therapy, prompting consideration of the diagnosis of AIFEC. The interleukin-1 receptor antagonist Anakinra (10mg/day increased to 20mg/day after 18 days) was trialed, again without clinical response, and a dose of tocilizumab (40mg) was given, but with only short-term improvement. The clinical situation deteriorated, with development of diffuse mucosal haemorrage, complicated by bladder clots and obstructive renal failure requiring surgical decompression. Eight weeks after presentation, after quantification of free IL-18 in the serum, a trial of recombinant IL-18 binding protein was initiated at 2 mg/kg subcutaneously every 48h (**Figure 1J**). Although there was slight improvement in diarrhea after five doses, thrombocytopaenia persisted and inflammatory markers remained elevated. Based on the

severity of symptoms, end-organ damage, and family wishes, active care was withdrawn and the patient died at age 11 weeks, 9 weeks after admission.

Case Two

Patient 2 (P2) presented at age 18 months with persistent fever despite treatment for bronchopneumonia with oral cephalosporin. The patient had a history of neonatal sepsis diagnosed at day 3 of life, two episodes of bronchopneumonia, as well as intestinal intussusception requiring surgical intervention at 11 months of age. In the 6 days prior to this admission, P2 experienced cough, dyspnea, wheezing, diarrhea, abdominal pain and maculopapular skin rash. On admission, the patient was febrile 38.8°C, with examination revealing symmetrical breath sounds with transmitted upper airway sounds and hepatomegaly. Chest X-ray confirmed bronchopneumonia and abdominal X-ray revealed an enlarged liver (**Figures 2A and 2B**). CRP was markedly elevated (**Figure 2C**) prompting commencement of treatment with intravenous ceftriaxone. Given the history of recurrent infections, primary immunodeficiency (PID) was suspected and intravenous immunoglobulin treatment was initiated. Evaluation for PID revealed elevated IgE levels and the diagnosis of HyperIgE syndrome was suspected (**Table S2**). Persistent fever and altered conscious state prompted consideration of central nervous system infection. CSF culture grew *Sphingomonas paucimobilis* and targeted antimicrobial treatment was commenced. Despite this, P2 remained febrile, with deterioration of conscious state, progressive hepatosplenomegaly, lymphadenopathy and thrombocytopaenia (**Figure 2D**). Subsequent CSF cultures remained sterile. The serum ferritin level at this time was markedly elevated at 16,500 μg/mL (normal range 7-140 μg/mL), as were transaminases and triglycerides, prompting a diagnosis of MAS. Treatment with methylprednisolone at 2.5mg/kg/day was commenced, however, the patient

died 16 days after admission. Retrospective analysis of serum from day 15 showed P2 had low levels of IL-1β and markedly elevated total IL-18 levels (**Figures 2E and 2F**).

Genetic Analysis

P1 underwent targeted Sanger sequencing of *NLRC4* due to clinical suspicion of AIFEC. Sequencing revealed heterozygous *NLRC4* c.1965G>C transition encoding for the p.W655C variant. P2 had the same variant detected by whole exome sequencing, subsequently confirmed by Sanger sequencing. The *STAT3* sequence of P2 was specifically reviewed and no mutations were identified. No immediate family members of either patient had this substitution, suggesting *de novo* mutations (**Figure 3A**). NLRC4 p.W655 is highly conserved across species (**Figure 3B**) and p.W655C has not been documented in the Genome Aggregation Database (gnomAD*)* (25). Although predicted to be benign (PolyPhen-2) or tolerated (SIFT), the suspicion of pathogenicity was such that further evaluation ensued (26, 27)}.

p.W655 is located in the leucine rich repeat domain of NLRC4.

NLRC4 consists of an N-terminal Caspase activation and recruitment domain (CARD), a nucleotide-binding domain (NBD), two hinge domains (HD1 and HD2), a winged helix domain (WHD) and a C-terminal leucine rich repeat (LRR) domain (**Figure 4A**). The structure of murine NLRC4, which shares 75% sequence identity with human NLRC4, suggests that NLRC4 exists in an ADP dependent auto-inhibited monomeric conformation, with the C-terminal LRR domain occluding the central NBD (23) (**Figure 4B**). Activation of NLRC4 results in a conformational change that exposes the NBD (24) **(Figure 4C)**. p.W655C resides in the LRR, distal to the currently known mutations, which are all located in the around the ADP binding regions (6-10, 28, 29) (**Figures 4A-4C**).

NLRC4 p.W655C results in increased ASC speck formation.

A hallmark of inflammasome activation within a single cell is the formation of the so-called ASC speck. We performed experiments to determine whether the expression of p.W655C NLRC4 resulted in increased ASC speck formation compared to wild type (WT) NLRC4. Flow cytometry was used to quantify ASC speck formation as a marker for inflammasome assembly and activation. HEK293T cells were transiently transfected with ASC-GFP and various mutant forms of *NLRC4*. We observed significantly increased ASC speck formation in cells transfected with p.W655C NLRC4 compared to WT NLRC4. The same was also true for the other known NLRC4 mutants, except p.H443P, when expressed at similar levels (**Figure 4D and 4E**). These data indicates that p.W655C increases inflammasome assembly.

NLRC4 p.W655C causes increased IL-1β, IL-18 and pyroptosis.

In order to model p.W655C NLRC4 in a relevant human cell line, *NLRC4*-deficienct THP-1 monocyte-like cells were generated using CRISPR/Cas9 gene editing techniques (**Figure S2**). THP-1 *NLRC4* KO cells were subsequently transduced with lentiviral constructs carrying *NLRC4* cDNA with various mutations. THP-1 cells transiently transduced with mutant NLRC4 exhibited increased cell death and released more IL-1β and IL-18 as compared to WT NLRC4 (**Figure 5A-C**). The cell death and cytokine response was similar in p.W655C NLRC4 as compared to other known disease causing mutations. p.H443P NLRC4 was expressed at lower levels, and released significantly less IL-1β than the other mutations (**Figure 5D**). Caspase-1 deletion significantly reduced IL-1β and IL-18 secretion, and lowered the cell death (**Figure 5E-G**).

Increased IL-18 secretion, but not cell death, is dependent on ASC.

369 The requirement of ASC in the NLRC4-AID was addressed by Romberg et al using a
370 HEK293T overexpression system, determining that ASC was required for mutant NLRC4
371 associated caspase-1 cleavage (30). To further explore the requirement of ASC in a monocytic
372 cell line, we transduced *ASC* KO THP-1 cells with WT or mutant NLRC4. Cell death seen in
373 p.W655C NLRC4 was not significantly decreased by deletion of ASC, but IL-18 was markedly
374 reduced to levels similar to that seen in *CASP-1* KO cells (**Figures 5E-5G)**. There was also a
375 trend towards reduced IL-1β secretion. This suggests that increased IL-18 release, and
376 potentially IL-1β, associated with this mutant, but not pyroptosis, is dependent on ASC. Thus,
377 cytokine release and cell death may either depend on distinct and individual pathways, or
378 require different thresholds of caspase-1 activity.
379
380 **Increased IL-1β and IL-18 secretion and cell death are not dependent on Caspase-8 or**
381 **NLRP3.**
382 Caspase-8 is a downstream effector of NLRC4-induced cytokine response in the context of *S.*
383 *Typhimurium* infection in a murine model (31). When transduced with NLRC4 constructs,
384 *CASP-8* KO THP-1 cells exhibit increased rather than abrogated cell death and IL-1β and IL-
385 18 secretion (**Figures 5E-5G**), indicating that caspase-8 does not contribute to the elevated
386 inflammatory response, but may rather potentially have a regulatory role. Stimulation of *CASP-*
387 *8* KO cells with nigericin, an activator of NLRP3, also resulted in increased IL-1β released
388 when compared to THP1-Cas9 control cells (**Figure S3D**), suggesting that the increase is not
389 specific to NLRC4. When THP-1 cells were stimulated with PrgI, a T3SS protein, ASC
390 contributed to cell death and cytokine response, and caspase-8 to cell death and IL-18 response,
391 suggesting that the mechanism of activation may differ between pathogenic NLRC4 mutations
392 and its physiological response to T3SS proteins (**Figure 5H-J**).
393

394 The presence of NLRP3 and NLRC4 in a single inflammasome complex has been reported in
395 the setting of *S. Typhimurium* infection (32, 33). We therefore explored the potential
396 contribution of NLRP3 to p.W655C NLRC4 associated inflammation by treating cells with the
397 specific small molecule NLRP3 inhibitor, MCC950 (34). However, cell death, and IL-1β and
398 IL-18 release were unchanged in response to co-culture with MCC950 (**Figure S3A-S3D**). As
399 MCC950 completely blocked NLRP3 activation by nigericin, the presented data indicate that
400 NLRP3 does not play a significant role in the autoinflammation seen in association with
401 p.W655C NLRC4.
402
403 **p.W655 does not tolerate substitution.**
404 To investigate the potential mechanisms of increased NLRC4 activation, we considered the
405 formation of a disulfide link, given that cysteine contains a sulfhydryl group which, when
406 oxidized, may create a disulfide bond. Western blots performed in reducing or non-reducing
407 conditions did not change the size of NLRC4, indicating that the molecule does not exist in
408 different conformations due to a new disulfide bond (**Figure S4A)**. This was further explored
409 through manipulation of the cysteine residue at position p.605, as this was considered to be the
410 most likely residue for p.W655C to form a disulfide bond. Mutation of p.C605 to alanine or
411 serine did not change the levels of ASC speck formation (**Figure S4B**), further arguing against
412 the formation of a disulfide bond as the mechanism of increased p.W655C NLRC4 activation.
413
414 In response to NLRC4 activation, p.W655 undergoes a small change in orientation (**Figure**
415 **6A**). We investigated the possibility that p.W655 interacts with an amino acid in close
416 proximity to keep NLRC4 in an autoinhibited conformation, and whether substitution with
417 cysteine results in loss of this interaction and subsequent activation (**Figure 6B**). Substitution
418 of the glutamic acid at p.600 to alanine or glycine to explore changes in polarity and size, or

419 the glutamine at p.657 to glutamic acid to explore changes in charge did not result in increased
420 ASC speck formation (**Figure 6C**). These findings suggests that disruption of local interactions
421 when NLRC4 is in an autoinhibited conformation are unlikely to explain the increased
422 activation in p.W655C NLRC4. Next, we asked whether increased NLRC4 activation was
423 specific to the cysteine substitution at this residue. Tryptophan was mutated to alanine (A),
424 aspartic acid (D) or serine (S) to explore changes in size, charge and polarity. Each of these
425 mutations resulted in increased ASC speck formation when compared with WT NLRC4
426 (**Figure 6C**), suggesting that p.W655 does not tolerate substitution, and that changes are not
427 specific to a cysteine at p.655.
428
429 **The LRR interface is important for inflammasome assembly.**
430 Next, we considered whether the LRR-LRR interface was important for increased
431 inflammasome activation seen with p.W655C. Several mutations to alanine (A) were made in
432 two separate alpha helices to remove potential binding surfaces in the adjacent LRR of the
433 activated NLRC4 oligomer (denoted LRR1: p.D1010, p.D1011, p.L1012, p.I1015 and LRR2:
434 p.R985, p.S988, p.Q989 , **Figure 7A**). Cell death and cytokine responses of LRR1 and LRR2
435 NLRC4 transduced into THP1-Cas9 cells showed no difference when compared with WT
436 NLRC4 (**Figure 7B**). Combining p.W655C with either LRR1 or LRR2 reduced cell death, and
437 IL-1β and IL-18 secretion to levels comparable with WT NLRC4 (**Figures 7B-7D**). This
438 suggests that the characteristics of the adjacent LRR, and potential interactions at this interface,
439 are important for the increased activation seen with p.W655C. Of note, the addition of LRR1
440 mutations to a representative disease-causing mutation involving either the NBD, HD1 or
441 WHD of NLRC4 resulted in significantly reduced cytokine secretion and cell death, with the
442 exception of p.H443P NLRC4, implying that LRR1 may be important for the oligomerization
443 of NLRC4. On the other hand, combining LRR2 with known disease causing mutations did not

444 reduce inflammasome activation, suggesting that LRR2 is an interface that may only be
445 engaged by the specific p.W655C NLRC4 mutation. This possibility was further explored
446 through stimulation of WT, LRR1, or LRR2 NLRC4 expressing THP1-Cas9 cells with T3SS
447 effector PrgI (**Figures 7E-7G, S4A-S4B**). Indeed, cells expressing LRR1 NLRC4 released
448 significantly less IL-1β in response to PrgI as compared to WT NLRC4 (**Figure 7F**), even
449 when increased amounts of LRR1 NLRC4 was expressed in the cells. We conclude that LRR1
450 is important for maximal physiological oligomerization of the NLRC4 inflammasome
451 complex.
452

DISCUSSION

We report the unfavorable clinical course of two children with MAS associated with c.G1965C/p.W655C mutations in *NLRC4*, and provide evidence of pathogenicity for this mutation in both a HEK293T overexpression model and genetically modified THP-1 cell models.

The clinical and biochemical evidence of MAS and gastrointestinal symptoms in our patients were similar to the original description of AIFEC (6, 10). Clinical symptoms and resistance to multiple immunomodulatory agents, together with increased serum levels of free IL-18, prompted consideration of the diagnosis of AIFEC and a trial of IL-18BP in P1. However, severe end organ damage after a prolonged disease course resulted in discontinuation of IL-18BP treatment and a change to palliative care. Although serum ferritin levels decreased in response to treatment with IL-18BP, effects cannot be adequately assessed after only five doses (**Figure 1G**). Thus, comparison to a recent case report of successful treatment of AIFEC with the IL-18BP tadekinig is problematic (35). The duration of clinical disease and/or the severity of illness with organ damage prior to treatment initiation, or differences in IL-18BP and free IL-18 may explain variable outcomes. Both patients with the p.W655C NLRC4 mutation succumbed to disease very early in life. Poor outcomes in the two reported patients with p.W655C NLRC4 mutations may reflect unfavorable genotype-phenotype correlations in this form of NLRC4-AID.

The detection of *Sphingomonas paucimobilis* in the CSF of P2 raises the question of an immunodeficiency associated with p.W655C NLRC4. This Gram-negative bacillus is generally considered to be of low virulence (36). A series of case reports of bacteremia with *S. paucimobilis*, usually from intravenous administration of contaminated solutions in a health

478 care setting, indicate that patients respond well to treatment and only one pediatric and one
479 adult case has been associated with mortality from this organism (37, 38). The possibility of
480 an associated immune deficiency is raised by the severity of illness as well as evidence of a
481 poor IL-1β, IL-18 and cell death response of AIFEC patient monocytes to *Salmonella*
482 *typhimurim* and *Pseudomoas Aeruginosa* when compared with healthy controls *(30).* There has
483 also been a report of activation of NLRC4 resulting in dampening of TLR5-induced antibody
484 response to flagella, raises the possibility of an associated immunodeficiency (39). However,
485 P2 cleared the organism with appropriate antimicrobial therapy as a repeat CSF culture was
486 negative. It is therefore difficult to conclude that the response to this flagellated organism was
487 impaired. Alternatively, it may be that this infection with *S. paucimobilis* triggered MAS. A
488 further consideration is the possibility of sample contamination rather than a true infection.
489
490 This previously unknown c.G1965C/p.W655C *NLRC4* mutation causes increased
491 inflammasome formation, cell death, and pro-inflammatory cytokine production in a caspase-
492 1-dependent manner (**Figures 5E-5G**). The role of ASC in the response of NLRC4 to infection
493 has been explored in murine gram-negative infection models including *Salmonella*
494 *typhimurium, Pseudomonas aeruginosa*, *Legionella pneumophila* and *Shigella*. In these
495 disease models, NLRC4 was required for both pyroptosis and cytokine production, but ASC
496 was only required cytokine responses (40-44). Here we show that in human cells, ASC is
497 required for IL-18 and possibly IL-1β production, but not cell death. The induction of cell death
498 and cytokine production by caspase-1 may therefore involve distinct pathways, with ASC
499 required for one, but not the other. Alternatively, the level of caspase-1 activity required for
500 cell death, and IL-1β production, may be lower than that required for IL-18 production. NLRC4
501 may associate with caspase-1 independently of ASC, as it contains a CARD domain. However,

ASC may still be required for optimal inflammasome assembly and caspase-1 activation, and the absence of ASC may result in cell death without a maximal cytokine response.

Interestingly, p.H443P NLRC4 caused less ASC speck formation in the HEK293T system, as well as lower cytokine response compared with other known disease-causing mutations in a THP-1 cell system. To date, p.H443P NLRC4 has been described in one Japanese family with familial cold autoinflammatory syndrome (8). No family member developed MAS, consistent with a less severe clinical presentation.

This is the first report of a mutation in the LRR of NLRC4 that causes disease. By exchanging p.W655 for aspartic acid, alanine or serine, we deduced that the size of tryptophan at p.W655 is potentially important for keeping NLRC4 in an autoinhibited conformation. Tryptophan is the largest amino acid and substitution with smaller amino acids resulted in similarly increased ASC speck formation, regardless of changes to charge or polarity.

Furthermore, our data suggests that a structural change at position 655 creates a binding interface together with LRR domain residues p.R985, p.S988 and p.Q989 in the active oligomeric structure (**Figure 7A**). Changes to the LRR adjacent to p.W655 disrupt this interface and abrogate increased inflammasome activation caused by the p.W655C mutation. The LRR1 interface appears to be important for oligomerization of NLRC4, as the cytokine and cell death response of known mutations is abrogated when combined with LRR1, as is the response to PrgI.

Taken together, this study highlights the broad spectrum of clinical features, and the severity of disease that can be seen in NLRC4-AID. We model the first LRR mutation in NLRC4,

p.W655C, to evaluate pathogenicity, and showed that the location of this residue is important in the mechanism of inflammasome assembly. Severe disease presentation and poor disease outcomes in both patients may reflect particularly unfavorable genotype-phenotype correlation for the p.W655C NLRC4 mutation in this syndrome.

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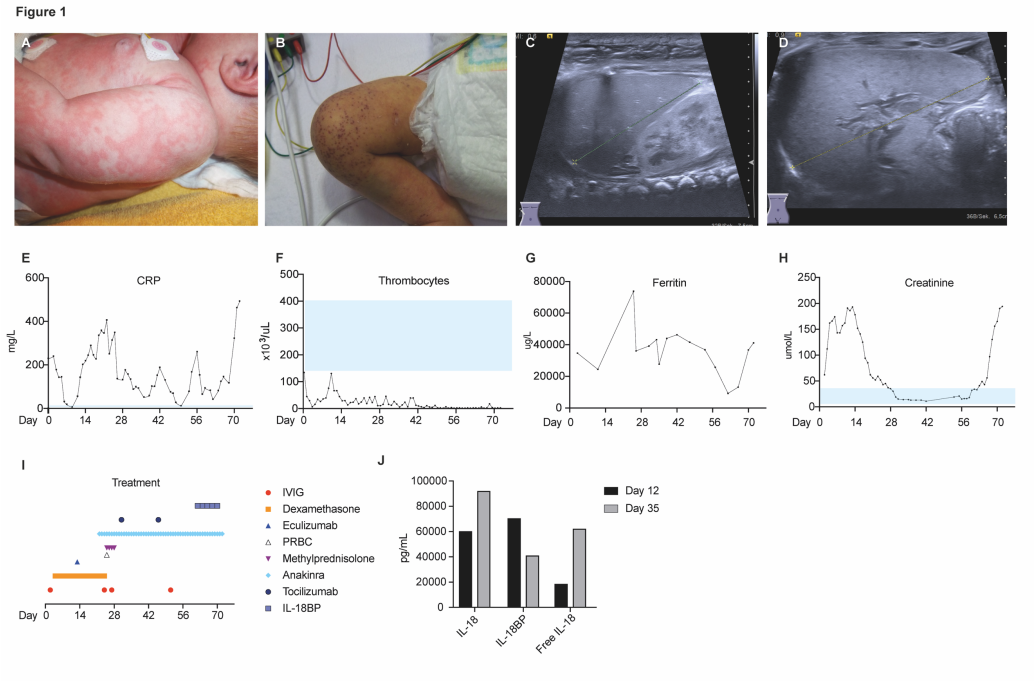

680

681 **Figure 1: Clinical manifestations and biochemical analysis of Patient 1.** Dermatological
682 signs evolved from the initial presentation of urticarial-like rash (A) to petechiae and
683 ecchymosis (B). Ultrasonographic images indicate hepatomegaly (C) and splenomegaly (D).
684 Raised C-reactive protein (CRP, E) and platelet count (F) were improved transiently with
685 intravenous immunoglobulin (IVIG) and dexamethasone. Ferritin (G) remained markedly
686 elevated despite treatment with numerous immunomodulatory agents (I). Acute renal injury
687 and response to peritoneal dialysis monitored with serum creatinine levels (H). Serum IL-18,
688 IL-18 binding protein (IL-18BP), chemokine (C-X-C motif) ligand 9 (CXCL9) and free IL-18
689 were assessed on days 12 and 35 (J). PRBC Packed red blood cells.

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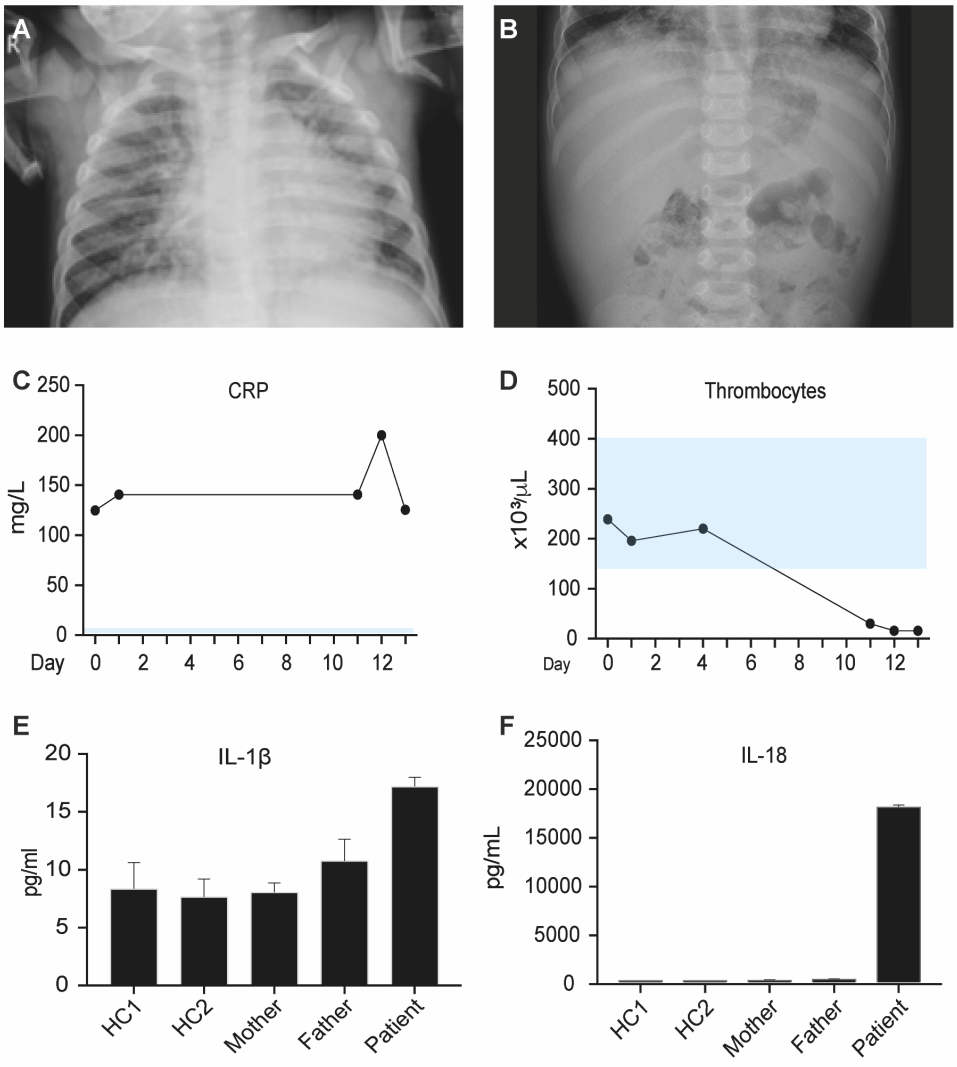
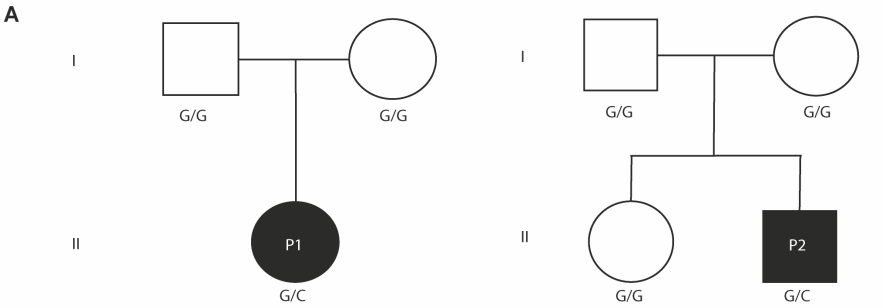
Figure 2



692

693 **Figure 2: Clinical manifestations and biochemical analysis of Patient 2.** Chest radiograph
694 documenting bronchopneumonia (A). Abdominal radiograph demonstrating hepatomegaly
695 (B). C-reactive protein (CRP, C) remained elevated throughout admission, with progressive
696 thrombocytopenia (D). Serum IL-1β (E) and IL-18 (F) from healthy controls, parents and
697 patient.

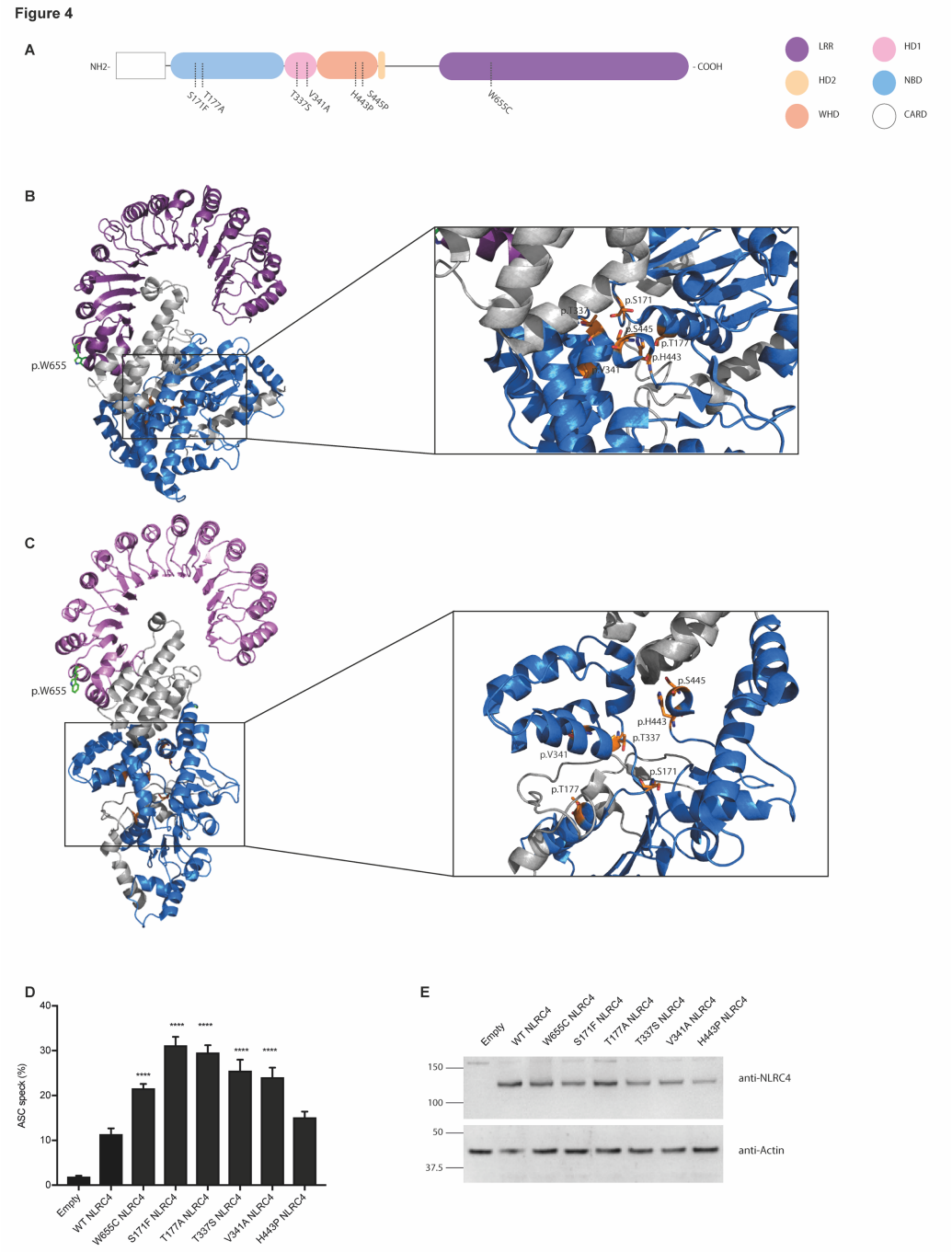
Figure 3



B

H. sapiens	636	MEEAPETYIPSRAVSLFFNWKQEFRTLEVTLRDFSKLNKQDIRYLGKIFS	685
P.troglodytes	636	MEEAPETYIPSRAVSLFFNWKQEFRTLEVTLRDFSKLNKQDIRYLGKIFS	685
M.mulatta	648	MEEAPETYIPSRAVSLFFNWKQEFRTLEVTLWDFSKLNKKDIKYLGKIFS	697
B.taurus	629	DQEFSGTYIPSRAVSLFFNWKQEFKTLDVTLRDFCKLSKKDIKYLEKIFS	678
M.musculus	636	TEGPSETYIPPRAVSLFFNWKQEFKTLEVTLRDINKLNKQDIKYLGKIFS	685
R.norvegicus	636	TEGPS-TYIPSRAVSLFFNWMQKFKTLEVTLRDISKLNKQDIKYLGKIFS	684
X.tropicalis	609	QSSVCDNYIPEKAVKLFFDWNQSIQTLEVTLRDFHQLNKKDIKYLGKICC	658

Figure 3: Pedigree of families. Patient 1 (P1) and Patient 2 (P2) both carried a G>C transition at c.G1965 *NLRC4* (A) encoding a tryptophan to cysteine substitution at p.W655. Solid symbols represent affected individuals and open symbols represent unaffected individuals. Squares=male, circles=females. Sequence alignment across species shows that this is a highly conserved locus (B).



706

707 **Figure 4: p.W655C is located in the leucine rich repeat (LRR) domain of NLRC4.** (A)

708 Schematic representation of NLRC4 domains, with variant of interest in green. Ribbon

709 representation of secondary structure of (B) autoinhibited (PDB Code 4KXF, (23)) and (C)

710 active (PDB Code 6B5B, (24)) NLRC4. p.W655C NLRC4 is displayed in stick format in green.
711 (D) ASC speck quantification of wild type (WT) and NLRC4 mutants using flow cytometry,
712 with NLRC4 expression in whole cell lysate assessed by western blot (E). Data pooled from
713 five independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. HD1=
714 hinge domain 1, HD2=hinge domain 2, NBD= nucleotide binding domain, WHD=winged helix
715 domain, CARD= caspase activation and recruitment domain.
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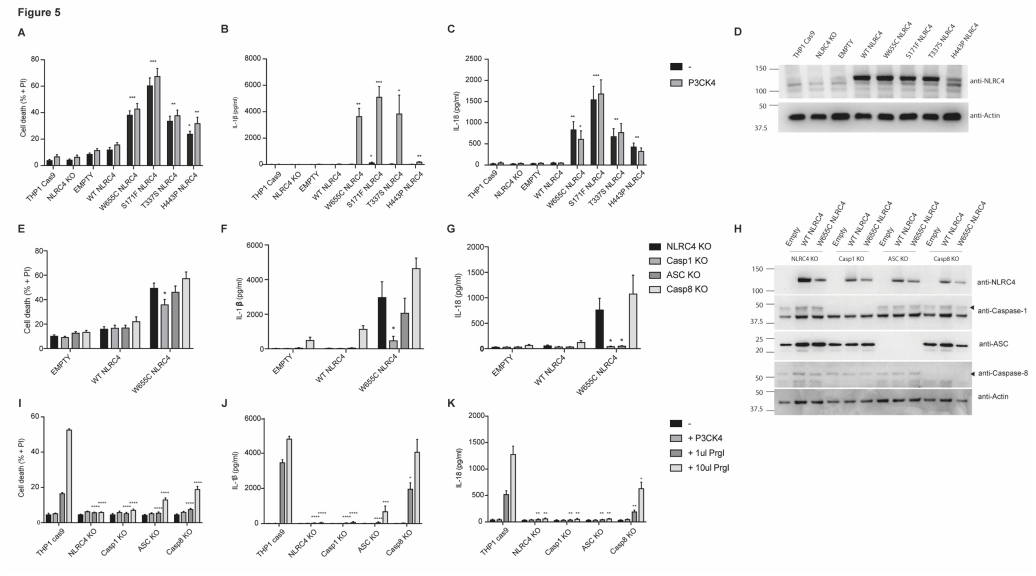

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720 **Figure 5: Effects of p.W655C NLRC4 on cytokine production and cell death.** *NLRC4*
721 knock out (KO) THP-1 monocytes were transduced with lentivirus coding for wild type (WT)
722 or various mutant *NLRC4* constructs (as indicated). 24 h after transduction, cells were plated
723 and treated with P3CK4. After 24 h, (A) propidium iodine (PI) staining was assessed by flow
724 cytometry to quantify cell death and (B) IL-1β and (C) IL-18 secretion were measured by
725 ELISA. (D) Expression of lentiviral *NLRC4* under each condition was qualified by western
726 blot of whole cell lysates. *CASP-1, ASC* or *CASP-8* KO THP-1 monocytes were transduced
727 with WT or p.W655C NLRC4 and assessment of (E) cell death, (F) IL-1β and (G) IL-18
728 secretion was undertaken after treatment with P3CK4, with expression of NLRC4 determined
729 by western blot (H). THP-1 cells, along with *NLRC4*, *CASP-1, ASC* or *CASP-8* KO THP-1
730 monocytes, were primed for 3 h with P3CK4 and infected with two amounts of retrovirus
731 expressing PrgI needle protein. After 24 h (I) cell death, (J) IL-1β and (K) IL-18 secretion were
732 assessed with flow cytometry and elisa respectively. Data pooled from at least three
733 independent experiments. **P <0.05, **P<0.01, ***P<0.001.*

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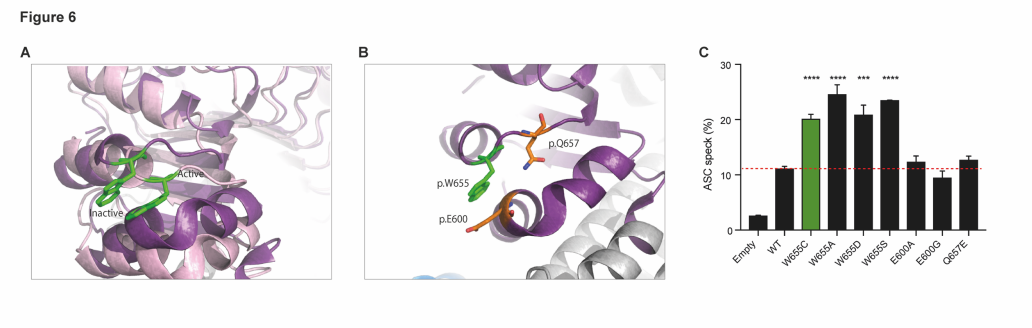

Figure 6: Assessment of local conformational changes in response to NLRC4 activation. (A) Ribbon representation of the region surrounding p.W655 displaying conformational changes between autoinhibited (dark purple) and active (light purple) NLRC4. NLRC4 p.W655 is highlighted in green. (B) Residues potentially interacting with p.W655 at rest, and maintaining NLRC4 in an autoinhibited conformation. (C) Flow cytometric ASC speck quantification of wild type (WT) and p.W655C NLRC4, along with mutations created to explore potential local interactions. Data pooled from three independent experiments. ****P* *<0.001, *****P* *<0.0001.*

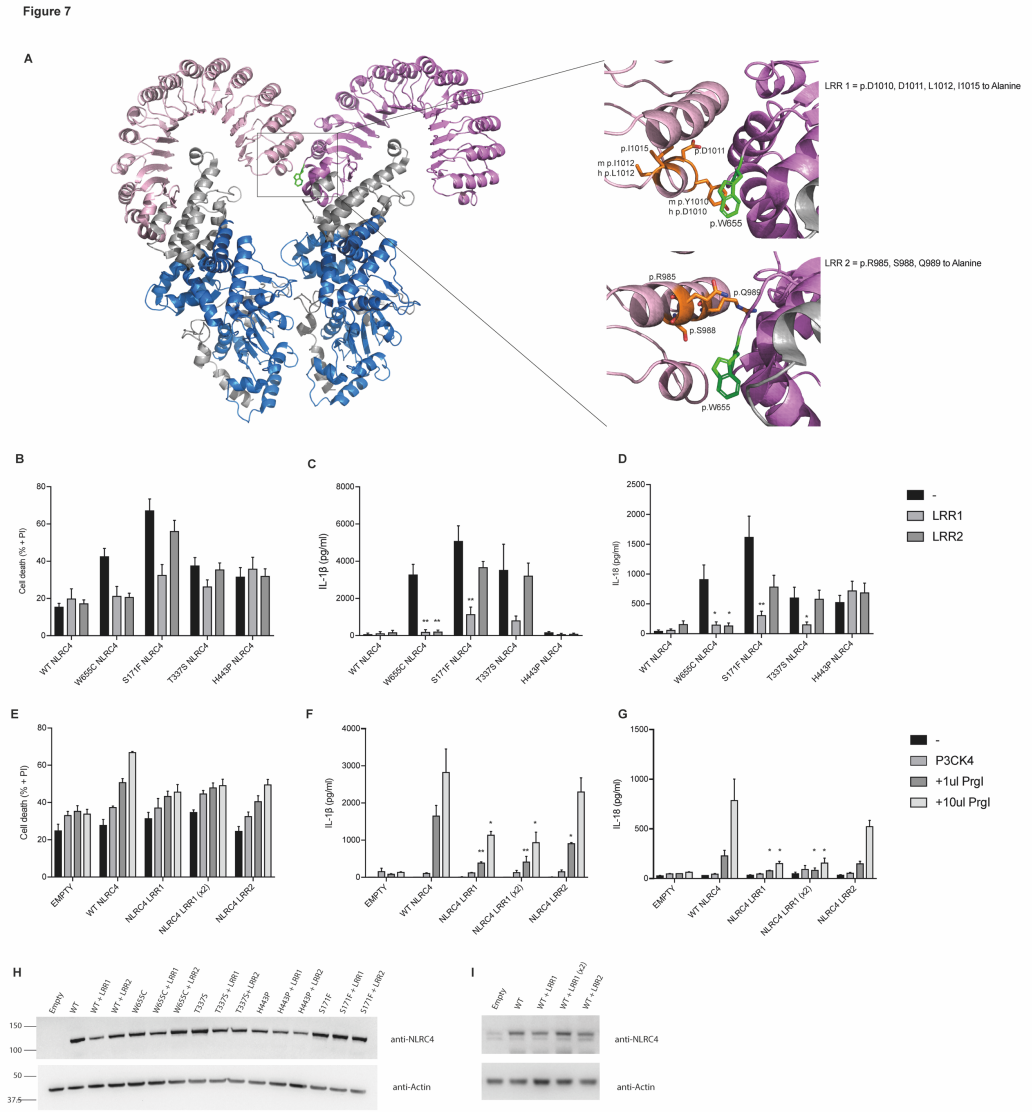

Figure 7: Assessment of p.W655 NLRC4 in oligomerised form. (A) Ribbon representation of NLRC4 in oligomerised state (PDB Code 6B5B, (24)) with magnified inset areas highlighting two potential regions of interaction with p.W655 (LRR1 and LRR2). *NLRC4 KO* THP-1 cells transduced with lentiviral constructs expressing wild type (WT) or mutant NLRC4 combined with LRR1 or LRR2 mutations. 24 h after transduction, cells were primed with P3CK4 for 24h then (B) propidium iodine (PI) staining was assessed by flow cytometry to quantify cell death and (C) IL-1β and (D) IL-18 secretion were measured by ELISA. *NLRC4*

KO THP-1 cells were transduced with 1ml of WT *NLRC4*, LRR1 *NLRC4* or LRR2 *NLRC4* virus, or 2ml of LRR1 *NLRC4* virus (LRR1 (x2)). After 24 h, cells were infected with a retroviral PrgI construct and (E) cell death, (F) IL-1β and (G) IL-18 secretion assessed the following day. NLRC4 expression was assessed by western blot analysis (H). Data pooled from at least three independent experiments. **P <0.05, **P<0.01.*

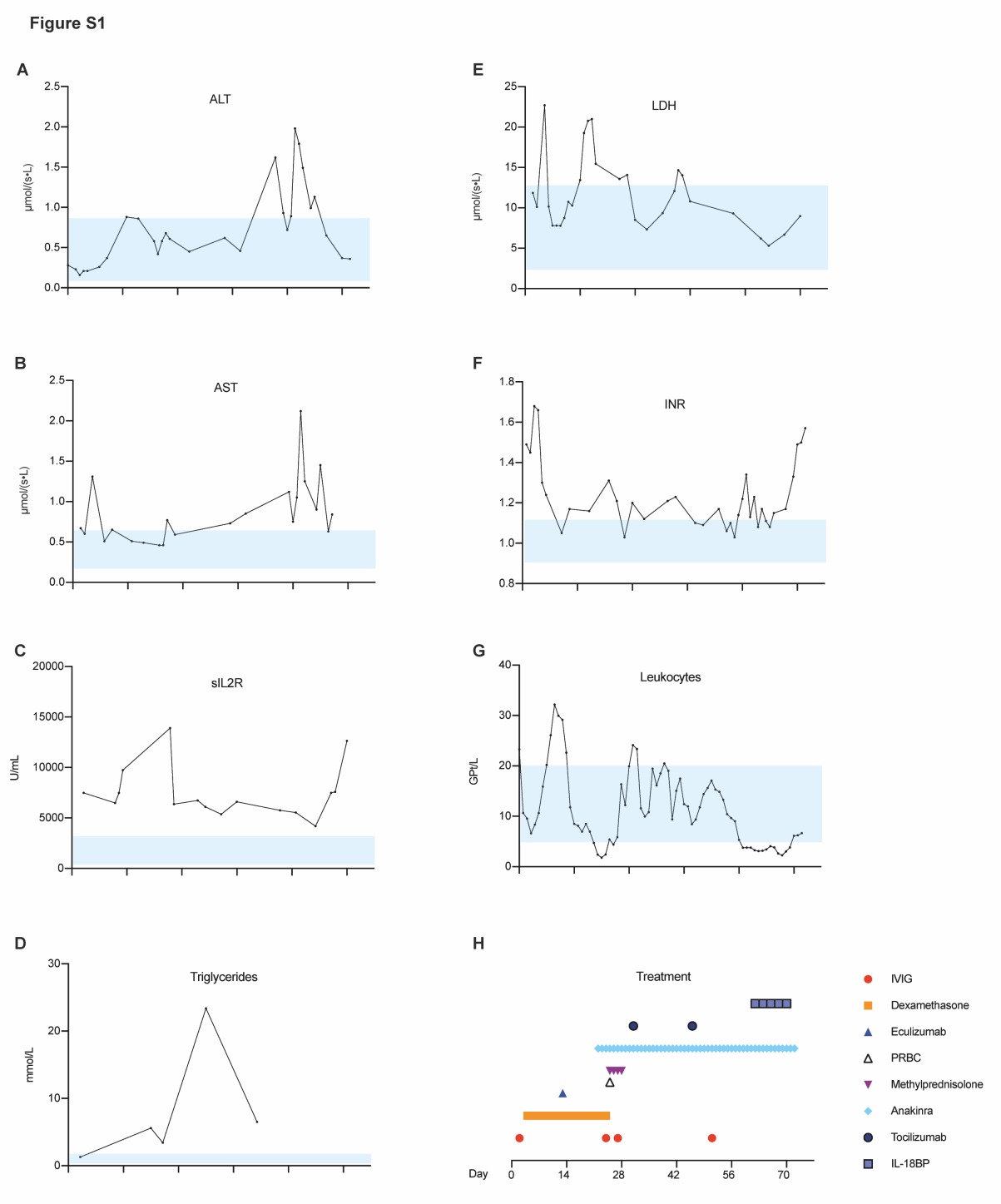

Figure S1: Biochemical and haematological profile of Patient 1. Alanine aminotransferase (ALT, A), aspartate aminotransferase (AST, B), soluble IL-2 receptor (sIL2R) (C), triglycerides (D), lactate dehydrogenase (LDH) (E), international normalized ratio (INR) (F) and leukocyte count (G) displayed over time. Treatment over the same time period is shown in (H). IVIG intravenous immunoglobulin, PRBC packed red blood cell.

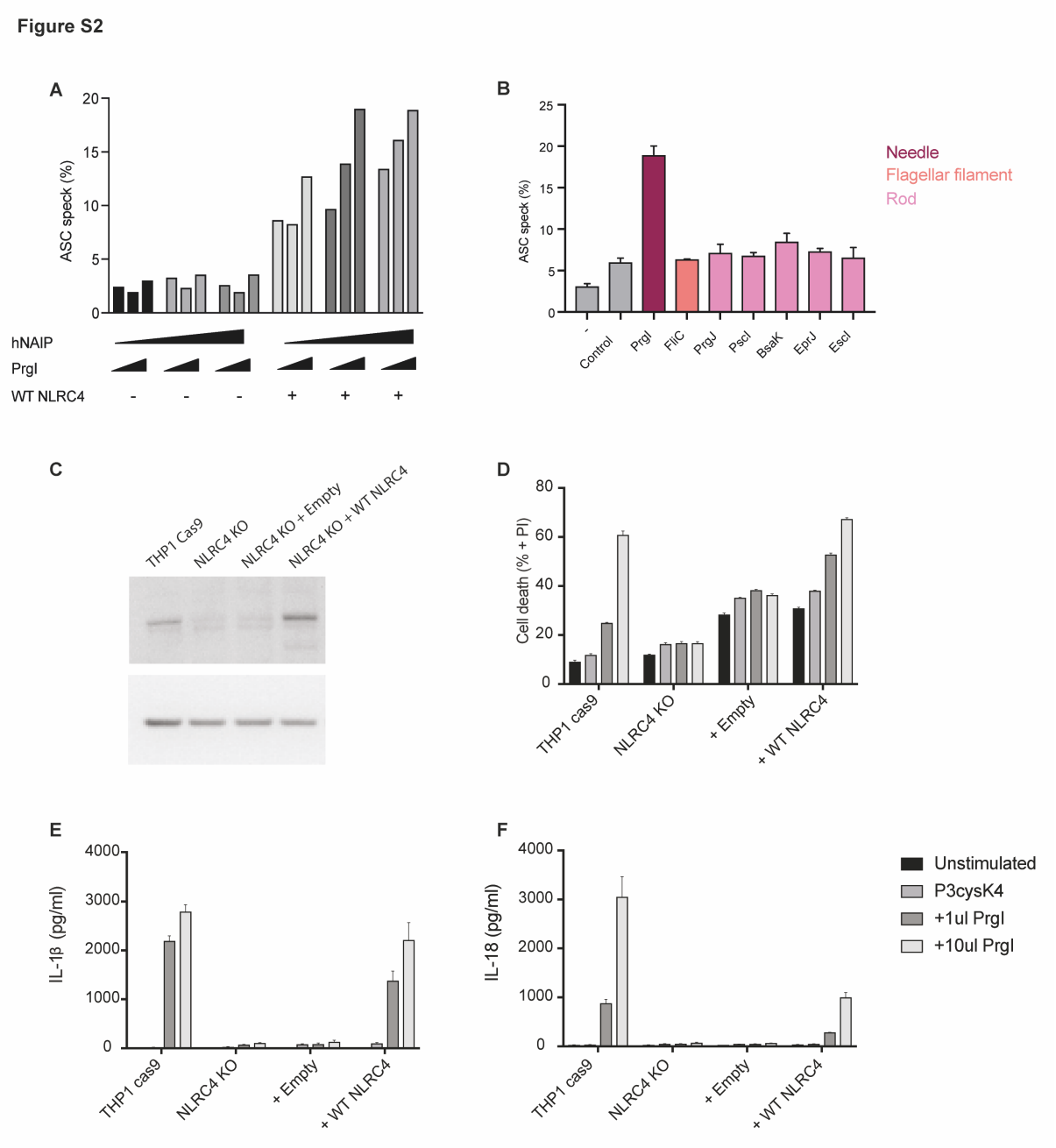

Figure S2: T3SS selection and validation of *NLRC4 KO* THP-1 Cas9 cells. HEK293T cells were transfected with wild type (WT) NLRC4 as well as increasing doses of human NAIP (hNAIP) and T3SS needle protein, PrgI. Flow cytometric analysis of ASC speck formation (A) shows that WT NLRC4 is needed for ASC specks to be formed, and that hNAIP is required for ASC specks to increase in response to low amounts of PrgI. To ensure specificity of response, various T3SS proteins were transfected into HEK293T cells in addition to WT NLRC4 and

hNAIP (B). The ASC speck response is specific to the only needle protein tested, PrgI. THP-1 Cas9 cells infected with inducible sgRNA targeting exon 2 of *NLRC4* were treated with doxycycline for 72 h and expression of NLRC4 assessed with western blot performed on whole cell lysate after 48 h rest (C). Cells were then primed with P3CK4 and infected with two amounts of retrovirus expressing PrgI needle protein. After 24 h assessment of (D) cell death, (E) IL-1β and (F) IL-18 secretion was undertaken.

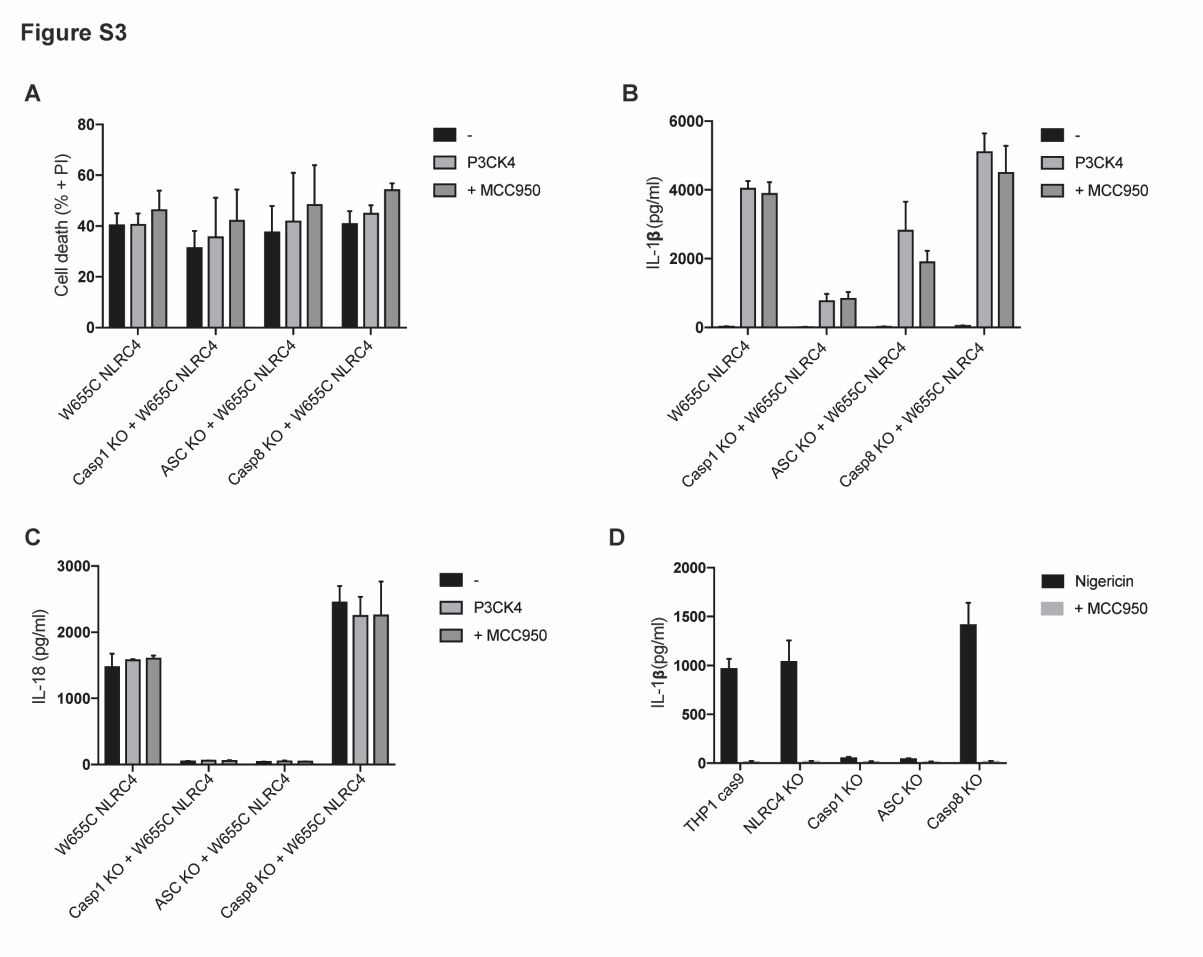

Figure S3: Role of NLRP3 in p.W655C NLRC4 associated inflammation. *NLRC4* knock out (KO) THP-1 monocytes were lentivirally transduced with wild type (WT) or p.W655C NLRC4. Contribution of NLRP3 to cell death and cytokine response was determined using MCC950 at 20ng/mL, with (I) cell death, (J) IL-1β and (K) IL-18 assessed after 24 hours of treatment. (L) IL-1β response to nigericin (10μM) for one hour with and without MCC950 pretreatment in cell lines used. Data pooled from at least three independent experiments.

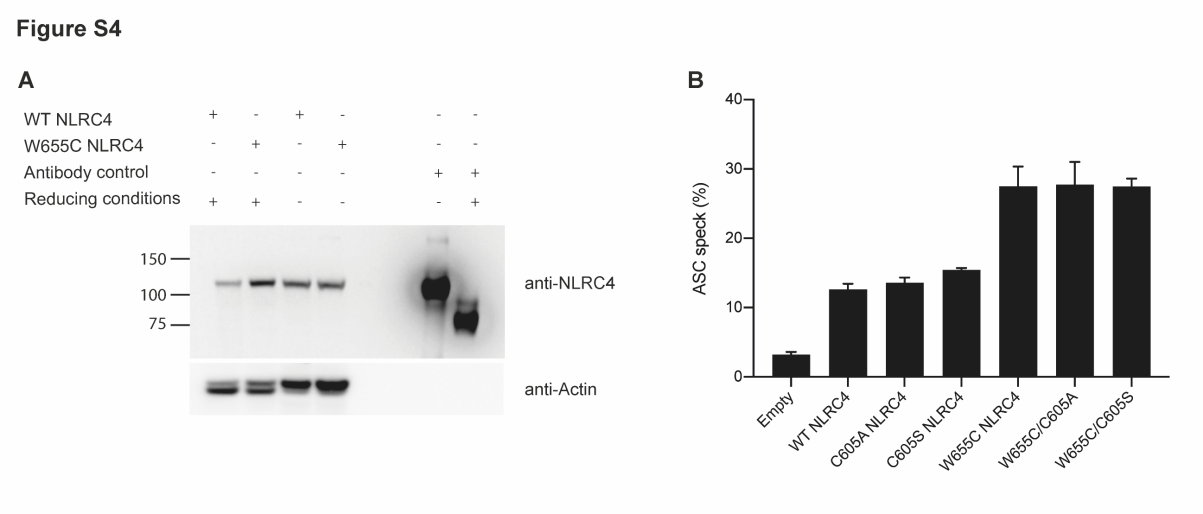

Figure S4: Investigation of potential disulphide bond. (A) HEK293T cells were transfected with wild type (WT) NLRC4 or W655C NLRC4. After 24 hours, cells were lysed in either reducing (presence of dithiothreitol, DTT) or non-reducing conditions. The size of the protein was compared between conditions. An antibody only control was used to ensure that a band could be detected in both conditions. Representative of three independent experiments. (B) ASC speck quantification using flow cytometry of wild type (WT) and p.W655C NLRC4 with and without manipulation of p.C605 NLRC4. Data pooled from three independent experiments.

4.3 Discussion

My submitted manuscript not only provides *in vitro* evidence of pathogenicity of a novel variant present in two unrelated patients, it also indicates that the mechanism of increased NLRC4 activation is distinct to that of mutations described to date. A number of key issues were not discussed in the paper due to word limit restrictions and are elaborated on here.

The LRR domain has previously been shown to be required for the autoinhibition of NLRC4, which is seemingly in conflict with the LRR data in **Figure 7**. HEK293T cells transfected with NLRC4 lacking the LRR domain resulted in spontaneous cleavage of Caspase-1 that was not seen with full length NLRC4 (Poyet et al., 2001). The LRR of NAIP is important in ligand recognition (X. Yang et al., 2018) and NAIP subsequently interacts with NLRC4 through NOD and WHD elements that are conserved between the two proteins (Diebolder, Halff, Koster, Huizinga, & Koning, 2015; Zhang et al., 2015). This would suggest that an NLRC4 lacking an LRR domain would potentially be in an active conformation, and still able to interact with NAIP. The relevance of such a deletion in health and disease is unclear. When a reference database was reviewed for genetic variations in NLRC4 in the healthy human population, a few frame shift and early termination variants are present in a heterozygous state (Lek et al., 2016). These variations occur at a number of positions throughout the LRR domain encoding DNA, including heterozygous p.Trp655Ter in three individuals. The likelihood of patients with an auto-active NLRC4 due to lack of LRR domain being present in a database of healthy controls is low given the severity of symptoms seen with disease (**Table 4.1**). It is possible that the NLRC4 in these patients is subject to nonsense mediated decay and therefore not expressed to cause auto-activation.

Furthermore, truncation of a domain is significantly distinct from an amino acid substitution. It is clear from structural models of NLRC4 in an oligomeric state that the LRRs are in close proximity. Although no formal interaction has been investigated in the past, results presented here suggest that an interaction does exist, requiring either one or all of p.Asp1010, p.Asp1011, p.Leu1012 and p.Ile1015 (**Figure 7A**). Patients with mutations in interface residues may actually have reduced, rather than increased, NLRC4 function.

Furthermore, data presented here also suggests that an amino acid substitution in the LRR region can result in an interaction between two LRR domains being formed. These findings may have implications for other NLRs and mutations in the LRR of other inflammasome forming proteins may have similar effects. However, there are limitations to modelling the effect of LRR mutations in other inflammasome forming proteins such as NLRP3. To date, the crystal structure of NLRP3 has not been elucidated. When comparing NLRC4 with NLRP3, there is less than 15% sequence identity ("Database Resources of the National Center for Biotechnology Information," 2017), with NLRP3 harbouring a PYD rather than the CARD present in NLRC4. Even when aligning the LRR specifically, the sequence identity ranges from 25% to 45%. This suggests that determining the regions in NLRP3 LRR that are analogous to the binding interface between NLRC4 LRRs will be challenging.

There are a number of other aspects of NLRC4-AID mutations that remain unexplored and warrant further evaluation, for example, determining whether the activation is dependent on phosphorylation of NLRC4, or on the presence of NAIP. A similar method to that described in the manuscript could be used to evaluate the role of phosphorylation of NLRC4. THP1 *NLRC4 KO* cells could be transduced with WT NLRC4 or p.Ser533Ala NLRC4 and the response to PrgI assessed, as performed in **Figure S2**. Furthermore, patient mutations combined with the

phospho-dead p.Ser533Ala, for example p.Trp655Cys/p.Ser533Ala NLRC4, could be evaluated as shown in **Figure 5**. These experiments would address two questions, (i) whether phosphorylation of NLRC4 is needed for its response to stimuli, and (ii) whether mutant NLRC4 acts independently of phosphorylation. Importantly, these would be performed in human cells. The requirement for NAIP in the activation of patient mutant NLRC4 could be evaluated through the generation of *NAIP KO* THP1 cells using CRISPR/Cas9 gene editing techniques. After confirmation of effective knock-out at a protein level as well as functionally, these cells could then be transduced with WT or mutant NLRC4 to determine whether a similar cell death and cytokine response results when compared to the same experiment performed in *NLRC4 KO* THP1 cells. If the response is indeed NAIP-dependent, it may suggest the need for a trigger such as an infection to induce inflammatory episodes. If independent of NAIP, it would suggest that the mutant NLRC4 protein can spontaneously oligomerise in the absence of NAIP.

Another area of interest is the potential link between NLRC4-AID and X-linked lymphoproliferative syndrome 2 (XLP2). Originally described in 2006 (Rigaud et al., 2006), XLP2 is a recessive X-linked disorder caused by mutations in *XIAP* (**Figure 4.3**) with features of EBV induced HLH and of inflammatory bowel disease (IBD) as well as recurrent infections (Latour & Aguilar, 2015). Although there is no known link between NLRC4-AID and XLP2, there are striking clinical similarities including HLH and marked gastrointestinal inflammation. Furthermore, NLRC4 associates with NAIP, which, like XIAP, is a member of the IAP family of proteins. This raises questions including: Does NAIP interact with, compete with, or regulate the expression of XIAP? If so, would increased NAIP-NLRC4 complex formation alter this relationship? Alternatively, could an increase in NAIP-independent NLRC4 complex formation, if it is indeed NAIP-independent in NLRC4-AID, affect NAIP and/or XIAP

expression? Addressing these questions may provide a link between these two phenotypically similar conditions.

Another consideration is of the markedly elevated serum IL-18 cytokine levels in patients with NLRC4-AID. Profound elevations in serum free IL-18, as well as increased CXCL9, an IFN-γ induced chemokine, have not been seen in other inflammasomopathies. How the increased activation of several inflammasome forming proteins results in such distinct clinical and serum cytokine profiles is not clear when the downstream effects are Caspase-1 activation and the cleavage of pro-IL-1β and pro-IL-18 to their mature forms. It is possible that these proteins engage in non-inflammasome pathways that account for differing disease presentations. Alternatively, there may be variability in cell specific expression of these inflammasome components or differing priming requirements that cause specific symptomatology, for example NLRC4 in IECs. Exploring this further may explain why patients experience the specific symptoms and complications they do, and whether more targeted treatment might improve their quality of life.

5 AUSTRALIAN AUTOINFLAMMATORY DISEASES REGISTRY (AADRY): A NATIONAL APPROACH TO THE GENETIC AND IMMUNOLOGICAL EVALUATION OF PATIENTS WITH MONOGENIC AUTOINFLAMMATORY DISEASES.

5.1 Introduction

Since the original publication in 1971 outlining two cases of FMF complicated by pulmonary amyloidosis (Pryor & Colebatch, 1971), there have been few studies looking at the status of monogenic AIDs in Australia (B. W. Robinson & Joske, 1980). The authors of a case series of six Australian children with FMF in 1989 suggested that with changing demographics the prevalence of this disorder in the population was likely to increase (Moore et al., 1989), however no formal effort has been made to monitor this. A recent cohort study documented 18 patients with CAPS across the country (Mehr et al., 2016). Importantly, only clinicians who were members of Australian Paediatric Surveillance Unit were surveyed, potentially neglecting an adult cohort of patients. The prevalence of TRAPS and HIDS in Australia is unknown, as is the status of more recently described AIDs.

Furthermore, clinicians caring for patients with an AID who do not have a pathogenic mutation in known disease-causing genes do not have a streamlined approach to the further genetic evaluation. The current diagnostic pathway, outlined in **Figure 5.1**, has recently included NGS techniques (**Section 1.3**) for the evaluation of a panel of genes. Clinicians suspecting a diagnosis for which Sanger sequencing is available will often opt for this due to lower cost and faster turnaround time. However, considering the broadening phenotype of patients with autoinflammatory disorders, a number of clinicians order a panel test first line. The most comprehensive panel offered in Australia includes 20 genes (**Appendix 3**) but lacks a number that have been recently described, such as *ADA2*, in part because of the lengthy process of

acquiring National Association of Testing Authorities, Australia (NATA) accreditation for inclusion.

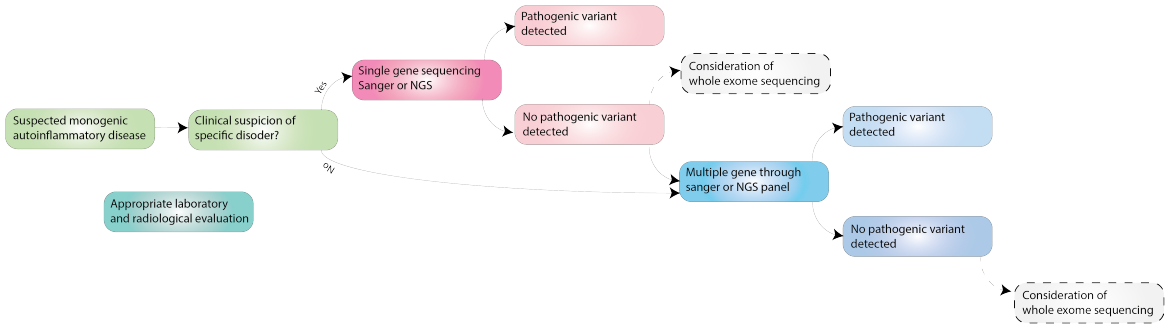

Figure 5-1 Pathway to a genetic diagnosis for autoinflammatory disorders in Australia.

A patient suspected of a monogenic autoflammatory disorder in Australia has access to Sanger sequencing technology for *MEFV, NLRP3, MVK* and *TNFRS1A* (pink) at four sites across the country. If one of the four disorders caused by mutations in these genes is not suspected, a patient may have multiple genes sequenced through a Next Generation Sequencing (NGS) panel (blue), currently performed at only two sites. If a pathogenic variant is not detected via either of these methods, and the clinical suspicion remains strong, AADRY provides the framework to recruit patients and provide further genetic evaluation (grey).

The Australian Autoinflammatory Diseases RegistrY (AADRY) was conceived to address limitations in the current knowledge of genetically defined AIDs in Australia as well as a means of providing clinicians with access to a research team to further evaluate patients without an established genetic diagnosis. Since the inception of AADRY, there have been two related projects established, the Melbourne Genomics Health Alliance (MGHA; HREC/13/MH/326) and the Australian Genomics Health Alliance (AGHA; 2016.224) which are addressing the question of diagnostic utility and economic viability of WES across multiple disease cohorts. These projects are primarily comparing current accredited diagnostic pathways with the use of WES and curation of a select gene list as first line genetic testing. AADRY has linked with both of these projects. Patients without a genetic diagnosis after curation of the approved gene

list are recruited to the research arm of AADRY with further interrogation of the WES results, as well as recruitment of family for segregation studies.

5.1.1 Approval by ethics committee

This project has been approved after rigorous review by two separate Human Research Ethics Committees (HRECs) with reference numbers HREC/15/MonH/31 and HREC/15/SCHN/346. A number of considerations were made in establishing the registry in terms of data confidentiality, access and storage, as well as the genetic evaluation of patients, in particular children. It is important to note that there is no international consensus on best practice regarding incidental findings, also known as secondary findings (Anastasova, Blasimme, Julia, & Cambon-Thomsen, 2013), unanticipated findings (Parker, 2008) or off-target findings (E. C. Hayden, 2013), arising from genomic research. The recommendations from the National Statement on Ethical Conduct in Human Research generated by the Australian Government (*National Statement on Ethical Conduct in Human Research* (2007) (Updated May 2015)) stipulates that participants have the choice of whether they are informed of significant findings or not. This is reflected in the Protocol and Patient Information and Consent Forms (PICFs) generated for this project (**Appendix 4** and **5**).

5.1.2 Recruitment process

Participants were recruited into one of three arms (**Figure 5.2)**. Group 1 included patients suspected of suffering from an AID who had undergone clinically appropriate genetic testing as determined by their treating clinician but remained without a genetic diagnosis. Group 2 defined family members of patients from Group 1 who were recruited for segregation studies. Patients with genetically confirmed autoinflammatory disorders were recruited into Group 3. Ascertainment of participants was through treating clinicians, most commonly a

rheumatologist or clinical immunologist. This chapter focuses on the results of recruitment to Groups 1 and 2.

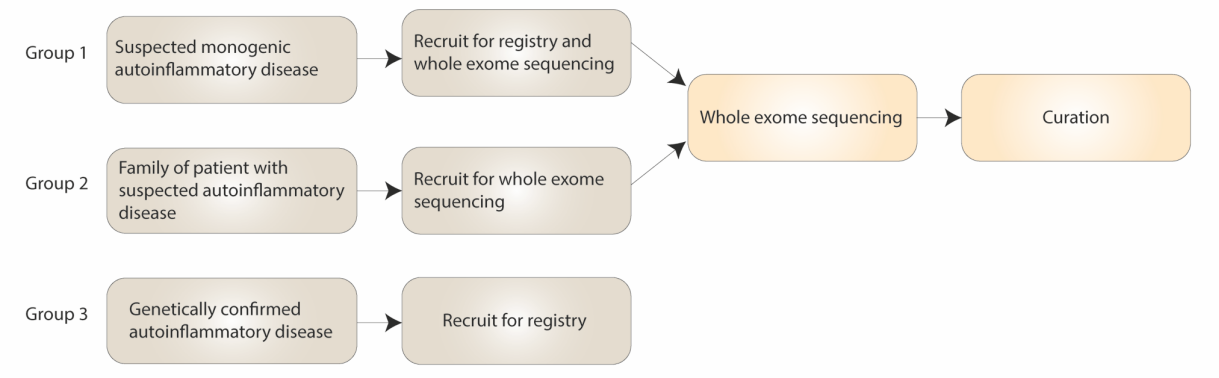

Figure 5-2 Recruitment arms of AADRY.

Participants were recruited to one of three arms. Group 1 included patients suspected of having an underlying monogenic autoinflammatory disorder without a genetic diagnosis despite diagnostic testing. Group 2 included, where possible, the parents of Group 1, as well as siblings. Patients with confirmed monogenic autoinflammatory disorders were recruited into Group 3.

5.1.3 Data collection

In order to streamline the potential collaboration with international registries, the data collected was in line with the EuroFever project (Ozen, Frenkel, Ruperto, & Gattorno, 2011) (**Appendix 6**). Furthermore, clinical diagnostic criteria for monogenic AIDs were included. The online REDCap platform was used to manage entry and de-identification of participants, and allowed for graded security and access to collection and storage of data.

5.1.4 Variant calling, curation and classification

The process of WES is described in **Section 1.3** and **Section 2.10**. A variant list was generated by collaborators at the Centre for Personalised Immunology (CPI, Canberra, Australia). Briefly, BAM files generated through Sequence/alignment Map (SAM) tools were subsequently run through a variant caller and filtering strategy as previously described (Field, Cho, Andrews, & Goodnow, 2015). Trio variant calling was undertaken using variant analysis

of sequenced pedigrees (VASP) as previously described (Field, Cho, Cook, et al., 2015). Initial filtering of the variants proceeded by excluding synonymous variants, variants with a dbSNP mean allele frequency (MAF) of >0.02, and intronic variants more than 50 base pairs from an intron-exon junction (**Figure 5.3**). Further filtering was undertaken for the analysis performed in **Section 5.2.3** and **Section 5.2.4** due to the volume of variants and resource restraints, and only variants with a MAF of <0.01 were included. The quality of the sequence data for each variant was assessed by viewing the BAM file on the Integrated genomics viewer (IGV) (J. T. Robinson et al., 2011) to ensure adequate depth and exclude potential artefact.

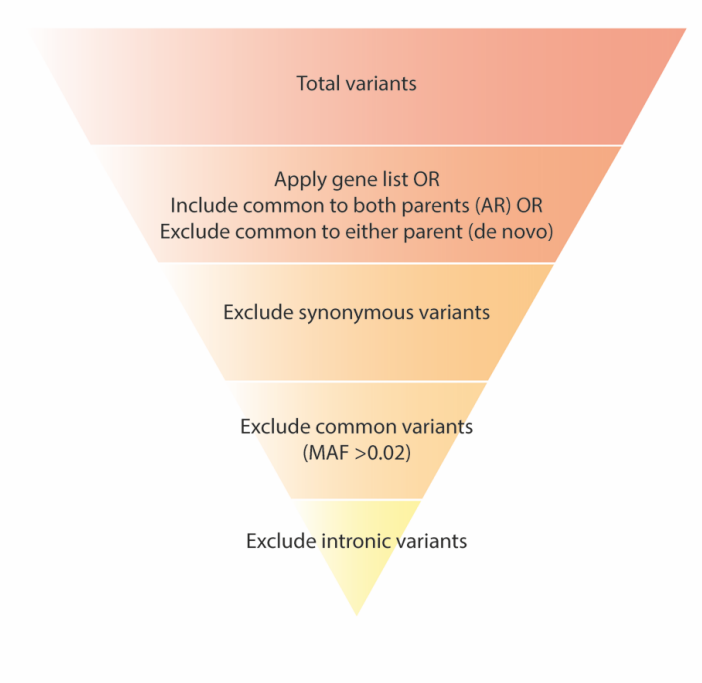

Figure 5-3 Filtering process of variants called.

The number of variants called was sequentially narrowed by stepwise filtering. A specific gene list (such as *Infevers*) was applied, or the mode of inheritance considered. Remaining variants were then excluded if synonymous, common as defined by a mean allele frequency of >0.02, or if found in an intronic region not proximal to an intron-exon junction.

The allele frequency of a variant was determined using 1000 Genomes database (Auton et al., 2015), dbSNP (Sherry et al., 2001), Exome Aggregation Consortium (ExAC) (Lek et al., 2016), and Genome Aggregation Database (gnomAD) (Lek et al., 2016). The coverage data on ExAC

was considered as a low allele frequency of a variant in a poorly covered gene may indicate inadequate sequencing data rather than true population data.

For the analysis of amino acid substitutions, a number of *in silico* tools were used including Polymorphism phenotyping version 2 (PolyPhen-2) (Adzhubei et al., 2010), Sorting Tolerant From Intolerant (SIFT) (P. Kumar, Henikoff, & Ng, 2009), MutationTaster2 (Schwarz, Cooper, Schuelke, & Seelow, 2014), Functional Analysis through Hidden Markov Models (fathmm) (Shihab et al., 2013) and Combined Annotation Dependent Depletion (CADD) (Kircher et al., 2014). Although most of these programs use a combination of allele frequency data as well as characteristics of amino acid change as determined by the Grantham Score (Grantham, 1974) and protein structure, there are differences in the algorithms that make direct comparison difficult. These algorithms were therefore used together to determine whether a variant is consistently predicted to be benign or pathogenic.

The tolerance of a gene or amino acid residue to variation was evaluated using multiple resources. Both ExAC residual variation intolerance score (RVIS) (Petrovski et al., 2015) and subRVIS (Gussow, Petrovski, Wang, Allen, & Goldstein, 2016) use allele frequency data to determine whether, when compared with a reference genome sequence, a gene of interest has more common functional variation, suggesting that it is tolerant to change. subRVIS extends this model by subdividing the gene into domain encoding regions. In both models, a lower score suggests less tolerance to change. Conservation of an amino acid residue across species was noted using HomoloGene ("Database Resources of the National Center for Biotechnology Information," 2017) and PhyloP (Pollard, Hubisz, Rosenbloom, & Siepel, 2010), where PhyloP provides phylogenic analysis to determine the rate at which residues are evolving. A positive

score indicates a site predicted to be conserved, and a negative score suggests a fast-evolving residue.

Variant classification was based on consensus guidelines published by the American College of Medical Genetics and Genomics (ACMG) (S. Richards et al., 2015), ranging from Pathogenic (5) and Likely Pathogenic (4) to Likely Benign (2) and Benign (1). Further subcategorization of variants of uncertain significance (VUS) was based on the weight of evidence towards pathogenicity (3a) or 'benign-ness' (3c) (**Appendix 7**).

5.1.5 Gene lists

5.1.5.1 Infevers gene list

As touched on in **Chapter 1**, the *Infevers* database is a freely accessible online resource that collates variants found in genes that are associated with AIDs. At its inception in 2001, the database documented disease causing variants in *MEFV, NLRP3, TNFRSF1A* and *MVK* with a dedicated editor responsible for assessing new entries for each gene. Since this time, the database has expanded, now including 30 genes and a total of 1590 variants implicated in AIDs. Whist the registry has kept abreast of most new autoinflammatory conditions described, certain limitations exist. *Infevers* relies on authors of publications or clinicians to submit the variant of interest for review. Importantly, the registry documents only the first report of the variant which, with more sequencing performed on a wider phenotypic spectrum, results in potentially benign polymorphisms remaining on the registry. For example, c.605G<A *MEFV* encoding p.Arg202Gln is listed on the registry (Bernot et al., 1998; Sarrauste de Menthiere et al., 2003) despite having a MAF of 0.2359 (Lek et al., 2016) with various variant classification guidelines interpreting it as benign or likely benign (Landrum et al., 2016). A comprehensive supplementary tool has been made available by Papa et al. on behalf of the Paediatric

Rheumatology International Trials Organisation (PRINTO) and the Eurofever project, highlighting the phenotype-genotype associations for the variants in *MEFV, MVK, NLRP3* and *TNFRS1A* based on patients recruited to the Eurofever registry (Papa et al., 2017). Whilst variants subsequently determined to be polymorphisms remaining on the registry is not necessary a significant issue, users must be cautious not to assume pathogenicity of all variants. Remarkable steps have been taken to maintain the currency of the list, with references updated if pathogenic mechanisms have been determined since the first report, as was done in the case of p.Ser242Arg *MEFV* (Masters et al., 2016).

5.1.5.2 International Union of Immunological Societies (IUIS) gene list

The *Infevers* gene list was until 2017 the most comprehensive list of AID causing genes. The IUIS' latest catalog of primary immunodeficiencies published in December of 2017 included interferonopathies under the category of AIDs for the first time (Bousfiha et al., 2018; Picard et al., 2018). These conditions were previously included under the category of 'disorders of immune dysregulation' (Picard et al., 2015) and no explanation for the change has been offered.

The IUIS classification now has a similar number of genes implicated in AIDs as the *Infever* database. However, as shown in **Section 1.2**, the gene lists are not the same. To ensure that all appropriate genes were assessed, both lists were used in the initial curation of WES results.

5.2 **Results and discussion**

5.2.1 Patient characteristics

The results presented in this chapter are of the first 20 participants recruited to Group 1 (summarized in **Table 5.2**). The mean age of participants was 12.4 years, with a median of 8.5 years and range of 3 to 51 years. The majority (14/20, 70%) of participants had previously undergone genetic testing. Both parents were recruited for 14 index cases and one parent for 5 index cases.

5.2.2 Gene lists

The *Infevers* and IUIS genes were curated for the first 20 participants recruited to Group 1 after ensuring adequate coverage on IGV. The filtering process was followed with a total of 55 variants in 23 genes for curation (**Figure 5.4**). Both *NLRP7* and *NOD2* had seven variants at the end of the filtering process, suggesting that these may by more polymorphic than other genes assessed. Although the mean number of variants for curation in an individual was 2.8, two participants did not have any variants for curation of the *Infevers* and IUIS genes. Four variants occurred in two participants (p.Phe4Cys *AP1S3,* p.Gln310Arg *NLRP7*, p.Leu1007Pro*fs2 *NOD2* and p.Gly8Arg *PSMB8*) and one variant was present in three participants (p.Glu601Lys *LPIN2*). The curation did not yield any pathogenic or likely pathogenic variants (**Table 5.3** and **Appendix 8**). The majority of variants (45/55, 82%) were classified as VUS. This is not unexpected as most patients had already undergone genetic sequencing of a select panel of genes prior to recruitment, and that had failed to detect a pathogenic variant (**Table 5.2**).

Study identifier	Age (years)	Gender	Clinical features	Family history	Previous genetic testing	Family recruited
AADRY10	51	Male	Severe psoriasis	NA	Nil	None
AADRY13	18	Male	Inflammatory encephalomyelitis, interstitial lung disease, serositis	No	*NLRP3, MEFV, TNFRS1A*	Trio (+)
AADRY15	3	Female	NOMID-like	No	*NLRP3*	Trio (+)
AADRY19	6	Male	Periodic fever, rash, diarrhea, developmental delay, rigidity	No	*MEFV, TNFRS1A*	Mother
AADRY25	19	Male	Periodic fever, polyarthritis, ischaemic events. Autoantibody +ve	No	*TNFRS1A*	Mother
AADRY26	18	Female	Periodic fever, pericarditis	No	*MEFV*	Trio (+)
AADRY27	9	Female	Periodic fever, periorbital oedema, hepatosplenomegaly. Autoantibody +ve. Tocilizumab responsive.	No	Panel	Trio
AADRY29	3	Female	Periodic fever. Prednisolone responsive. Not responsive to IL-1β antagonism.	No	Panel	Trio (+)
AADRY30	8	Male	NOMID-like, not responsive to IL-1β antagonism.	No	*NLRP3*	Trio
AADRY31	8	Male	Atypical PFAPA	No	Nil	Trio (+)
AADRY32	20	Male	Recurrent uveitis, synovitis and myalgia	No	Nil	Trio (+)
AADRY33	8	Female	Periodic fever, associated with epigastric pain, headache and sore throat. No response to tonsillectomy.	No	Nil	Trio (+)
AADRY34	9	Male	Periodic fever.	No	*MVK*	Trio (+)
AADRY60	4	Female	Periodic fever, mucosal ulceration, conjunctivitis and abdominal pain.	No	Nil	Trio
AADRY68	15	Female	Periodic fever associated with rash and arthritis, chest pain and conjunctival injection.	No	*NLRP3, MEFV*	Trio
AADRY71	5	Female	Periodic fever, mucosal ulceration, conjunctivitis, arthritis, myalgia.	No	*MEFV*	Trio
AADRY74	26	Female	Periodic fever, mouth ulcers, sore throat, urticarial, lymphadenopathy, syncope.	No	Nil	Mother (+)
AADRY78	4	Male	Periodic fever associated with sore legs, back and abdomen.	No	*TNFRS1A*	Trio (+)
AADRY82	9	Female	Febrile episodes on background of multiple comorbidities. Rash, diarrhea. Response to colchicine.	No	*MEFV, TNFRS1A*	Trio
AADRY91	5	Female	Periodic fever associated with abdominal pain. Recurrent urticaria	No	Panel	Trio

Table 5-1 Patient characteristics.

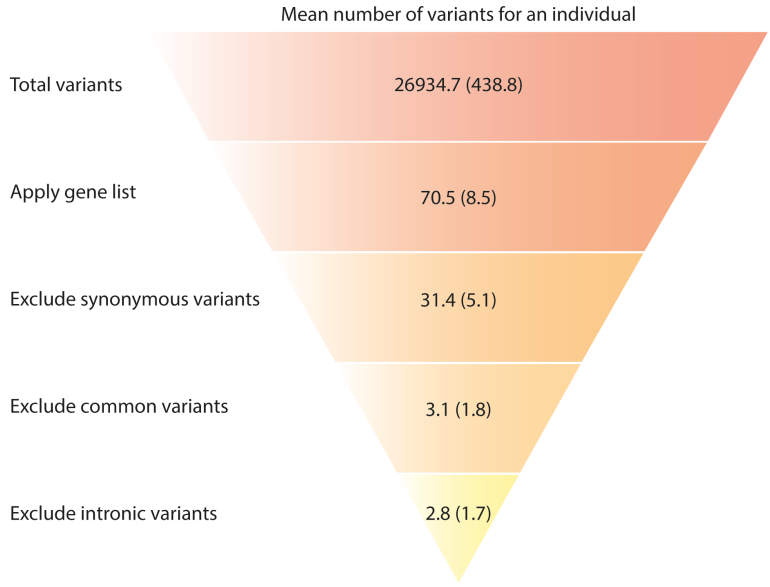

Figure 5-4 Infever and IUIS gene list filtering results.

After application of the Infever and IUIS gene list, the average number of variants per participant was 70.5, which was reduced to 2.8 after sequential filtering. Standard deviation of each filtering step is given in parentheses.

Importantly, only four of the variants were in genes that caused diseases related in some way to the phenotype of the participant. AADRY25 was found to have a heterozygous p.Val939Met *NLRP1* variant. There has been a single publication of three patients with mutations in *NLRP1* with a systemic disorder including autoimmune manifestations **(Section 1.1.1.1.2.1)** (Grandemange et al., 2017). Unlike AADRY25, however, the patients presented suffered from diffuse skin keratosis. The skin involvement is also highlighted in an earlier publication of recurrent skin inflammation and cancer susceptibility in a large Tunisian family with heterozygous GoF mutations in *NLRP1* (Zhong et al., 2016). These conditions are, however, newly described and the full phenotypic spectrum of disease is not clear. Importantly, the patient's asymptomatic mother harbours this variant, and it is present with a frequency of more than 1/100 in healthy cohort databases, further decreasing the likelihood of its pathogenicity.

AADRY60 harboured the p.Pro75Leu *TNFRSF1A* variant (previously p.Pro76Leu *TNFRSF1A*). Early work suggested to possibility of this variant being a low-penetrant mutation for TRAPS rather than a benign polymorphism (Aksentijevich et al., 2001). Monocytes from patients with this variant were stimulated with PMA and, unlike healthy control monocytes, the clearance of TNFR1 was reduced, suggesting defective receptor shedding. Since this time, the population prevalence of 1 in 35 (Auton et al., 2015), rising to 1 in 10 in sub-Saharan West African populations (Tchernitchko et al., 2005), and lack of segregation with disease, has raised questions about the pathogenicity of this variant. Additionally, this variant was detected prior to the participant's enrolment in AADRY through Sanger sequencing, and was interpreted as likely benign by the reporting laboratory.

The p.Phe4Cys *AP1S3* variant was found in a patient with severe plaque psoriasis, AADRY10. Literature to date suggests that AP1S3 encodes a subunit important for the stability of the adapter protein complex (AP)-1 which is involved in endosomal transport (Hirst et al., 2011). This particular variant was linked to GPP in 2014 after a single publication documented the WES results of 9 individuals (8 females and 1 male) with GPP, with 6 noted to have heterozygous p.Phe4Cys AP1S3 variants (Setta-Kaffetzi et al., 2014). In vitro, expression of p.Phe4Cys AP1S3 in HEK293T cells was reduced when compared with WT AP1S3. Knock-down of *AP1S3* using shRNA in HaCaT immortalised keratinocytes resulted in reduced induction of *IFNB1* in response to TLR3 signalling when compared with WT AP1S3 (Setta-Kaffetzi et al., 2014). It was hypothesised that, given reduced protein expression seen, p.Phe4Cys AP1S3 would behave in a similar manner to the knock-down model. How this protein linked to GPP, previously associated with mutations in *IL36RN* (Marrakchi et al., 2011), was explored by Capon and colleagues, who noted that expression of *IL36A* was increased in *AP1S3* deficient cells, as was *IL-1β* and *IL8* (Mahil et al., 2016). There are still

questions regarding the pathogenicity of p.Phe4Cys *AP1S3* in GPP. The MAF of up to 0.008 of a variant supposedly causing a rare autosomal dominant condition is quite high. This, along with the coexistence of *IL36RN* mutations in some of the patients with *AP1S3* mutations (Mahil et al., 2016), suggests that other genetic factors may be at play in patients with p.Phe4Cys *AP1S3.* The presence of this variant in another participant (**Table 5.3**) without dermatological features supports the idea that p.Phe4Cys *AP1S3* may be an association rather than a cause of GPP. Importantly, the data on variants in AP1S3 in psoriasis have been limited to GPP, a rare form of psoriasis characterised by pustules, widespread erythema and systemic symptoms (Setta-Kaffetzi et al., 2014). The role of AP1S3*,* if any, in plaque psoriasis, which is present in 0.5 to 11.4 percent of the population (Michalek, Loring, & John, 2017), remains to be investigated, and the significance of this variant in AADRY10 is unclear.

Of note, AADRY10 also harbours a p.Arg682Trp *CARD14* variant. CARD14 has been shown to interact with BCL10 and is involved in NF-κB signalling (Bertin et al., 2001). Variants in *CARD14* have been associated with susceptibility to psoriasis (C. T. Jordan, Cao, Roberson, Pierson, et al., 2012). Association studies on two large psoriasis pedigrees with an autosomal dominant trait linked p.Ser116_Gly117ins22 *CARD14,* generating an aberrant splice variant, with disease. In a HEK293T model, cells transfected with mutant CARD14 led to a 3- to 4-fold increase in NF-κB reporter levels when compared with WT CARD14. Another study examined a cohort of 6000 psoriasis patients and 4000 healthy controls (C. T. Jordan, Cao, Roberson, Duan, et al., 2012). The authors found that p.Arg682Trp *CARD14* had a frequency of 0.013 in cases and 0.012 in controls. Furthermore, p.Arg682Trp CARD14 did not increase NF-κB activity in a HEK293T luciferase assay when compared with WT CARD14, and did not result in upregulation of genes found in psoriatic keratinocytes. Taken together, these data suggest that this variant is not pathogenic. Whether having both p.Arg682Trp *CARD14* and

p.Phe4Cys *AP1S3* results in an increased risk of psoriasis is uncertain. Segregation studies would be interesting to determine whether asymptomatic family members harbour the same variants. If so, this would strengthen the argument that these variants are unlikely to be significantly contributing to the phenotype seen in AADRY10.

Table 5-2 Infever and IUIS gene list variant classification.

Variant classification was performed according to American College of Medical Genetics and Genomics guidelines. (1) Benign, (2) Likely Benign, (3) Variant of Uncertain Significance.

Study identifier	Variants	Gene	Amino acid change	Phenotypic match	Segregation with disease	Classification
AADRY10	3	*AP1S3*	F4C	Possible	NA	3a
		CARD14	R682W	Possible	NA	3c
		NOD2	L1007Pfs*2	No	NA	3c
AADRY13	6	*CARD14*	P506L	No	No	1
		NLRP12	R971G	No	No	3c
		NLRP7	K511R	No	No	1
		NOD2	R684W	No	No	3c
		NOD2	G908R	No	No	3b
AADRY15	3	*CARD14*	R682W	No	No	3c
		CECR1	V349I	No	No	3c
		TNFAIP3	T647P	No	No	2
AADRY19	3	*AP1S3*	T32I	No	No	1
		LPIN2	E601K	No	NA	2
		PSMB8	G8R	No	No	3b
AADRY25	2	*ADAM17*	V673I	No	NA	3c
		NLRP1	V939M	Possible	No	3c
AADRY26	5	*APS13*	F4C	No	No	3c
		LPIN2	E601K	No	No	2
		PLCG2	P522R	No	No	3c
		PSMB4	E197V	No	No	3a
		PSMB8	G8R	No	No	3b
AADRY27	0					
AADRY29	4	*IL10RB*	V276M	No	No	3c
		NLRP7	Q310R	No	No	3b
		NLRP7	L311I	No	No	3b
		SH3BP2	A212V	No	No	3b
AADRY30	0					
AADRY31	2	*NLRP7*	Q310H	No	Yes	3b
		SAMHD1	V112D	No	Yes	3c
AADRY32	1	*SAMHD1*	D585N	No	No	3a
AADRY33	3	*IFIH1*	D112G	No	No	3a
		NOD2	L1007Pfs*2	No	No	3c
		PLCG2	M28L	No	No	3c
AADRY34	2	*TNFAIP3*	T108A	No	No	3b
		TNFAIP3	I207L	No	No	3c
AADRY60	6	*CARD14*	P506L	No	No	1
		IL10	G15R	No	No	2
		LPIN2	E601K	No	No	2
		NLRP7	R156Q	No	No	2
		RNASEH2A	D205E	No	No	3c
		TNFRSF1A	P75L	Possible	No	3c
AADRY68	2	*ADAM17*	Q30R	No	No	3c
		PSMB9	V32I	No	No	3c
AADRY71	0					
AADRY74	3	*IL36RN*	N47S	No	No	3b
		LPIN2	D891N	No	No	3c
		NOD2	R703C	No	No	3b
AADRY78	4	*NOD2*	V793M	No	No	3c
		NOD2	S431L	No	No	3a
		PLCG2	I671V	No	No	3b
		SAMHD1	P22S	No	No	3c
AADRY82	3	*AP1S3*	R33W	No	No	3a
		NLRP1	T656M	No	No	3c
		PSMB9	R173C	No	No	3b
AADRY91	3	*ACP5*	R272C	No	No	3a
		NLRP7	Q310R	No	No	3b
		NLRP7	L311I	No	No	3b

5.2.3 De novo

There were 14 index cases for whom both parents' WES was available for analysis. Following the filtering process set out in **Section 5.1.4**, a total of 119 variants were available for analysis with an average of 12.5 variants per participant (**Figure 5.5**). This list was further narrowed by manually assessing the quality of reads in IGV as well as excluding variants with a MAF of greater than 0.01 in the ExAC or gnomAD database (Lek et al., 2016). These variants were excluded from formal curation for now given time limitations. A total of 46 variants with *de novo* inheritance pattern in 14 participants were assigned for formal curation (**Table 5.4** and **Appendix 9**).

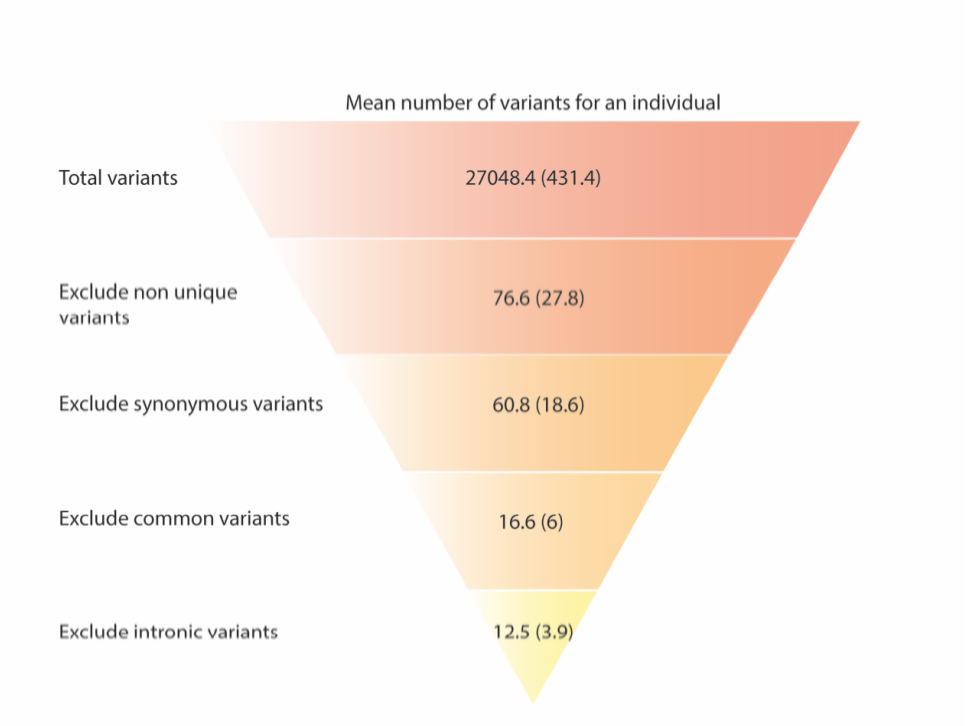

Figure 5-5 Filtering flow chart of de novo variants.

Excluding variants present in either parent reduced the average number of variants to 76.6 , which was reduced to 12.5 after sequential filtering. Standard deviation of each filtering step given in parentheses.

Only one variant was in a gene known to cause a monogenic disorder. Mutations in *SIX1* have been reported to cause autosomal dominant deafness and branchio-oto-renal syndrome (OMIM

605192) in cases of p.Glu125Lys, p.Trp129Cys, and p.Arg110Trp mutations (Ruf et al., 2004). The variant p.Arg119Cys *SIX1* in AADRY27 is consistently predicted to be pathogenic, resides in a highly conserved region and has a significant amino acid change. As it is novel and not a phenotypic match, it has been designated as a VUS.

5.2.3.1 Novel SHARPIN variant

Of the *de novo* variants, one was in a gene with a defined role in the innate immune system. The heterozygous p.His315Asn *SHARPIN* variant was identified in AADRY15. Briefly, this participant is a 17-month-old girl with a clinical diagnosis of CAPS completely responding to IL-1β antagonism, in whom no pathogenic mutation in *NLRP3* was identified. During the first year of life, she was noted to have a cranial nerve VII palsy and subsequent magnetic resonance imaging revealed hyperintensities in both facial nerves, both trigeminal nerves and the right oculomotor nerve. Further history revealed recurrent febrile episodes of long duration associated with cold induced urticaria. Serum markers of inflammation were elevated, prompting trial of multiple immunomodulatory agents including intravenous immunoglobulin, high dose corticosteroids, cyclophosphamide and methotrexate without success. Full clinical and biochemical response was seen after administration of anakinra therapy.

To date, no human disease-causing mutations have been identified in *SHARPIN*. *SHARPIN* encodes the protein SHARPIN, a key component of LUBAC involved in the regulation of the NF-κB pathway (**Section 1.1.2.1**). Prior to this function being elucidated, spontaneous homozygous frame shift mutations in *Sharpin* (referred to as $Sharpin^{cpdm}$) had been identified in mice resulting in multi-organ inflammation with eosinophilic dermatitis as well as underdeveloped secondary lymphoid organs (Seymour et al., 2007). The essential role of SHARPIN in LUBAC, as well as the importance of linear ubiquitination in the regulation of

the NF-κB pathway was subsequently defined (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). MEFs from *Sharpin*^{cpdm} mice demonstrated increased susceptibility to TNF induced cell death (Ikeda et al., 2011). More recently, optimal NLRP3 activation was reported to require SHARPIN (Gurung, Lamkanfi, & Kanneganti, 2015; Rodgers et al., 2014). Caspase-1 and pro-IL-1β cleavage was reduced in BMDMs from *Sharpin*^{cpdm} mice compared with WT BMDMs after priming with LPS and subsequent stimulation with NLRP3 agonist ATP (Gurung et al., 2015). In response to LPS stimulation alone, *Sharpin*^{cpdm} expressed less pro-IL-1β and NLRP3 when compared with WT BMDMs, suggesting that SHARPIN is important for the priming of the NLRP3 inflammasome (**Section 1.1.1.1.1**). Interestingly, this does not explain the inflammatory picture seen in *Sharpin*^{cpdm} mice and suggests that the phenotype is NLRP3-independent. What these data suggest, however, is that, at least in the murine model, SHARPIN is involved in the regulation of the NF-κB pathway and may be important for the priming of NLRP3.

The variant itself encodes a Histidine to Asparagine substitution at position 315 of SHARPIN, located five amino acid residues from the interaction with SHANK1 domain. This variant has not been identified in any healthy cohort databases, although p.His315 is substituted with Glutamine in 20 individuals and Tyrosine in one individual (Lek et al., 2016). Five independent in-silico algorithms predicted this variant to be benign and the amino acid change is only moderate. However, given the CAPS-like phenotype of AADRY15, the possible link between SHAPRIN and NLRP3 is interesting and strengthens the argument to perform *in vitro* functional assessment. The decision to investigate this variant further considered the factors above, as well as feasibility including access to relevant reagents.

5.2.3.1.1 Confirmation of SHARPIN variant

A fresh DNA sample was obtained from AADRY15. Primers were designed to amplify exon 7 of *SHARPIN* including regions flanking the intron to ensure that the PCR product was from patient genomic DNA (**Table 2.6**). The product was sequenced by Sanger method and the chromatogram viewed (**Figure 5.6**). A heterozygous C>A transition at c.1380 *SHARPIN* encoding p.His315Asn SHARPIN was confirmed.

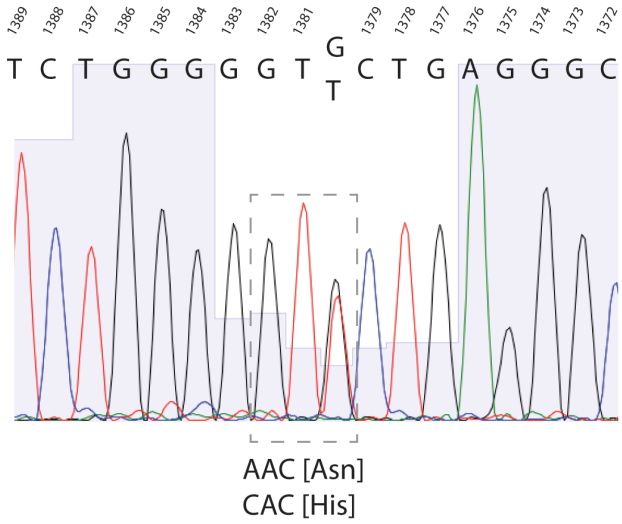

Figure 5-6 Confirmation of novel variant in SHARPIN.

Sanger sequencing of exon 7 of *SHARPIN* with a reverse sequencing primer revealed a double peak at c.1380 with nucleotides G and T present in equal number of reads. This confirmed heterozygosity with C and A present in a 1:1 ratio.

5.2.3.1.2 Future validation of SHARPIN variant

A number of *in vitro* experiments can be performed to determine the functional consequences of p.His315Gln *SHARPIN*. To explore the role of this variant on the NLRP3 inflammasome specifically, the TOFIE assessment of ASC speck formation, optimized in HEK293T cells transiently or stably expressing fluorophore labeled ASC (**Section 2.6**), may be used. HEK293T cells expressing ASC will be transiently transfected with a plasmid expressing NLRP3, and ASC speck formation subsequently quantified using flow cytometry. The effect

of transfection of increasing amounts of SHARPIN will be assessed, as will be the effect of co-expression of the remaining LUBAC components, HOIL1 and HOIP. An important control would be the use of an alternative inflammasome forming protein such as pyrin to ensure that any effects seen are specific to NLRP3. The NF-κB pathway can be explored initially through the use of an NF-κB luciferase assay in HEK293T cells transfected with mutant and WT SHARPIN. The baseline NF-κB response may require the transfection of other components of the pathway and potentially stimulation with a ligand, such as TNF. Remaining on transient transfection experiments, the relationship between SHARPIN, HOIL1 and HOIP can also be explored in HEK293T cells with co-transfection of components of LUBAC and immunoprecipitation of SHARPIN. This would allow exploration of whether the variant in SHARPIN alters the interaction of these proteins. SHARPIN is expressed in a wide range of cells (Uhlen et al., 2015), suggesting that human monocyte-like THP1 cells can provide a more physiological model than in HEK293T cells. THP1 monocytes can be stably transduced with a lentiviral construct so that endogenous SHARPIN is expressed in addition to mutant SHARPIN. In this model, both the NF-κB and NLRP3 pathways can be examined with the use of TLR ligands such as Pam3CysK and LPS as well as NLRP3 activator nigericin (Mariathasan et al., 2006). Ideally, *ex vivo* assessment of PBMCs from AADRY15 would be performed. Serum cytokine analysis is complicated by the use of anakinra in this patient, but assessment of PBMCs may provide useful information such as baseline increase in cytokine secretion, altered ubiquitination profile or abnormal response to stimulation, with both healthy donors and parents used as controls.

5.2.3.2 Other variants

There were three variants curated encoding Human Leukocyte Antigen (HLA), the major histocompatibility complex (MHC) that is specific to humans. These genes are highly

polymorphic and although involved in antigen presentation, they were not likely to be disease causing when formally curated (**Appendix 9**). Furthermore, the participants with these variants did not have features of adaptive immune dysregulation. Variants in genes involved in microtubule regulation, cell adhesion and actin cytoskeleton (p.Ser378Ter *CLASP2* and p.Arg130Gln *TTLL3*) are interesting given that known alterations in these pathways may result in immunodeficiencies, as well as autoinflammation. Although the variant in *TTLL3* is consistently predicted to be benign, and is in a region of only moderate conservation with a low amino acid alteration score, the truncation of CLASP2 is a variant to consider following up in the future.

Patient Id	*Gene*	Amino Acid Change
AADRY13	*CLASP2*	S378Ter
	PSKH2	Q47E
AADRY15	*CAMTA2*	S491P
	SHARPIN	H315N
AADRY26	*PCDHB11*	L496P
	PCDHB11	G532S
AADRY27	*EPPK1*	V2291M
	SIX1	R119C
AADRY29	*FAM8A1*	S153L
	FAM8A1	L135P
	FAM8A1	Q149R
	FAM8A1	A140T
	FAM8A1	G155S
	HLA-B	A182T
AADRY30	*DDX31*	R835K
	DDX31	Q838K
AADRY32	*CACNA2D2*	E1058Ter
AADRY33	*BTBD7*	C245fs*Ter1132
	C16orf78	R78Q
	ZNF626	A349D
	ZNF814	A158V
AADRY34	*IFNA14*	T179K
	IFNA17	T179K
	KDM2B	T28Sfs
	MCC	S25_S26insA
	NCOA6	R1652Q
	PNPLA5	S82N
	TRDN	E168_K169del
	UBE2QL1	L10P

Patient Id	*Gene*	Amino Acid Change
AADRY60	*CCRN4L*	R62P
	DMXL2	Q1329Ter
	MUC16	I7261M
	MUC16	S7025L
AADRY68	*HLA-DRB5*	R54Q
	INTS8	S755R
	MYCBP2	R3271H
	SELENOO	S184P
	SRRM4	R582P
AADRY78	*DLG2*	R888W
	NBPF10	R3484S
	ZBTB41	D21fs
AADRY82	*MAGI1*	Q421_T422insL
	SIRPA	T50S
	VAV1	R276T
AADRY91	*HLA-A*	I218V
	TTLL3	R130Q

Table 5-3 List of de nono variants formally curated.

Highlighted in red is a novel variant in *SHARPIN* for further investigation.

5.2.4 Autosomal recessive

There was no history of consanguinity in any of the families recruited. A total of 55 homozygous variants in 13 participants remained after initial filtering. Again, this list was further narrowed by manually reviewing the quality of the read and excluding variants with a MAF of greater than 0.01 in the ExAC or gnomAD database (Lek et al., 2016). A total of 12 homozygous mutations in 7 participants were formally curated (**Table 5.8** and **Appendix 10**).

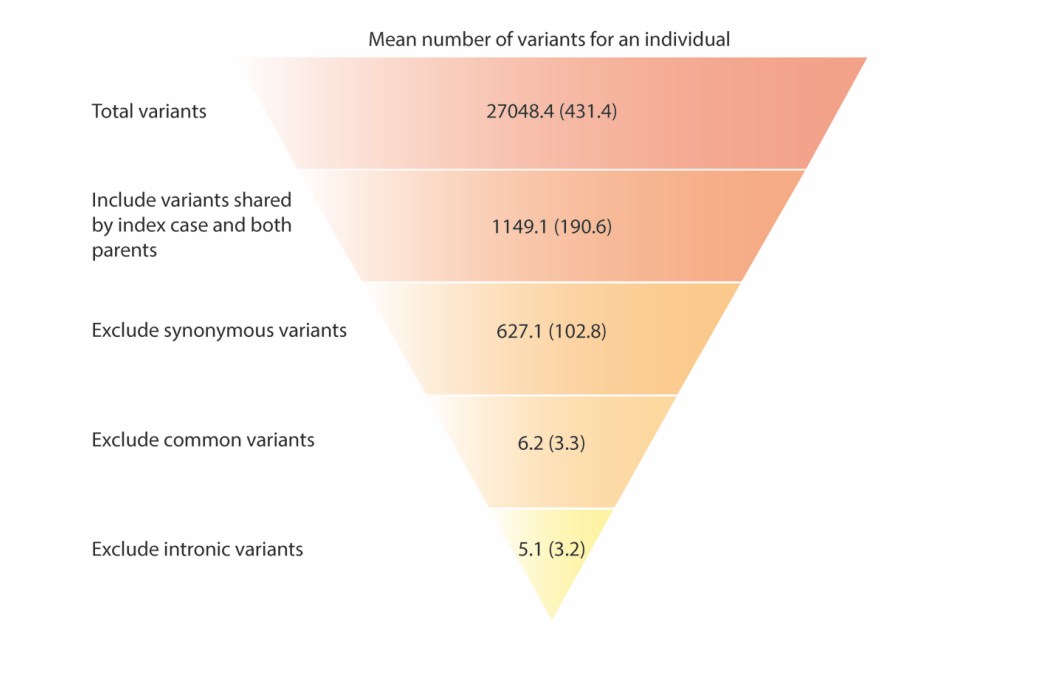

Figure 5-7 Filtering flow chart of autosomal recessive variants.

Including variants present in both parents in a heterozygous state and in the index case in homozygous state reduced the average number of variants to 1149.1 , which was reduced to 5.1 after sequential filtering. Standard deviation of each filtering step given in parentheses.

Although the majority of the variants reviewed were uncommon in the homozygous state in the healthy population database gnomAD, p.Val352Ala *CEP128* was present in a homozygous state in 24 individuals, and p.Pro488Ser *EPOR* in 34 individuals, reducing the likelihood of their pathogenicity.

The autosomal recessive variants listed were not in genes known to cause monogenic disorders of the immune system. Mutations in *ALDH1A3* have been linked to microphthalmia (OMIM 615113) (Yahyavi et al., 2013), however the variant detected in AADRY26 is consistently predicted to be benign and is in a region of low conservation. According to the ACMG guidelines this variant would be classified as likely benign (2). More importantly, AADRY26 does not have features of microphthalmia.

Of possible interest is the variant in *CCND3* in AADRY29, a participant with cyclical fevers and raised inflammatory markers, with symptomatic and biochemical response to corticosteroid therapy but not IL-1β antagonism. The gene *CCND3* encodes one of three D-type cyclins that form a complex with the proline-directed serine/threonine cyclin-dependent kinase (CDK) 4 or 6 that are involved in cell cycle transition (Matsushime et al., 1994). There have been multiple reports of the role of CDKs in malignancy, most recently by Sicinski and colleagues linking high expression of cyclin-D3 to pro-survival of T-cell acute lymphoblastic leukaemia (T-ALL) cells (H. Wang et al., 2017). In the context of T-ALL cells, the authors determined with immunoprecipitation and mass spectroscopy that the cyclin-D3-CDK complex associated with mediators of glycolysis and suggested that cyclin-D3 overexpression in malignant cells resulted in altered cellular metabolism leading to increased survival and proliferation. The role of CDKs in inflammation has also been explored. In an siRNA screen performed in A549 cells stably expressing NF-κB luciferase reporter, CDKs 5 and 7 were determined to be important for NF-κB regulation (Choudhary et al., 2011). A549 cells with CDKs 5 and 7 knocked down displayed reduced fold-increase in mRNA expression of NF-κB inducible genes in response to TNF stimulation when compared with a scrambled siRNA control, suggesting that these two CDKs are important for optimal NF-κB gene expression. The cyclin-D3 associated CDK6 is also implicated in the NF-κB pathway, with knock-down

of *CDK6* in synchronised HeLa cell cycle cultures revealing a defect in TNF induction of *IL8* mRNA expression (Handschick et al., 2014). CDK6 and the p65 subunit of NF-κB were shown to co-localise in transfection experiments and chromatin immunoprecipitation sequencing (ChIP-seq) revealed co-recruitment to the *IL8* promotor.

The potential implications for p.Pro134Ser CCND3 on CDK6 function and NF-κB target gene induction is unknown, but could be explored *in vitro*. Preliminary luciferase assay experiments include exploration of the ability of transfected mutant CCND3 or WT CCND3 to induce NF-κB activity. This would require the co-expression of the relevant CDK. The effect of p.Pro134Ser CCND3 on the stability of CDK6 could be explored in overexpression or knock-down experiments followed by WCL and western blot. The ability of p.Pro134Ser CCND3 to interact with CDK6 could be assessed in co-immunoprecipitation experiments. Experimental design will need to take in to consideration a number of factors, including the nuclear location of interactions, as well as the possible impact of the mutation on cell cycling and survival, potentially complicating the evaluation of this variant.

Patient ID	*Gene*	Amino acid change
AADRY26	*ALDH1A3*	R15G
	DMKN	G270delG
AADRY27	*GPR137C*	H120R
AADRY29	*CCND3*	P134S
	BEND6	H167D
	EFHC1	I401T
AADRY30	*PHLDA1*	Q195delQ
AADRY33	*MUC6*	P1906S
	FAM231B	F71del*fs
AADRY34	*CEP128*	V354A
AADRY82	*GEMIN5*	R1016C
	EPOR	P488S

Table 5-4 List of autosomal recessive variants formally curated.

5.3 Discussion

AADRY was established to streamline the evaluation and follow up of patients with suspected or confirmed monogenic AID. There were a number of hurdles in establishing AADRY. A distinct ethics committee for each of the Australian states required multiple applications to be submitted. This has since been streamlined with the National Mutual Acceptance approval. The lack of consensus on, and possibly experience with, genetic evaluation of participants with research intent led to different requirements from each committee that reviewed the project. What has resulted from this rigorous review, however, is a robust ethical framework under which participants can be recruited for WES and evaluated for potential disease-causing variants.

The focus of this chapter has been the genetic evaluation of the first 20 participants with a genetically unconfirmed syndrome. Perhaps not surprisingly, no known disease-causing mutations were identified in participants. Besides a variant of interest in *SHARPIN* explored in **Section 5.2.3.1**, a number of variants could possibly be implicated in autoinflammatory diseases, such as those involved in the cytoskeleton. However, unlike *SHARPIN* and most likely due to limited research rather than negative data, there is no evidence directly showing that these variants are involved in innate immune physiology. There were also variants in genes where the function in normal physiology has not yet been determined, such as *NBPF10.* The decision to pursue these variants *in vitro* therefore requires consideration of resources required and their availability, as well as the likely benefit to the participant if a genetic cause was identified.

The phenotype of participants impacts which, and how, variants are evaluated. As suggested in **Section 5.2.3.1**, a variant in a participant with an IL-1β driven disorder as determined by

response to anakinra could be evaluated in assays assessing inflammasome function. Some participants were recruited with the working diagnosis of 'atypical' periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis (PFAPA) (**Table 5.1**). These patients deviated enough from the expected clinical course that their clinician sought genetic evaluation. Determining the significance of genetic variants in participants with possibly self-limiting symptoms is challenging. The interpretation of MAF from population data requires caution, as it is foreseeable that a healthy cohort may include individuals who, at some point in time, had symptoms similar to the participant. Furthermore, there is also the possibility of a parent having similar symptoms as a child but due to failure to recall and report this, the appropriate segregation studies may not be performed. This is probably less likely with a more persistent and severe phenotype.

It is not only in instances of a mild phenotype that the MAF has the potential to mislead. A true MAF requires a gene to be sequenced with adequate coverage. A number of variants listed in **Section 5.2.3** and **Section 5.2.4** were in genes that have low ExAC percentage coverage. For these genes, a low MAF may be a reflection of failure to capture a variant that is actually present in a healthy cohort. This has implications for the curation process. Allele frequency data from healthy cohorts is used in the *in-silico* prediction algorithms, which can only be as robust as the input data.

The curation process outlined is time intensive. As no family member recruited had similar symptoms to the index participant, a *de novo* or autosomal recessive inheritance pattern was assumed, although work on determining potential compound heterozygous mutations is underway. The variability in penetrance of a number of immunological conditions has broadened the clinical manifestations compared to original publications, or indeed family

members harboring the same variant. A future curation pathway will be undertaken in these exomes, as well as all future exomes, using variants in all 320 genes listed by the IUIS for Primary Immunodeficiency (Picard et al., 2015). A number of participants recruited bridged the autoimmune-autoinflammatory spectrum and assessing an extended gene list would ensure that all known disease-causing genes affecting immune function were considered regardless of the ability to recruit family members. Although some genes have only been associated with immunodeficiency to date, for example *MYD88*, a GoF variant may have a different, possibly autoinflammatory, presentation.

The long-term viability of this project will depend on ongoing collaboration with clinicians as well as regular review of the NATA approved diagnostic services available. Genetic curators would be invaluable to reduce the volume of analysis performed by any one individual. This division of effort would also allow more focus to be placed on ascertainment of participants with genetically confirmed monogenic AIDs (Group 3), which has the potential to provide important insights for clinicians managing patients with these conditions.

6 CONCLUSION

This thesis documents previously unreported GoF mutations in the inflammasome forming receptors pyrin and NLRC4. The novel p.Glu244Lys pyrin mutation (**Chapter 3**) is only the second to be described in the literature to cause PAAND. The novel p.Trp655Cys NLRC4 mutation (**Chapter 4**) is the first variant in the LRR domain of an NLR to have its pathogenicity investigated and validated through *in vitro* modelling.

For the patients and families involved, the implications are clear. The index case presented in **Chapter 3** has been able to end a 30-year investigative odyssey into the cause of her difficult to control suppurative dermatological condition, now diagnosed as PAAND. Furthermore, she and her family have the opportunity to engage in appropriate genetic counseling which will include discussion of the possible implications for future generations. Younger family members who develop symptoms can undergo early molecular evaluation and have treatment instituted to prevent the persistence of significant systemic inflammation seen in the index case. Having said this, whether this early treatment would indeed result in a reduction in symptoms and improved outcomes is entirely speculative as there have been no longitudinal studies of PAAND patients, and an optimal treatment regimen is yet to be determined. The cohort of patients formally diagnosed with PAAND is small and deficiencies in our knowledge remain. For the families involved in **Chapter 4**, both lost a child to MAS secondary to a *de novo* mutation in *NLRC4*. The molecular findings in the first child resulted in a change in therapy to include IL-18BP, but the patient still succumbed to disease. This genetic finding, and confirmation of its pathogenicity, in these cases is still important despite the unfortunate sequelae. Given that both mutations were *de novo,* parents can be appropriately counselled about the implications for future pregnancies, or indeed the sibling of the second child.

Beyond the individual cases, validating the pathogenicity of novel findings has broader implications. Identification of either c.*G730A MEFV* or c.*G1965C NLRC4*, encoding p.Glu244Lys pyrin and p.Trp655Cys NLRC4 respectively, in the genetic evaluation of patients with symptoms and signs consistent with an inflammatory disease should prompt consideration of a definitive diagnosis. These SNPs would previously have been considered as VUSs, but with *in vitro* evidence supporting pathogenicity, should now be considered as pathogenic or likely pathogenic. Following this, laboratories engaging in Sanger sequencing must now ensure appropriate coverage of exons encoding these regions. Importantly, for teams involved in the curation of genetic results, the experimental evidence provided in **Chapter 4** highlights the limitations of *in silico* prediction algorithms. Further investigation of a variant should be considered if does not reside in a domain previously associated with disease.

Further questions regarding the mutations investigated in this thesis remain to be addressed. The experimental evidence provided in **Chapters 3** and **4** do not address the phenotypic variability seen amongst patients with the *same* mutation. The presented family with PAAND suffered a range of dermatological manifestations, with the index case at the more severe end of the spectrum. The two patients with mutations in *NLRC4* had MAS as well as gastrointestinal symptoms, but the differences are marked. One patient had acute renal impairment requiring peritoneal dialysis. The second patient had a presumed CNS infection on a background of pneumonia, suggesting a potential immune deficiency. Addressing differences in clinical presentation associated with the *same* genotype, or the penetrance, is complex. Qualification of immunological differences could be addressed through assessment of serum, as well as primary patient cells. Differences in serum cytokine profile as determined by multiplex analysis may provide insight into key cytokines driving disease and, if different between

patients, may guide further investigation. However, such profiles may be affected by the patient's current therapeutic regimens including immunosuppressive agents. Another option is the quantification of ASC speck formation in primary monocytes from each affected member (as shown in **Chapter 3**) to investigate if phenotype correlates with inflammasome formation. In the case of PAAND, the quantification of ASC specks at baseline and after LPS stimulation was undertaken in the three affected family members (**Chapter 3, Figure 2C** of manuscript). The percentage of monocytes with ASC specks formed was comparable amongst the patients. Even with LPS stimulation, although increased when compared with healthy controls, there was little variability in ASC speck formation amongst patients with PAAND. This suggests that the differences in clinical presentation are not necessarily accounted for by differences in ASC speck formation in monocytes. The formation of ASC specks in primary cells of the patients presented in **Chapter 4** has not been assessed. The quantification at baseline would be informative, as would the response to the natural NLRC4 ligand, PrgI. Given the possibility of immune deficiency in the second patient, it would be interesting to determine if there is an adequate response to stimulation of NLRC4.

There are alternative explanations for differing phenotypes amongst patients with the same genetic variant. One consideration is the presence of another genetic variant that affects disease presentation. A true digenic disorder requires the inheritance of a distinct heterozygous mutation in two genes that, when inherited separately, do not cause a phenotype (A. Gazzo et al., 2017; Lupski, 2012). The broader term of epistasis refers to possible interactions between genes (Cordell, 2002). Determining genetic epistasis is complex and requires an appropriate pedigree, which includes more than one gene mutated in a single pedigree, a range of genetic permutations and at least one member with WT alleles in both genes (Ameratunga et al., 2017;

A. M. Gazzo et al., 2016). Unfortunately, neither of the families investigated here allows for determination of an epistatic effect on disease.

Another possible explanation for the phenotypic heterogeneity amongst patients is epigenetic differences. Epigenetic processes such as DNA methylation, histone modifications, chromatin remodelling and non-coding RNAs can alter the activity of a gene without changing the DNA sequence. For example, a transcriptionally active gene has minimal DNA methylation and an open chromatic structure. Monozygotic and dizygotic twin studies of concordance have been used for some time to determine the contribution of a particular genotype to phenotype (Ben-Zvi, Brandt, Berkun, Lidar, & Livneh, 2012). More recently, this has been combined with methods of quantifying epigenetic changes. In a study of monozygotic twins disconcordant for the clinical diagnosis of CVID, a DNA methylation array performed on CD19+ B cells revealed that both switched and unswitched memory B cells of the twin with CVID had higher DNA methylation in genes relevant to B cell function (Rodríguez-Cortez et al., 2015). This finding highlights that epigenetic factors could account for phenotypic variations. There have been two publications assessing DNA methylation in patients with monogenic AIDs, but in each case the diseased population was compared with a healthy control (Kirectepe et al., 2011; Vento-Tormo et al., 2017). Vento-Tormo et al. assessed the DNA methylation status of genes encoding various components of the inflammasome in monocytes of patients with CAPS and compared this with healthy controls (Vento-Tormo et al., 2017). They noted that genes such as *IL1B, IL1RN* and *ASC* were demethylated more efficiently in CAPS monocytes when compared with healthy controls, a feature that normalized when patients were treated with anti-IL1 therapy.

Determining the epigenetic factors contributing to the phenotypic variability of a particular genotype is not simple. With a small number of patients, one possible approach is to compare the methylation status of genes potentially involved in the phenotype observed. An alternative approach is genome-wide DNA methylation profiling (Zuo, Tycko, Liu, Lin, & Huang, 2009). However, drawing conclusions from only two individuals with different genetic backgrounds may not be feasible.

The genotype-phenotype correlation also requires further investigation. Many questions are left unanswered when comparing patients with PAAND to those with FMF. Both groups of patients have mutations in *MEFV* and evidence of increased pyrin activation, but they are clinically distinct. Although evidence is provided in **Chapter 3** that the FMF mutation p.Met694Val *MEFV* is not associated with loss of 14-3-3 binding in an overexpression model, this result alone does not explain the differences in clinical presentation. Importantly, the experimental data presented only assessed the behavior of WT compared with mutant pyrin in the context of inflammasome formation. There may be alternative pathways in disease pathogenesis. Exploration of the NF-κB or apoptosis pathways, for example, may shed light on differences between p.Glu244Lys and p.Met694Val pyrin. The choice of pathway/s explored could be guided by techniques such as gene expression profiling. In an analysis of 22 patients with CAPS, gene expression profiling of PMBCs using an RNA based microarray was able to determine 270 genes that were differentially expressed compared with healthy controls (Balow et al., 2013). To date there are 20 patients reported to have PAAND including those presented in **Chapter 3**. Gene expression profiling of these patients along with healthy controls and patients with FMF may provide information on genes differentially expressed in these conditions. It would be important to select a specific cell type for analysis that is relevant to the phenotype of patients. As patients with PAAND have significant skin involvement when

compared to the more systemic FMF, it would be reasonable to interrogate keratinocytes in addition to monocytes.

Given that the residues affected in PAAND are located in a different domain to the majority of FMF-associated mutations, it is possible that there are protein interactions that are affected in only one of the conditions. As discussed in **Chapter 3**, PSTPIP1 is a key candidate to be involved in the pathophysiology of PAAND, but not FMF, as there is significant overlap between the PAPA syndrome phenotype and PAAND. A number of experiments were outlined in the discussion section to address this possibility. There are supplementary approaches that could be employed when there is a conceivable *unknown* interactor. Mass spectrometry was performed by Masters et al. to compare interacting proteins pulled down with WT or PAAND pyrin (Masters et al., 2016). The addition of an FMF mutant pyrin to this experiment may reveal a unique gain or loss of an interactor when compared with PAAND pyrin.

Furthermore, CRISPR/Cas9 gene editing techniques would allow for CRISPR screens to be performed in primary human cells or iPSC to determine genes encoding proteins of importance in disease. A CRISPR screen allows for the simultaneous testing of thousands of genetic deletions using a lentiviral library of sgRNAs targeting the host genome in cells expressing Cas9 (Koike-Yusa, Li, Tan, Velasco-Herrera Mdel, & Yusa, 2014; Shalem et al., 2014; T. Wang, Wei, Sabatini, & Lander, 2014). To date, most CRISPR screens are performed in cells isolated from Cas9-expressing transgenic mice or in human cell lines stably expressing Cas9. It is feasible for this technique to be used on, for example, the iPSC cells generated by Kawasaki et al. from a patient with NLRC4-AID (Kawasaki et al., 2017). Kawasaki et al. successfully knocked out *NLRC4* in this cell line and in doing so determined that NLRC4 was the driver for the cytokine response seen. iPSC could instead be transduced with the CRISPR

library sgRNAs in order to identify genes important to the cytokine response are evaluated. The screen would, if successful, show that *NLRC4* is indeed important for disease phenotype. It should also validate other known genes of importance, such as *CASPASE1* and *ASC*. The screen may, however, also uncover genes not previously known to be involved in the pathogensis of NLRC4-AID. This technique could theoretically be used to compare genes involved in FMF and PAAND, using iPSC from patients with each condition generated. This is not a simple process and using iPSC from different individuals creates significant challenges as the genetic background on which the screen is performed is not the same. An appropriate human cell line or murine model of FMF and PAAND would be a suitable approach. However, there is no murine PAAND model published to date, and the THP1 cell lines generated in this thesis had already undergone CRISPR/Cas9 genetic deletion of *MEFV,* which complicates the experimental approach. Alternatively, THP1-Cas9 cells stably expressing mutant pyrin (either FMF or PAAND) without deletion of endogenous pyrin could be used for a CRISPR/Cas9 screen. Unfortunately, these cells were not able to be generated during the course of this PhD (transient transduction was used in experiments presented in **Chapter 3**).

The techniques discussed here and throughout this thesis will be important when investigating novel genetic variants that are discovered through AADRY (**Chapter 5**). Variants presumed to be LoF can be investigated through genetic inactivation of the candidate gene using CRISPR/Cas9 techniques. The confirmation of the knock-out may be difficult as availability of resources to validate a loss of protein expression, such as a specific antibody, may be limited. In this case, validation of loss of gene transcription can be performed using quantitative PCR methods (Baker & Masters, 2018). Variants considered to be likely GoF, such as heterozygous alterations, could be investigated using a construct expressing the gene with the variant of interest.

The experiments performed should be guided by the patient phenotype. A variant discovered in a patient with an IL-1β targeted therapy-responsive disease could be investigated through, for example, expression of the variant of interest in THP1 cells and determining whether there is greater spontaneous IL-1β release, or IL-1β release with priming alone, when compared with WT. If the IL-1β phenotype was recapitulated *in vitro*, the same experiment could be performed on *CASPASE1 KO* and *ASC KO* THP1 cells to determine the role of the inflammasome in IL-1β release. This highlights the importance of detailed information from the clinician in order to direct accurate experimental design.

Besides response to IL-1β targeted therapy, the patient's response to other biological agents may be important. *In vitro* modelling of a variant found in a patient with response to infliximab, a chimeric monoclonal antibody targeting TNF, would be focused on assessment of the NF-κB pathway. Not all patients will have had a trial of such agents, or indeed a therapeutic response. In these cases, guidance may be sought from analysis of serum cytokines, or indeed literature on the gene of interest. As discussed in **Chapter 5**, the patient with a variant in the gene *CCND3* did not respond to IL-1β antagonism, but literature on this gene and the encoded protein implicates the NF-κB pathway. This would be a logical pathway to interrogate first. The cell type in which *in vitro* modelling would be performed may also be guided by the patient phenotype. AADRY13 is an 18 year old male with interstitial lung disease, amongst other symptoms. Given the pulmonary manifestations, the A549 human lung epithelial cell line is a reasonable first choice.

The patient phenotype and detailed clinical history guides the choice of variants investigated, cell type used and experimental approach adopted. The selection of patients in the initial stages

of AADRY avoided strict inclusion criteria. Whilst there are no plans to change this, more attention needs to be given to patients with a clear family history or distinct phenotype as this will allow for more confident assessment of a variant's segregation with disease. Furthermore, a severe phenotype allows for comparisons with previously described AIDs and engagement with appropriate collaborators to determine whether other patients with similar phenotypes exist. A clinical history of periodic fevers and abdominal pain alone, for example, may not be specific enough for such studies.

One approach to the recruitment of patients suffering this severity of disease is to liaise with clinicians who have trialled biologic agents such as anakinra, infliximab or tocilizumab (targeting IL-6) with or without success. The only biologic agent approved under the Pharmaceutical Benefits Scheme in Australia for an AID is anakinra, for the treatment of moderate to severe CAPS. A clinician requesting a trial of this medications for any other indication is required to apply for access through a drug committee at the hospital, suggesting that the clinician has reasonable evidence that the agent will be of benefit, or that alternative agents have failed. Most of these patients suspected of having an AID would have undergone extensive prior testing, including multiple genetic tests, and are hence ideal candidates for AADRY.

Tests for the more 'common' AIDs such FMF and CAPS are available in Australia through NATA accredited services, and as such patients who test negative to these present a diagnostic challenge. The patients recruited to AADRY have been educated about the genetic testing performed, the limitations that exist, and the likelihood of determining a genetic cause for their condition, which is low. Appropriate consenting is vital so that patients or parents are not expecting a definitive genetic diagnosis from participation in AADRY, but that this is only one

of the possible outcomes. The consenting process is also important to highlight the possibility of uncovering an incidental finding. Such a finding would not only have implications for the patient themselves in terms of medical screening and health insurance, but also family members. In establishing the consent forms for AADRY, a lack of consensus on the reporting of these findings was apparent. Although the ACMG Working Group on Incidental Findings has determined that incidental findings must not only be reported but actively looked for (Green et al., 2013), neither Europe or Australia have followed suite. With the increased use of broader sequencing techniques in research, and eventually in clinical practice, stakeholders should reach a consensus on the reporting of incidental findings.

These stakeholders could also facilitate a streamlined approach to the establishment of nationwide research projects that involve the genetic sequencing of patients. Projects such as AADRY not only facilitate the translation of research into clinical care, but also serve as a prompt for clinician interaction and collaboration. Despite such advantages, the challenges of meeting distinct requirements at each involved site are substantial. An agreed set of guidelines would educate researchers on the minimum required information needed to establish such projects and it would facilitate the linking of researchers to clinicians and patient populations that would be of mutual benefit.

The work presented in this thesis highlights techniques that can be employed to validate novel genetic findings in patients with AIDs. The formation of AADRY provides a platform to discover such genetic changes, and will serve as a key link between clinicians and researchers. Moreover, the robust foundations established ensure that AADRY continues to recruit and be of value to patients with confirmed or suspected AIDs into the future.

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8 APPENDICES

8.1 Appendix 1: Review article

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Monogenic autoinflammatory diseases: Cytokinopathies

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ABSTRACT

Rapid advances in genetics are providing unprecedented insight into functions of the innate immune system with identification of the mutations that cause monogenic autoinflammatory disease. Cytokine antagonism is profoundly effective in a subset of these conditions, particularly those associated with increased interleukin-1 (IL-1) activity, the inflammasomopathies. These include syndromes where the production of IL-1 is increased by mutation of innate immune sensors such as NLRP3, upstream signalling molecules such as PSTPIP1 and receptors or downstream signalling molecules, such as IL-1Ra. Another example of this is interferon (IFN) and the interferonopathies, with mutations in the sensors STING and MDA5, the upstream signalling regulator AP1S3, and a downstream inhibitor of IFN signalling, ISG15. We propose that this can be extended to cytokines such as IL-36, with mutations in IL-36Ra, and IL-10, with mutations in IL-10RA and IL-10RB, however mutations in sensors or upstream signalling molecules are yet to be described in these instances. Additionally, autoinflammatory diseases can be caused by multiple cytokines, for example with the activation of NF-κB/Rel, for which we propose the term Relopathies. This nosology is limited in that some cytokine pathways may be degenerate in their generation or execution, however provides insight into likely autoinflammatory disease candidates and the cytokines with which newly identified mutations may be associated, and therefore targeted.

1. Introduction

In the decade since Hawkins, Lachmann and McDermott described the remarkable efficacy of recombinant human interleukin-1–receptor antagonist anakinra in the treatment of a patient with Muckle–Wells Syndrome [1], the prognosis of patients with cryopyrin-associated periodic fever syndromes (CAPS) has changed dramatically. Prior to the elucidation of IL-1 signalling in CAPS, non-specific immunosuppressive medications were trialled with a relatively poor response. Once the genetic basis was shown to be mutations in *NLRP3*, the gene encoding cryopyrin [2], the dominant role of IL-1 in CAPS was established and the theoretical and subsequent practical benefit of anakinra confirmed. Since this time, there has been a focus on determining the genetic basis of inflammatory diseases in general, and exploring potential benefit of biologic agents. Here, we categorise and use monogenic autoinflammatory diseases to illuminate cytokine pathways, and highlight the complexity and areas of uncertainty in the pathophysiology of these diseases (Fig. 1).

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2. IL-1

The role of the IL-1 family in innate and adaptive immunity has been well explored. A total of 11 members have been identified, whose various effects are mediated via four signal receptor complexes and two decoy receptors [3,4].

The first of these cytokines, IL-1, has many and widespread biological functions including mediation of inflammatory and acute phase responses. The inactive precursor to IL-1β (pro-IL-1β) is found predominantly in the cytoplasm of haematopoietic cells and is produced in response to toll like receptor signalling, complement cascade, cytokines and IL-1 itself [3–7]. Although there is evidence of extracellular cleavage of pro-IL-1β by neutrophil proteinase-3 and elastase, routes of recent interest are both the canonical and non-canonical cytoplasmic inflammasome complexes [4,6].

2.1. Sensing

The inflammasome complex formed by NLRP3 (Nalp3, cryopyrin), adaptor protein ASC and caspase-1 senses danger caused by signals such as ATP, amyloid, monosodium urate crystals, calcium pyrophosphate dehydrate crystals and cholesterol crystals. These

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danger signals lead to opening of ion channels and potassium efflux from cells, with a possible role of changes in intracellular calcium and ROS levels [3–7]. Once activated, the NLRP3 inflammasome cleaves pro-caspase-1 to its active proteolytic form caspase-1 and subsequently cleaves pro-IL-1β to IL-1β [3,4,6].

The importance of the inflammasome in IL-1β formation is highlighted in CAPS, a group of diseases with a spectrum of clinical severity. These monogenic disorders are caused by mutations in *NLRP3* (also known as *CIAS1*), in regions predominantly coding the nucleotide binding domain [2,8]. The mutant NLRP3 of these patients exhibits enhanced pro-IL-1β processing activity [8,9]. Recombinant human interleukin-1–receptor antagonist (Anakinra) is profoundly beneficial for these patients, with reduction in the long term complications of chronic inflammation such as amyloidosis [10]. A human monoclonal antibody targeting IL-1β (Canakinumab) and a dimeric fusion protein that neutralised IL-1β (Rilonacept) are also extremely effective and now have FDA approval for use in the management of patients with CAPS [10,11].

Despite being one of the earliest autoinflammatory syndromes to be described, the exact pathophysiology of Familial Mediterranean Fever (FMF) is uncertain. The largely autosomal recessive disease is caused by a mutation in *MEFV*, which encodes the pyrin protein [12,13]. The role of pyrin remains debated and the possibility of both pro-inflammatory and anti-inflammatory effects complicates the understanding of this protein in health and disease. There are a number of theories, including that wild-type pyrin acts as an inhibitor of IL-1β production, or that it is itself prevented from activating IL-1β by various interactions [8,14,15]. It has been shown that wild-type pyrin can bind to ASC and make it unavailable for use in the inflammasome, but that it can also form caspase activating inflammasomes [5,8,14–16]. It may be the balance of these two functions that is important. There are certainly alternative roles for pyrin as colchicine, a microtubule polymerisation inhibitor, is very effective in the management of FMF. Anti-IL-1 agents, whilst helpful, are usually considered as therapy if response to first line treatment is not complete [8,10]. Recent data indicates that pyrin is a sensor of bacterial effectors that target RhoGTPases or a factor downstream of this [17]. This suggests that FMF mutations may have been positively selected due to a protective effect against certain species of bacteria that encode these effectors, and that targeting IL-1 may be beneficial during infections where pyrin is activated.

Three recently published papers on clinical syndromes resulting from mutations in NLRC4 highlight the importance of, and differences between, inflammasome platforms. A mutation resulting in p. Thr337Ser substitution affecting the nucleotide-binding domain of NLRC4 was found on whole exome sequencing of a patient with recurrent febrile and macrophage activating syndrome NLRC4-MAS [18]. This defect leads to constitutive caspase-1 cleavage, and increased secretion of IL-18 from monocytes and macrophages [18]. In the same edition of *Nature Genetics*, a gain in function mutation in NLRC4 encoding p. Val341Ala in the HD1 domain of the protein leading to a phenotype of enterocolitis and autoinflammation was described [19]. Macrophages from patients with the syndrome of enterocolitis and autoinflammation associated with mutation in NLRC4 (SCAN4) showed spontaneous formation of ASC foci and increased pyroptosis [19]. In subsequent literature, a Japanese family with a phenotype consistent with Familial Cold Autoinflammatory Syndrome (FCAS) was shown to have a mutation in NLRC4 encoding a p. His443Pro substitution in the nucleotide binding domain [20]. Although one patient with NLRC4-MAS has been successfully treated by IL-1 blockade [18], a significant role for IL-18 in this and indeed in the other intrinsic inflammasomopathies cannot be discounted. The variability in the phenotypes of patients with mutations NLRC4, and the predominant enteric pathology in the first two descriptions, may yet be explained by IL-18, an effect of commensals, or the NLRC4 bacterial trigger flagellin.

There are regulatory proteins termed NLR family, Apoptosis Inhibitory Proteins (NAIPs) that are involved in the NLRC4 response. Although only one human NAIP has been found, the multiple mouse NAIPs have been shown to dictate specificity for NLRC4 [21]. NAIP2 is involved upstream of NLRC4 in the recognition of bacterial PrgJ and NAIP5 and 6 respond specifically to bacterial flagellin [21]. In a series of experiments involving transfected cells and combinations of wild-type and constitutively active NAIP5 and NLRC4, it was determined that constitutively active NAIP5 could signal wild-type NLRC4 and hence activate caspase 1 [21]. Though there have not been any reports of mutations resulting in gain of function of NAIPs, it is not unreasonable to predict a similar phenotype to those with NLRC4 activating mutations.

The formation of inflammasomes is tightly regulated by proteins with inhibitory or assisting roles. The deubiquitylation of NLRP3 required as an activating step is mediated by BRCC3, however there is no evidence to support gain of function of BRCC3 leading to constitutively activated NLRP3 [22]. There are numerous negative regulators of NLRP3 that could be inactivated in autoinflammatory disease [23], for example, nitric oxide (NO), micro-RNAs (miR-223), E3 ligases (TRIM30, March7). The possible involvement of these negative regulators is highlighted by a paper identifying a novel negative regulator of NLRP3 inflammasome activity A20 [24]. Myeloid specific deletion of A20 in mice causes increased caspase 1 activation and a phenotype reminiscent of rheumatoid arthritis [24].

2.2. Upstream signalling

There are a number of other autoinflammatory conditions linked to defects upstream of IL-1β, albeit less well defined. Pyogenic arthritis, pyoderma gangrenosum, and acne (PAPA) syndrome also may involve pyrin and IL-1β, but the association is not exclusive. This autosomal dominant condition results from a mutation in PSTPIP1 (also known as *CD2BP1*) which encodes proline–serine–threonine phosphatase-interacting protein 1 (PSTPIP1) [5,8,25]. The mutated PSTPIP1 has stronger and longer binding interaction with pyrin [5,8,25]. Depending on whether pyrin is considered pro or anti-inflammatory, there are different explanations of how the mutation causes effect, either through conformational change to pyrin by the process of binding allowing oligomerisation of pyrin with adapter proteins and formation of an active inflammasome [8]. Alternatively, the mutated PSTPIP1 may prevent the inhibitory function of pyrin on the NLRP3 inflammasome [5]. Whilst most patients experience benefit with IL-1 inhibition, the ongoing flare in some despite high doses suggests more than one cytokine could be involved [5,8].

Caspase 12, caspase recruitment domain family, member 8 (CARD8), CARD16, CARD17 and CARD18 focus on inhibition of caspase 1 activity [26]. Interestingly, caspase 12 exists in the majority of the population in truncated form, and full length protein due to a single nucleotide polymorphism seen in approximately 20% of African, Asian and South American population renders them hyporesponsive to endotoxin and susceptible to severe sepsis [27]. CARD8 provides negative regulation of NLRP3 [28]. CARDs 16, 17 and 18 are induced by pro-inflammatory signals and inhibit caspase 1 activity as part of a negative feedback loop [29,30]. Furthermore, three pyrin-only proteins (POP1, POP2 and POP3) have been identified and have been shown to inhibit inflammasome formation [31,32]. Although deficiencies in these CARDs and POPs have not been described in humans, an inflammasomopathy would be a foreseeable consequence.

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2.3. Receptor or downstream signalling

To ensure appropriate homeostasis, there are endogenous antagonists to IL-1 which are secreted in response to pro-inflammatory stimuli. IL-1R antagonist (IL-1Ra) is a naturally occurring competitive inhibitor of IL-1β binding to its receptor [3,6,7].

The consequence of lack of inhibition is exemplified in patients with Deficiency of the IL-1 Receptor Antagonist (DIRA), a syndrome characterised by neutrophilic pustular dermatosis, periostitis, aseptic multifocal osteomyelitis, and high acute-phase reactants [33]. These patients have a mutation in IL1RN, the gene encoding IL-1Ra [5,33,34]. The lack of antagonism leads to unopposed IL-1 receptor activation and increased response to IL-1α and IL-1β. Not surprisingly, these patients show an impressive response to anakinra [10,33].

Once released, IL-1β binds to a receptor subunit of IL-1R, prompting recruitment of a second receptor subunit. The two TIR domains allow recruitment of myeloid differentiation primary response protein 88 (MYD88), IL-1R-associated kinase 4 (IRAK4), TNFR-associated factor 6 (TRAF6) to initiate nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways [3]. All of these are prime candidates for the identification of activating mutations that drive autoinflammatory disease, however so far, only loss of function mutations associated with immune deficiency are known.

2.4. Not yet known

Although the exact pathophysiology is unknown, the shared clinical characteristics between Schnitzler syndrome and CAPS prompted a trial of IL-1β antagonists with great effect [35]. There has been one report of *NLRP3* V198M mutation resulting in the phenotype consistent with the syndrome, but this has not been a uniform finding [36]. Schnitzler syndrome is characterised by a monoclonal IgM gammopathy and features suggestive of an autoinflammatory condition such as fever, arthralgia, lymphadenopathy, hepatosplenomegaly and increased inflammatory markers [8,35]. Although a number of cytokine disturbances have been reported, clinical response within hours of treatment with anakinra implies that IL-1β is a key player [35]. There are varying reports of the efficacy of TNF-α inhibition in Schnitzler syndrome. A case report of exacerbation of symptoms with adalimumab and etanercept treatment suggested the deterioration in clinical picture after initial improvement was causal [37]. In cases where remission was achieved, longer interval between initiation and resolution of symptoms, as well as the necessity of co-administration with systemic immunosuppressive therapies [38], implies that IL-1β is more directly linked to the pathophysiology and phenotype than TNF-α. More recently, anti-IL-6 treatment with tocilizumab was trialled successfully in three patients with Schnitzler syndrome who failed to respond to IL-1 inhibition [39]. There have been attempts to clarify the pathophysiology of this condition, with studies showing a dominant role for IL-1β and IL-18 [40,41], with anomalous processing and spontaneous release of IL-1β from PBMCs [41]. Whether this is causal, and how this links in with a monoclonal gammopathy remains uncertain.

Majeed syndrome is an autosomal recessive disease caused by a mutation in LPIN2, coding protein lipin-2 [42,43]. This protein is a phosphatidate phosphatase (PAP) that is important in glycerolipid biosynthesis, acting as a transcription co-activator regulating lipid metabolism [42,44] and is upregulated in macrophages during stress [44]. Patients with Majeed syndrome have chronic recurrent multifocal osteomyelitis, inflammatory neutrophilic dermatoses and congenital dyserythropoitic anaemia [45]. Depletion of lipin-2 in murine and human macrophages lead to an increased expression of proinflammatory genes by saturated fatty acids through alteration in phosphorylation of the JNK/c-Jun pathway [44]. However, the full extent of the role of this protein in vivo and the mechanism of the phenotypic consequences of it being defective is not known. Two patients showed improvement with a trial of IL-1 antagonists but did not respond to anti-TNF-α, linking this disease to the IL-1 pathway by uncertain means [10,46].

The inflammasomopathies are the paradigm for cytokinopathies, with genetic lesions observed throughout all levels of IL-1β production and signalling, and frequently negative regulators there of. This can also now be established for the interferonopathies, as described later in this review, however future work may also flesh out a similar network of diseases involving IL-10, or as discussed next, IL-36 (see Table 1).

3. IL-36

The IL-36 cytokines, comprising of IL-36α, IL-36β and IL-36γ, are part of the IL-1 family, agonists exhibiting proinflammatory effects via the IL-36R (IL-1Rrp2) [4,47]. Once released, IL-36 cytokines lead to a number of factors that induce Th1 and Th17 polarisation [4,47]. Whilst initially thought to be primarily produced by innate immune cells and lymphocytes, there is evidence of its release from epithelial cells in the skin and lungs as well as brain tissue [47]. Interestingly, despite IL-36R being widely expressed in the brain, studies thus far have failed to show a proinflammatory response with the addition of IL-36β and IL-36γ [48].

3.1. Sensing

The expression of IL-36 is tissue dependent. The regulation in skin is controlled by epidermal growth factors and expression in bronchial epithelium enhanced by IL-1, TNF, IL-17 and TLR3 ligands. One potential danger signal triggering IL-36γ production is the alarmin cathelicidin-related antimicrobial peptide (CAMP) LL37 [49], however a sensor for this is still controversial.

IL-36 cytokines require processing of the N-terminal methionine to have optimal affinity for IL-36R but the proteases responsible have not yet been elucidated [47,50]. Such activators would be excellent candidates for activating mutations in autoinflammatory disease.

3.2. Upstream signalling

Some determinants of LL37 signalling have been established, which may be common signalling mechanisms leading to IL-36 upregulation. These include G-proteins and p38 [49].

3.3. Receptor or downstream signalling

The actions of IL-36 are negatively controlled by IL-36Ra, an IL-36R antagonist [4,47,50], which blocks recruitment of the second receptor IL-1RAcP (the common co-receptor to IL-1 and IL-33) [4,47,50]. Unlike classic antagonists, IL-36Ra has been shown to induce IL-4 mRNA expression and it needs processing for full antagonistic activity [47,50]. This antagonist is constitutively expressed in keratinocytes [51].

A recently described new autoinflammatory syndrome is that of interleukin-36-receptor antagonist deficiency (DITRA) [33]. Mutations in IL36RN result in decreased IL-36Ra antagonistic effects due to defects in both protein stability and affinity for its receptor [33]. The patients with this condition have diffuse pustular erythematous rash associated with high fever, general malaise, systemic inflammation and occasionally a 'geographic tongue' and nail dystrophy [33]. Although there is no established treatment,

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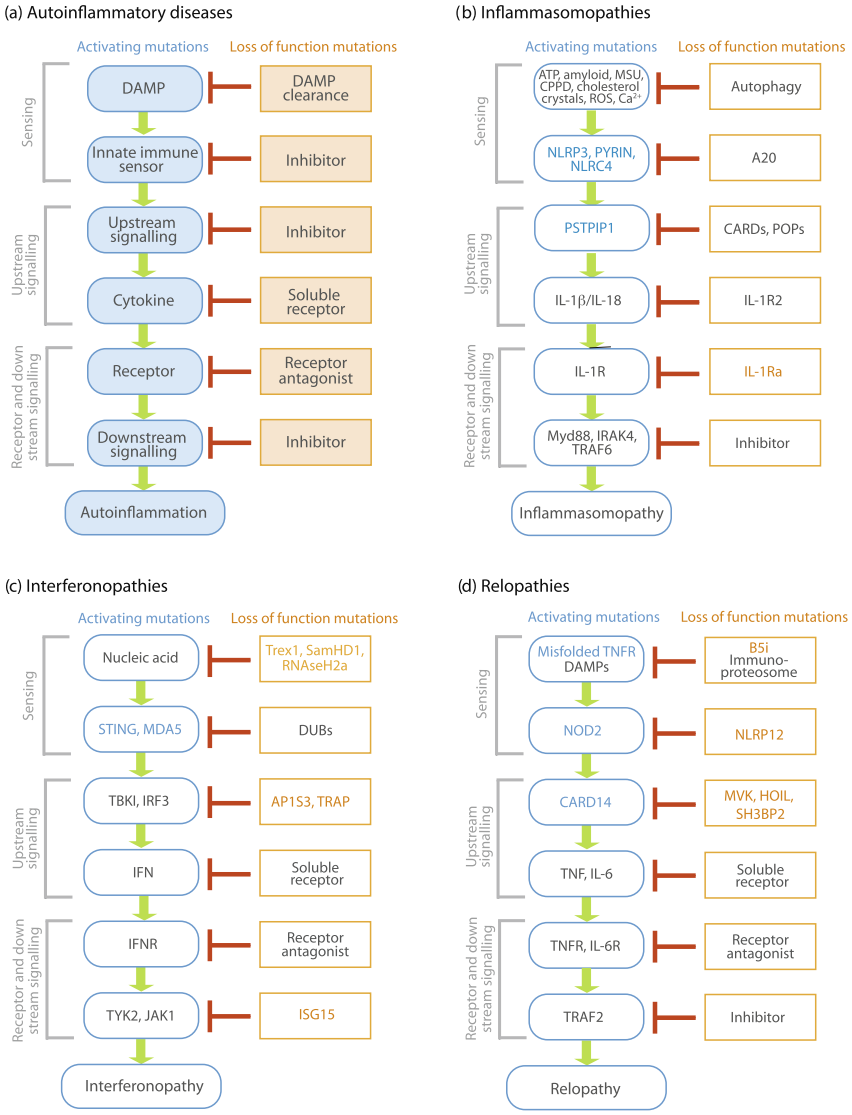

Fig. 1. Schematic representation of the Cytokinopathies. (a) A proposed classification to integrate autoinflammatory diseases within a framework of cytokine signalling. (b) This has been applied to the inflammasomopathies, (c) interferonopathies and (d) relopathies, demonstrating examples of proteins that may be involved in autoinflammation. Highlighted in blue are proteins that display activating mutations in human autoinflammatory disease, and in orange those that have loss of function mutations. DUBs Deubiquitinating enzymes.

one patient trialled on anakinra responded positively [52], and various other treatment regimens including corticosteroid therapy, acitretin, and anti-TNF-α have shown some efficacy [33]. The lack of universal response to IL-1 antagonist therapy, as well as the delay of weeks in clinical improvement, suggests that the actions of IL-36 are non-redundant. Whilst there is a positive feedback loop between IL-1 and IL-36, IL-36 also increases TNF and interleukins 6 and 8, and treatment strategies must address these also [53,54]. Interestingly, a subset of patients with generalised pustular psoriasis (GPP) without preceding psoriasis vulgaris were also noted to have mutations in IL36RN [55,56], and overexpression of IL-36α in basal keratinocytes in mice lead to acanthosis and hyperkeratosis, highlighting the importance of regulation of IL-36 in keratinocytes [47].

4. IL-10

IL-10 is widely produced by many immune cells and displays potent anti-inflammatory effects [57–60]. The function of this cytokine is complex, and depends on the cell type on which it is acting [58–60].

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Table 1
Classification of known autoinflammatory diseases and the involved genes based on signalling pathway, as shown in Fig. 1. AGS Aicardi–Goutieres syndrome, CAPS cryopyrin associated periodic syndrome, DIRA deficiency of interleukin 1 receptor antagonist, DITRA deficiency of interleukin 36 receptor antagonist, FMF familial mediterranian fever, EOIBD early onset inflammatory bowel disease, GPP generalised pustular psoriasis, HIDS HyperIgD syndrome, HLH haemophagocytic lymphohistiocytosis, NAPS12 NLRP12 associated periodic syndrome, NLRC4-MAS NLRC4 mediated macrophage activation syndrome, PAPA pyogenic arthritis, pyoderma gangrenosum and acne, PRAAS proteasome associated autoinflammatory syndromes, PRP pityriasis rubra pilaris, RHM recurrent hydatiform mole, SAVI STING associated vasculopathy with onset in infancy, SCAN4 syndrome of enterocolitis and autoinflammation associated with mutation in NLRC4, SPENCD spondyloenchondrodysplasia, TRAPS TNF receptor associated periodic syndrome.

	Cytokine				
	IL-1 family		Other		
	IL-1 (Inflammasomopathies)	IL-36	IL-10	IFN Interferonopathies	Multiple; TNF, IL-6 etc (Relopathies)
Sensing	CAPS (NLRP3) FMF (MEFV) NLRC4-MAS (NLRC4) SCAN4 (NLRC4) RHM (NLRP7)			SAVI (*TMEM173*) AGS (IFIHI,TREX1, RNASEH2A, SAMHD1)	PRAAS (*PSMB8*) TRAPS (TNFRS1A) Blau (NOD2) NAPS12 (NLRP12)
Upstream signalling	PAPA (PSTPIP1)			GPP (AP1S3) SPENCD (*ACP5*)	PRP (CARD14) LUBAC deficiency (HOIL1) HIDS (MVK) Cheburism (SH3BP2)
Receptor or downstream signalling	DIRA (IL1RN)	DITRA (IL36RN) GPP (IL36RN)	EOIBD (1L10RA, *IL10RB*)	AGS-like (ISG15)	Familial HLH (multiple)
Not yet known	Schnitzler (unknown) Majeed (LPIN2)				

4.1. Sensing

In an excellent review of both production and regulation of IL-10, Saraiva and O'Garra detail the complexity of release of IL-10 [61]. Both TLR and non-TLR signalling pathways may lead to IL-10 production. They note that the strength of stimuli and the cell on which it acts are variables leading to a different level of IL-10 being released [61].

4.2. Upstream signalling

The predominant signalling pathways leading to IL-10 production are the extracellular signal regulated kinase (ERK), p38 and NF-κB pathways [61]. As these are coincident with both IL-10 and pro-inflammatory cytokines there are no specific autoinflammatory diseases yet known to be caused by mutations in these genes.

There are also a number of pathways leading to the down regulation of IL-10 production. Both the TLR-induced and non-TLR signalling pathways are inhibited by IFN-γ. IL-10 has negative feedback on itself, with expression of Dual Specificity Phosphatase 1 (DuSP1) resulting in decrease in p38 phosphorylation and hence decreased IL-10 production [61]. Again, mutations in these pathways are not yet known.

4.3. Receptor or downstream signalling

Once released, IL-10 binds to IL-10R, a JAK/STAT3 class of receptor comprised of two subunits IL-10R1 and IL-10R2 encoded by *IL10RA* and *IL10RB* respectively [57,60]. Once bound to its receptor, the anti-inflammatory effect of IL-10 is dependent on the induction of heme oxygenase-1 [60] and eventually inhibits translocation of NF-κB to the nucleus [58]. The downstream effect is inhibition of TLR induced MyD88 translation, STAT1 phosphorylation and INFα and γ gene transcription [58].

Furthermore, association studies of patients with very early onset inflammatory bowel disease (EOIBD) revealed homozygous mutations in *IL10RA* and *IL10RB* [60]. This was replicated in review of patients with early onset enterocolitis, with mutations in *IL10RA* found in 7 of the 14 children developing symptoms before one year of age [62]. These mutations decreased IL-10 signalling with evidence of decreased STAT3 phosphorylation and decreased expression of suppressor of cytokine signalling 3 (SOCS3) [60]. Numerous pro-inflammatory cytokines including but not limited to TNF-α and interleukins 1α, 1β, 2 and 6 were increased [60]. Case reports of bone marrow transplantation resulting in clinical remission have been published [63], but as yet no targeted cytokine treatment option has been shown to be effective in patients with mutations in *IL10RA/IL10RB*. Given the complexity of action of the cytokines, this is not entirely surprising.

5. Interferons

Interferons have a variety of effects with well described antiviral, antitumor and immunomodulatory activity. Type I interferons (IFN-α, -β, -ω, -ε, -κ) are produced by most cells, whereas NK, NKT and T cells are the primary sources of type II interferons (IFN-γ) [64]. Type I and type II interferon receptor components (IFNAR1/2 and IFNG1/2 respectively) are expressed on most nucleated cells, suggesting that both have the potential for widespread activity [64].

5.1. Sensing

Both TLR dependent and independent pathways may be engaged to induce IFN production. The TLR independent pathway involves retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5) acting as cytoplasmic sensors of nucleic acid [64,65]. Furthermore, STimulator of INterferon Genes (STING) and DNA-dependent activator of IFN-regulatory factors (DAI) induce type I IFN in response to cytosolic DNA, either from external pathogens or self [65,66].

STING is an evolutionarily conserved endoplasmic reticulum transmembrane protein with the downstream effect of activation of IFN regulatory factor 3 (IFR3) transcription factor, translocating to the nucleus and, with NF-κB, transcribes *IFNA* and *IFNB* genes and produce type I IFN [65,66]. A recently described monogenic autoinflammatory disorder termed STING-associated vasculopathy with onset in infancy (SAVI) documented early onset vasculitis

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localised to cheeks, ears, nose, and digits with the absence of thrombocytopaenia and autoantibodies typically associated with antiphospholipid syndrome [67]. These patients were noted to have a gain of function mutation in *TMEM173* gene encoding STING which resulted in a constitutively active STING and positive feedback loop of interferon production and interferon receptor binding [67].

Patients with Aicardi–Goutières syndrome (AGS) have an upregulated IFN gene signature. AGS is a genetically heterogeneous disorder characterised by inflammation of skin and brain, with phenotypes resembling an overlap of sequelae of congenital infection and SLE [68]. Mutations in genes encoding three prime repair exonuclease 1 (TREX1), Ribonuclease H2 subunits A, B and C (RNASEH2A/B/C), Sam domain- and HD domain containing protein (*SAMHD1*) [68] as well as adenosine deaminase (*ADAR1*) have all been implicated in AGS [69]. Extensive inflammatory features including lymphocytic vasculitis, mouth ulcers, deforming arthropathy and cerebral vasculopathy are seen in patients with SAMHD1 mutations [68]. Both TREX1 and RNASEH2 are nucleases and it is hypothesised that dysfunction of the enzyme activity leads to accumulation of endogenous nucleic acids that are sensed through RIG-I and MDA5 lead to a type I IFN response [68,70]. Although the exact function of SAMHD1 has not been elucidated, a similar mechanism has been proposed [68].

The role of MDA5 in the immune response and IFN release was highlighted in a mouse model published by Funabiki et al. in 2014 [71]. Mice with a missense mutation in *IFIH1* leading to G821S in MDA5 had increase in unstimulated MDA5 signalling leading to upregulation of IFN signature as well as a lupus phenotype [71]. Two case series, one of eleven patients [72] and another of six [73], were published soon after, describing heterozygous mutations in *IFIH1* associated with dysregulation of signalling, increase in IFN and AGS phenotype [72,73]. Although mutations have not yet been described, similar consequences resulting from gain of function of RIG-I and possibly LGP-2 may be expected.

5.2. Upstream signalling

Although the exact pathophysiology is still under investigation, patients with spondyloenchondrodysplasia (SPENCD) have elevated type I IFN levels [68,74,75]. This disorder is characterised by skeletal dysplasia, cerebral calcification and spasticity, and an increased susceptibility to the development of SLE [68,74,75]. SPENCD is caused by biallelic mutations in *ACP5*, a gene encoding tartrate-resistant acid phosphatase (TRAP) [68,74,75]. In health, TRAP functions to hydrolyse substrates including nucleotides, phosphoproteins, and osteopontin (Opn). Opn has been shown to have a role in type I IFN production acting as an intracellular signal transduction molecule, and it is hypothesised that patients with defective TRAP have accumulation of Opn and dysregulation of IFN production [68,74,75]. TRAP may also have a role in removing nucleic acid that would otherwise have signalled IFN production via RIG-I [68,74,75], and thus could be classified with the other sensor mediated inflammasomopathies, however ongoing studies should inform as to the dominant pathway of IFN regulation.

Both SPENCD and AGS patients have a propensity for SLE. Monogenic forms of SLE have been described with mutations in *C1q, C1r, C1s, C4, DNase1,* TREX1 *and ACP5* leading to a spectrum of lupus phenotypes [68]. Several lines of evidence exist for the role of IFN in SLE, including the induction of lupus like features in patients on IFN therapy and almost all children with lupus having an upregulated IFN gene signature [68]. However, given the plethora of genes linked to SLE in mouse models and the diverse spectrum of phenotypes, it is likely that the pathogenesis of the disease is complex [68].

Interestingly, a form of monogenic GPP has been described that disrupts the TLR dependent pathway of IFN induction. A mutation in *AP1S3*, encoding adaptor protein complex 1 (AP1), was noted during a whole exome sequencing of patients with GPP [76]. The destabilised 3D structure of the protein disrupts endosomal translocation of TLR3 and leads to inhibition in downstream signalling [76]. In knockdown keratinocytes, there was a decrease in TLR3 mediated IFN-β induction and subsequent reduction in the anti-inflammatory effects of this cytokine, including failure to downregulate IL-1 [76]. It is the deregulation of IL-1 that is targetable with current medications, with some patients responding to IL-1 blockade [56,77,78].

5.3. Receptor and downstream signalling

Type I IFN acts via heterodimeric type I IFN receptor (IFNAR) which is expressed in most nucleated cells [64]. The dimerization of IFNAR1 and IFNAR2 leads to phosphorylation of TYK2 and JAK1 with subsequent activation of STAT members and transcription of IFN stimulated genes [64]. The downstream result is increased killing of virally infected cells, and increased susceptibility to cell death inducing stimuli [64]. The self-amplification of IFN allows for rapid effect, with type I IFNs increasing expression of molecules that increase IFN production [64]. The potential role of JAK inhibition in limiting this positive feedback loop and hence in treatment of patients with the autoinflammatory disorder SAVI has been explored in vitro, with reduction in *IFNB1* transcription in patients' lymphocytes noted [67]. Further analyses of mouse models may result in clinical trials.

The negative regulation of IFN involves three main processes. Firstly there is downregulation of IFNAR expressed on cell surfaces, achieved through internalisation that is promoted by proinflammatory cytokines, TLRs, as well as oxidative and metabolic stress [79]. Secondly, there is induction of negative regulators SOCS1, SOCS3 and USP18 [79]. These mediators are induced by type I IFN as part of a negative feedback loop [79]. SOCS1/3 compete with STAT, whilst USP18 displaces JAK1, both acting on the IFN receptor [79]. Thirdly, microRNAs are induced and provide regulation of gene transcription [79].

There has been a recent description of three siblings with idiopathic basal ganglia calcification (IBGC), a feature also seen in AGS and SPENCD, with whole exome sequencing determining an autosomal recessive mutation in *ISG15* gene encoding the intracellular ubiquitin-like modifier ISG15 [80]. These patients were deficient in ISG15, leading to increased S-phase kinase-associated protein 2 (SKP2) mediated proteolysis of USP18 and subsequently an upregulated IFN gene signature [80]. Prior to this, three unrelated patients with loss of function mutations in ISG15 were reported with a phenotype of disseminated BCG post vaccination attributed with insufficient IFN-γ production [81]. When reviewed, they also demonstrated IBGC and upregulation of IFN-α/β [80].

6. Multiple cytokines

Not all monogenic autoinflammatory syndromes have their mechanisms linked to one cytokine alone. Despite this, pleiotropic cytokinopathies tend to have a single well defined defect in physiology and can respond remarkably well to targeted therapy.

6.1. Sensing

Toll like receptors (TLRs), type I transmembrane receptors with an extracellular domain comprised of leucine rich repeat (LRR) motifs, and a cytoplasmic Toll/IL-1R (TIR) domain, are essential in the eventual transcription of inflammatory related genes

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through their ability to recognise pathogen associated molecular patterns (PAMPs) [82,83]. Cell surface TLRs (TLR1, 2, 4, 5 and 6) recognise PAMPs such as LPS, triacyl lipoprotein, diacyl lipopeptides and flagellin [82,83]. TLRs 2, 4 and 6 have the additional functional capability of recognising danger associated molecular patterns (DAMPs) [83,84]. A number of endogenous ligands have been elucidated, including but not limited to molecules released from dying cells (HMGB1, heat-shock proteins and extracellular matrix components), amyloid-β, oxidative-LDLs and oxidative-phospholipids [83,84]. TLRs 7 and 9 recognise self DNA and self RNA respectively [83,84]. The downstream response of all TLRs except TLR3 is activation of the MyD88 dependent pathway and activation and translocation of NF-κB to the nucleus and alteration in transcription of genes encoding IL-6, IL-12 and TNF [82,83]. TLR3 along with TLR4 initiate the TIR domain containing adaptor protein inducing IFNB (TRIF) pathway and subsequent type I interferon transcription [82,83]. Perhaps somewhat surprisingly, monogenic diseases due to upregulation of these elements has not yet been described.

A vital feature in the homeostasis of cells is the recognition of DAMPS released during host stress and injury. An increase in DAMPS, or inability to clear them, will ultimately lead to increase in inflammatory cytokines. The proteasome associated autoinflammatory syndromes (PRAAS) are a group of disorders including chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE) syndrome, Nakajo–Nishimura syndrome (NNS), joint contractures, muscle atrophy, microcytic anaemia and panniculitis-induced lipodystrophy (JMP) syndrome, Japanese autoinflammatory syndrome with lipodystrophy (JASL), and autoinflammation, lipodystrophy, and dermatosis syndrome (ALDD) characterised by severe lipodystrophy and muscle weakness and linked by mutations in *PSMB8* [33,85,86]. *PSMB8* encodes the b5i subunit of the immunoproteasome, and mutations in this gene lead to a failure of formation of a complete immunoproteasome. There is subsequent accumulation of DAMPS and hence activation of NF-κB [85,86], and patients with these disorders have increase in IL-6 [85,86]. There is also constitutively hyperresponsiveness of STAT-1 to IFN and increased IFN signalling, which goes some way to explain why IL-6 antagonists alone have not been effective. JAK inhibitors, targeting the STAT-1 hyperphosphorylation in these patients, and treatment antagonists to the inhibitors of alternative proteolytic proteins are currently being investigated [85].

Despite the name, the clinical features of TNF Receptor-Associated Periodic Syndrome (TRAPS) are not mediated by TNF alone. Patients with TRAPS harbour an autosomal dominant mutation in *TNFRSF1A* which results in abnormally folded TNFR1 [8,86]. Mutant TNFR1 are retained in the endoplasmic reticulum (ER) and do not progress to function at the cell surface [86]. The receptors may signal in ER during stress and activate JNKs, adding to the transcription of inflammatory mediators [86]. Elevated serum TNF levels are not seen in most patients and there have been reports of infliximab and adalimumab causing inflammatory attacks in this population [10]. There have been positive results with anakinra, with decrease in disease activity and relapse rates, suggesting a role for IL-1 in the pathophysiology of the phenotype. One patient treated with tocilizumab, and IL-6 antagonist, responded [10].

Blau syndrome (BS), a disorder characterised by granulomatous uveitis, arthritis, skin rash and camptodactyly, is caused by autosomal dominant mutations in *NOD2*, encoding the NACHT domain of NOD2 (also known as caspase recruitment domain family member 15, CARD15) and leading to subsequent upregulation of NF-κB activation [8,87]. It is possible that the mutations lead to a constitutively active NOD2, with further enhanced signalling in response to PAMPs [8,88]. No single agent has proven effective in patients with BS, TNF inhibition leading to partial control in some, and variable reports of response to IL-1 inhibition [10,87,88], highlighting the complexity of this condition and the induction of various cytokines by NF-κB.

The regulation of NF-κB has been explored in experiments involving NLRP12. Long considered to have an inhibitory role on NF-κB, there is ongoing debate as to the mechanism of this effect, as well as possible stimulatory functions [89,90]. HEK293T cells transfected with wild type NLRP12 demonstrated strong inhibition of NF-κB [91]. Knock out mice studies have implicated NLRP12 as an inflammasome component involved in the recognition of Yersinia Pestis and processing of IL-18 and IL-1β after infection [92]. Examining the canonical pathway of NF-κB activation in patients with NLRP12 mutation and cold induced autoinflammatory disease (NLRP12-associated periodic syndrome or NAPS12) demonstrated altered kinetics rather than peak level of activation, reaching a plateau earlier than healthy counterparts [93]. Possible mechanisms and alternative pathways have been investigated. In NLRP12$^{-/-}$ mice, there was increased non-canonical activation of NF-κB and a phenotype of colitis and increased colon cancer [90]. Another study demonstrated no effect on NF-κB activation, but rather increased ASC speck formation and caspase-1 activation [90]. This suggests a role of the inflammasome and may explain why patients present with the same variability and similar phenotype to CAPS. Whilst this seems a promising possible pathophysiologic mechanism, the inconsistent response and development of tolerance to IL-1 antagonism suggests that there may be a more complicated and likely dual role of NLRP12 [10,33].

6.2. Upstream signalling

The binding of DAMPs to pattern recognition receptors including TLRs and NLRs triggers a signal cascade involving the adaptor molecules MyD88 and TIR domain-containing adaptor-inducing interferon (TRIF). The end result is activation of NF-κB pathway (via MyD88) and interferon (via TRIF).

HyperIgD syndrome (HIDS) is a disease caused by mutations in MVK leading to accumulation of mevalonic acid (MA). In excess, MA is toxic, resulting in deficient biosynthesis, perturbation of signals and defective autophagy [8,94]. Furthermore, the defective MVK pathway leaves patients unable to produce anti-inflammatory isoprenoids [5,8]. Decreased isoprenoids has been related to increased GTPase Rac1 activity and subsequent activation of caspase-1 [5,8]. Supporting the role of Rac1 are in vitro studies showing that the inhibition of this enzyme in mononuclear cells from patients with HIDS leads to lower IL-1β production [5,8,95]. A number of cytokines are elevated in patients with HIDS, including IL-1α, IL-1β, TNF-α and IL-6 [96]. Recent in vitro studies indicate that the hyperresponsiveness of patients' peripheral blood mononuclear cells and resultant increase in cytokinesis is dependent on stimulation of TLR2, TLR4 and NOD2 [96]. Although the TNF-α levels are elevated in these patients, their response to IL-1 inhibition with anakinra is greater than to TNF-α antagonists [10]. Furthermore, a trial of IL-6 antagonist tocilizumab in two patients with ongoing symptoms despite anakinra therapy resulted in clinical improvement in both [96].

The familial form of Pityriasis Rubra Pilaris (PRP) is a rare autosomal dominant condition caused by mutations in CARD14, encoding caspase recruitment domain family, member 14 (CARD14) [33,97]. The gene is specifically expressed in keratinocytes and its protein functions as a regulator of NF-κB signalling. The altered structure of the mutated CARD14 coiled-coil domain increases activation of NF-κB [97], leading to a phenotype of follicular hyperkeratosis, palmoplantar keratoderma, and erythema [33,97].

Cherubism is an autosomal dominant condition caused by heterozygous mutations in SH3BP2, encoding the signalling adaptor protein SH3-domain binding protein 2 (SH3BP2) [8,34,98]. This

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inflammatory bone condition is characterised by bony swelling of upper and lower jaws, typically with regression during puberty [8,34,98]. In health, tankyrase, a member of the polyADP-ribose polymerase (PARP) family, interacts with SH3BP2 with modulation of downstream pathways. Knockout mice models have demonstrated that mutations in SH3BP2 lead to reduced tankyrase binding, reduced ADP-ribosylation of SH3BP2 and decreased degradation and overproduction of TNF-α [98]. The inflammation in cherubism is thought to be MYD88 dependent, after engagement of TLR2 and TLR4 by DAMPS. One hypothesis for the regression seen with age is the reduction in DAMPS after jaw stabilisation [98]. The limitation to the jaw is difficult to explain. Osteoblasts with this mutation show increased responsiveness to M-CSF and RANKL [8], and in heterozygous mouse models, bone marrow derived M-CSF dependent macrophages have been shown to undergo TNF-α-induced osteoclastogenesis independently of RANKL, through spleen tyrosine kinase (SYK) and phospholipase Cγ2 (PLCγ2) phosphorylation [99]. Interestingly, case reports of patients with cherubism treated with TNF alpha inhibitors did not show significant clinical response, suggesting that TNF alone is not responsible for the phenotype [100].

Activation of and signalling via NF-κB is the downstream effect of multiple cytokines and the perpetuation of the inflammatory response. Linear ubiquitination of NEMO and RIP1 with a polyubiquitin chain generated via linear ubiquitin chain assembly complex (LUBAC) is thought to play a key role in the regulation of the canonical pathway of NF-κB [101,102]. LUBAC is an ubiquitin ligase (E3) composed of heme-oxidized IRP2 ligase-1 (HOIL-1L, also known as RBCK1), HOIL-1L- interacting protein (HOIP), and SHANK-associated RH domain interacting protein (SHARPIN) [101,102]. Although much remains to be elucidated with regards to the mechanism of action of the individual components, together in LUBAC, they have a non-redundant role in NF-κB pathway and signalling via TNFR1 [101,102]. LUBAC is abundantly expressed in both thymus and spleen and it is suggested that the complex is required for CD40 and B cell function [101,102].

In order to elucidate the function of HOIL-1L, a number of experiments with HOIL1$^{-/-}$ mouse model have been documented. The first defect in humans was published in 2012, with loss of function mutations in HOIL1 (RBCK1) reported in three individuals from two unrelated families [103]. The phenotype of autoinflammation, invasive bacterial infections and muscular amylopectinosis was due to mutations in HOIL1 leading to LUBAC instability [103]. Interestingly, the effect of this defect varied depending on cell type. NF-κB induction in response to IL-1β and TNF was impaired in fibroblasts. Conversely, monocytes of HOIL-1 deficient patients were hyperresponsive to IL-1β [103]. The susceptibility of these patients to invasive pyogenic infections could be attributed to the impaired response to TLR stimulation. Response to stimulation of TLRs 2, 4 and 6 were partially affected, but IFN-β production via TLR3 was eliminated completely. TLR 7 and 8 stimulation didn't produce TNF [103]. The mechanism of intracellular glycogen inclusions in their muscles (amylopectinosis) remains to be elucidated.

6.3. Receptor and downstream signalling

Perforin and the cytotoxic CD8+ cells have been shown to be vital in the control of inflammation and underlines the intimate relationship between the adaptive and innate immune system. Genetic defects in this pathway have led to familial haemophagocytic lymphohistiocytosis (fHLH), characterised by expansion of T lymphocytes and macrophages and haemophagocytosis, as well as creation of "cytokine storm" [104,105]. The proposed mechanism is ongoing antigenic stimulation as a result of impaired killing of infected cells, with a central role of IFN-γ producing CD8+ T cells. Elevated levels of IFN-γ, IL-1β, TNF-α, IL-18 and IL-6 have also been consistently reported [104,105]. Whilst the mainstay of treatment remains corticosteroid therapy, there have been various results of success and failure with treatment of patients with TNF-α inhibition [104]. Trials of IL-1 and IL-6 antagonism in patients with systemic juvenile idiopathic arthritis (SJIA), a disorder complicated by macrophage activating syndrome (MAS), have been successful [104,106–108]. The role of these agents in reducing the frequency and/or severity of MAS, or indeed in the treatment of primary HLH is less certain. Case reports of successful resolution of SJIA related MAS after treatment with anakinra and conventional immunosuppression have been published [109–111]. However evidence for use of anakinra monotherapy, canakinumab or tocilizumab in the treatment of MAS is less conclusive [107,112–115]. Phase II trials looking at tocilizumab and anti-IFN- γ therapy in HLH are currently underway, and results may further assist in elucidating of primary cytokine driving the syndrome [104].

7. Not yet known

Two recently described autoinflammatory conditions involve the PLCγ2. PLCγ2-associated antibody deficiency and immune dysregulation (PLAID) is characterised by cold induced urticaria, granulomatous disease, autoimmune disease and hypogammaglobulinemia [116,117]. Patients with this condition have an autosomal dominant inframe deletion in *PLCG2* resulting in gain of function of PLCγ2 and increased PLCγ2-dependent signalling after receptor crosslinking [116,117]. Chronic stimulation and negative feedback of this pathway may have a role in the pathophysiology of hypogammaglobulinemia [117]. Autoinflammation and PLCγ2-associated antibody deficiency and immune dysregulation (APLAID) patients have a decreased threshold for triggering PLCγ2, without constitutive activation, leading to a phenotype of recurrent blistering skin lesions, bronchiolitis and recurrent sinopulmonary infections, arthralgia, ocular inflammation, enterocolitis, and mild immunodeficiency without autoantibodies [116,118]. Like PLAID, APLAID is an autosomal dominant condition caused by a missense mutation in *PLCG2* [116,118]. Treatment with non-steroidal anti-inflammatory drugs and TNF inhibitors have been unsuccessful and there was only a partial response to an IL-1 inhibition [118]. The mutation in APLAID affects an inhibitory domain of the enzyme leading to enhanced activity and subsequently more intracellular Ca^{++} release and activation of ERK in CD19+ B cells [119]. This increase in intracellular calcium may be a trigger of NLRP3 activation, linking this pathway with inflammasome and IL-1 and possibly explaining a partial response to anti-IL-1 therapy [119].

The H syndrome is characterised by hyperpigmentation, hypertrichosis, hepatosplenomegaly, heart abnormalities, hearing loss, hypogonadism, low height and occasionally hyperglycaemia. It has been linked to mutations in *SLC29A3*, a gene encoding the human equilibrative nucleoside transporter 3 (hENT3), important for passive transport of nucleosides [120]. Patients may also present with recurrent fevers, in addition to classic features of H syndrome [121]. The relationship between severity and SLC29A3 expression is unclear, as is the mechanism of hENT3 dysfunction and phenotype [121]. Cytokines IL-1, IL-6 and TNF are normal in knock out mice, and IL-1 treatments in patients with SLC29A3 spectrum disorders have been unsuccessful [121].

The description of a new autoinflammatory condition linked to deficiency adenosine deaminase 2 (DADA2) was published at the same time as mutations in *CECR1* (encoding ADA2) were linked to familial cases of polyarteritis nodosa [122,123]. In health, ADA2 has both catalytic and growth factor activities, deactivating extracellular adenosine and terminating signalling through adenosine receptors [122,124,125]. Deficiencies in ADA1, an intracellular

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adenosine deaminase, have been known to cause severe combined immunodeficiency. The phenotype of DADA2 is strikingly different, highlighting the unique roles, with patients with autosomal recessive loss of function *CECR1* mutations presenting with intermittent fevers, systemic inflammation, lacunar strokes, hepatosplenomegaly, and hypogammaglobulinemia of isotype IgM [123]. These patients had impaired endothelial development and a defect in macrophage development, with abnormal anti-inflammatory M2 differentiation [123]. The mechanism of recurrent fevers and systemic inflammation as well as endothelial dysfunction has not been clarified.

8. Conclusion

The recent description of unusual phenotypes with features of autoinflammation as well as autoimmunity has blurred the distinction between the two classes of syndromes and has highlighted the intimate link between innate and adaptive immune responses. Furthermore, diseases limited to specific organ systems suggest that autoinflammation need not be systemic. The response to targeted treatment is profound in patients where the specific cytokine involved can be identified. For this reason, it is important to focus on the pathophysiology of diseases, and the dominant cytokine leading to phenotype, to determine whether specific pathways can be targeted to provide therapeutic benefit for patients.

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8.2 Appendix 2: Monogenic AIDs summary table

CONDITION	GENE/S	PROTEIN	MOI	GOF/LOF	PATHWAY	CYTOKINE GROUP	SYSTEM INVOLVED	OTHER FEATURES	HUMAN CELL MODEL	POTENTIAL MURINE MODEL	REF
ADAM17 DEFICIENCY	*ADAM17*	ADAM17	AR	LOF	Unknown	Unknown	Skin GIT	ID[#]	**PBMC**: ↓TNF-α response to LPS, PMA + anti-CD3/anti-CD28 antibodies.	Nil	(1)
AGS1	*TREX1*	TREX1	AR or AD	LOF	Interferon	T1IFN	CNS	AI	**Human neural stem cell-derived astrocytes, primary astrocytes, brain-derived endothelial cells**: shRNA knock down → IFN gene signature + ↑ proinflammatory cytokines.	*Trex1*$^{-/-}$ mice	(2-5)
AGS2	*RNASEH2B*	RNASEH2B	AR	LOF	Interferon	T1IFN	CNS	AI		*Rnaseh2b knockout first* [*KOF*] mice	(6, 7)
AGS3	*RNASEH2C*	RNASEH2C	AR	LOF	Interferon	T1IFN	CNS	AI		*Rnaseh2c*$^{-/-}$ mice (8)(8)(8)	(6, 7)
AGS4	*RNASEH2A*	RNASEH2A	AR	LOF	Interferon	T1IFN	CNS	AI	**Human neural stem cell-derived astrocytes, primary astrocytes, brain-derived endothelial cells**: shRNA knock down minimal change in ISG/interferon cytokine profile.	*Rnaseh2a*$^{G37S/G37S}$	(2, 6, 7)
AGS5	*SAMHD1*	SAMHD1	AR	LOF	Interferon	T1IFN	CNS	AI	**HeLa cells**: transfection of mutant SAMHD1 showed abnormal localisation. **Human neural stem cell-derived astrocytes, primary astrocytes, brain-derived endothelial cells**: shRNA knock down → IFN gene signature + ↑ proinflammatory cytokines.	*Samhd1*$^{-/-}$ mice	(2, 9-13)
AGS6	*ADAR1*	ADAR1	AR	LOF	Interferon	T1IFN	CNS	AI	**HEK293Tcells**: Interferon reporter assay. **Lymphoblastoid cell line**: ↓ ADAR1 expression of mutant c/w WT. **Human neural stem cell-derived astrocytes, primary astrocytes, brain-derived endothelial cells**: shRNA knock down minimal change in ISG/IFN cytokine profile.	*Adar*$^{f/-}$ *SCL*-Cre-ER^{T} * mice	(2) (14, 15)
AGS7	*IFIH1*	MDA5	AD	GOF	Interferon	T1IFN	CNS	AI	**HEK293T**: Interferon reporter assay.	*Ifih1*$^{gs/+}$ mice	(16) (17, 18)
AIADK	*NLRP1*	NLRP1	AD	GOF	Inflam	IL-18 ?IL-1β	Multiple			*Nlrp1a*^{Q593P} mice*	(23, 24)

AIFEC	*NLRC4*	NLRC4	AD	GOF	Inflam	IL-18	Multiple	ID# Dysreg	**Monocytes**: ↑IL-1β in response to PrgI. **Monocyte derived macrophages**: ↑cell death, ↑IL-1β, IL-18 with LPS priming + flagellin. **HEK293T cells**: ASC speck analysis and inflammasome reconstitution. **iPSCs**: ↑IL-1β, IL18 to LPS.	mu-NLRC4 transgenic mice	(76-80)
AILJK	*COPA*	COPA	AD	Dominant negative	? NF-kB ? Interferon		Multiple incl lungs, kidney	AI ++	**CD4 T cells**: skewing to Th17 response. **BLCL**: ↓ autophagy, ↑ transcription IL-1 β, IL-6, IL-23. **HEK293T cells**: siRNA knockdown → ↑ER stress.	Nil	(35, 36)
APLAID	*PLCG2*	PLCγ2	AD	GOF	Unknown ?Inflam ?NF-kB	Unknown ?IL-1β	Multiple	ID	**PBMC**: ↑response to NLRP3 activation. **B cells**: ↑ERK phosphorylation.	Multiple*	(21, 22)
BLAU SYNDROME	*NOD2*	NOD2	AD	GOF	NF-kB	Multiple TNFα	Multiple		**HEK293T cells**: NF-kB luciferase assay. ↑ activity with transfection of mutants. **PBMC**: single patient w novel variant ↓ NF-kB response.	*Nod2*^{2939ic} mice	(25) (26, 27)
CAPS	*NLRP3*	NLRP3	AD	GOF	Inflam	IL-1β	Multiple		**PBMC**: constitutively high IL-1β secretion, as well as IL-6 + TNF. **THP1 cells**: ↑ IL-1β and IL-18 when transduced with mutant c/w WT. **CD4 T cells**: α-CD3 + α-CD46 stimulation →↑ IL-1β in patient cells c/w WT.	*Nlrp3*$^{A350VneoR/+}$ *Nlrp3*$^{L351PneoR/+}$ mice *Nlrp3*^{R258W} mice	(28-32)
CHERUBISM	*SH3BP2*	SH3BP2	AD	? GOF ? dominant negative	? NF-kB ? NFATc1	TNF-α	Bone			*Sh3bp2* $^{P416R/+}$ mice	(33, 34)
DADA2	*CECR1*	ADA2	AR	LOF	Unknown	?T1IFN ?TNFα	Multiple incl vascular	AI ID Dysreg	**PBMC**: ↑ B cell death when cultured without stimulation. **Monocytes**: differentiate into M1>M2.	Nil	(37-40)
DIRA	*IL1RN*	IL-1Ra	AR	LOF	IL-1β	Multiple	Multiple Bone		**Mononuclear cells**: stimulation with IL-1β → ↑ IL-1α, MIP1α, TNFα, IL-8, IL-6 c/w WT.	*Il1ra*$^{-/-}$ mice *(41, 42)(40, 41)	
DITRA	*IL36RN*	IL-36Ra	AR	LOF	Other	IL-36	Skin		**PBMC**: IL-36A stimulation → ↑ IL-1α, IL-6, IL-8, TNFα c/w WT.	*IL1F6* transgenic, *IL1F5*$^{-/-}$ mice	(43)
EOIBD	*IL10, IL10RA, IL10RB*	IL10, IL10RA, IL10RB	AR	LOF	Other	IL-10	GIT		**PBMC**: Failure of IL-10 to ↓ LPS induced ↑TNF α in patients with receptor mutations. More rapid TNF α response to LPS. Failure to phosphorylate STAT3 in response to IL10.	*IL10*$^{Trunc/Trunc}$ mice* *IL-10*$^{-/-}$ *Cx3cr1*$^{gfp/+}$ mice	(44-47)

FCAS2	*NLRP12*	NLRP12	AD	LOF	NF-kB ? Inflam	TNFα, IL-6, IL-1β	Skin Multiple		**HEK293T cells**: NF-kB luciferase assay **PBMC**: ↑ spontaneous TNF-α, IL-6, IL-1β c/w WT.	*NLRP12*$^{-/-}$ mice	(48-50)
FMF	*MEFV*	Pyrin	AR>>AD	GOF	Inflam	IL-1β	Multiple		**PBMC**: no spontaneous IL-1β secretion when cultured. ↑ IL-1β in response to LPS (inconsistent). Anti-CD3/CD28 stimulation → ↑ IL-17 and IL-22. **Neutrophil**s: possible release of IL-1β through NETS.	*Mefv*$^{M680I/M680I}$ mice *Mefv*$^{M694V/M694V}$ mice *Mefv*$^{V726A/V726A}$ mice	(51-54)
H SYNDROME	*SLC29A3*	SLC29A3	AR	LOF	Unknown	Unknown	Multiple	ID O		*ENT3*$^{-/-}$ mice	(55, 56)
HA20	*TNFAIP3*	A20	AR	LOF	NF-kB Inflam		Multiple	AI	**HEK293T cells**: NF-kB luciferase assay **PBMC, fibroblasts**: ↑ nuclear translocation p65 at rest + with TNF stimulation. **PBMC**: LPS → ↑ inflammatory cytokines. Polarisation to Th9, Th17 CD4 T cell linage. LPS → NLRP3 inflam activation.	*A20*$^{-/-}$ mice*	(57)
HIDS	*MVK*	MVK	AR	LOF	Inflam	IL-1β	Multiple	Dysreg	**PBMC**: ↑ IL-1β, IL-6 and TNFα at rest and with stimulation. **EBV-LCL**: accumulation unprenylated Rab proteins (temperature dependent)	*Mvk*$^{-/+}$ mice	(58-61) (62)
HOIL1 DEFICIENCY	*RBCK1*	HOIL1	AR	LOF	NF-kB	Multiple	Multiple	ID	**Fibroblasts, B cells**: ↓NF-kB activation. JNK phosphorylation normal. Impaired response to IL-1β>TNF **CD3+, CD19+, CD56+ cells:** No response to TNF or IL-1β stimulation. **Monocytes**: IL-1β stimulation → ↑IL-6 and MIP-1α c/w healthy control.	*Rbck1*$^{-/-}$ mice* (overtly normal)	(63)
HOIP DEFICIENCY	*RNF31*	HOIP	AR	LOF	NF-kB	Multiple	Multiple	ID	**Fibroblasts**: ↓IKK phosphorylation, IL-6 production in response to TNF or IL-1β stimulation. **B cells**: ↓ CD80 upregulation with CD40L + IL-21 or IL-4. **Monocytes**: IL-1β stimulation → ↑IL-6 and IL-1β c/w healthy control.	*Hoip*$^{-/-}$ mice (embryonically lethal). Various crosses.	(64)
HYDM1	*NLRP7*	NLRP7	AR	Unknown	? Inflam ? NF-kB	Unknown	Placenta	O	**HEK293T cells**: transient transfection → abnormal methylation. **PBMC**: ↓IL-1β and TNF in response to LPS. Conflicting data on secretion as well as effect on pro-IL-1β expression.	Nil (no murine orthologue)	(65-70)

MAJEED SYNDROME	*LPIN2*	Lipin 2	AR	LOF	Inflam	IL-1β	Bone Skin Multiple	O	**HEK293T cells**: PAP activity assay. Hepa1-6 cells: PPARα luciferase assay.	*Lpin2*$^{-/-}$ mice	(71-74)
MSPC	*NLRP1*	NLRP1	AD	GOF	Inflam	IL-1β	Skin	O	**HEK293T cells**: ASC speck assay, reconstitution of inflam ↑pro-IL-1β cleavage. **Primary keratinocytes**: spontaneous inflam activation. **PMA differentiated THP1 cells**: doxycycline induced *NLRP1* expression constructs. Mutants ↑IL-1β and cell death. ASC dependent.	*Nlrp1a*^{Q593P} mice*	(24, 75)
ORAS	*OTULIN*	Otulin	AR	LOF	NF-kB	TNF	Multiple	ID O	**HEK293T cells**: transfection of mutant ↑NF-kB pathway c/w WT. NF-kB luciferase assay showed ↓inhibitory effect of mutant Otulin c/w WT. **T cells**: normal proliferation and NF-kB response to TCR stimulation **B cells**: normal proliferation and NF-kB response to BCR stimulation **Fibroblasts**: expression undetectable. ↑p-IKBa, p-IKKa/b, p-P38 and P-JNK with TNF stimulation. ↓ ability to deubiquitinate linear chains. **PBMC**: ↓ ability to deubiquitinate linear chains.	CreERT2-*Otulin*$^{LacZ/flox}$ mice	(81, 82)
PAAND	*MEFV*	Pyrin	AD	GOF	Inflam	?Multiple	Skin Multiple		**Monocyte**: ↑ASC speck formation, caspase-1 activity, c/w healthy control **PBMC**: ↑ IL-18 and IL-1Ra with LPS stimulation c/w healthy control **HEK293T cells**: ASC speck assay. 14-3-3 binding in overexpression model. **THP1 cells**: retroviral reconstitution and lentviral reconstitution of *MEFV* KO cells. Mutants ↑cell death, ↑IL-1β, IL-18.	Nil	(83, 84)
PAPA SYNDROME	*PSTPIP1*	PSTPIP1	AD	Unknown	Inflam	?IL-1β	Skin, joints Multiple		**HeLa cells**: Transient cotransfection. Mutant PSTPIP1 hyperphosphorylated and ↑binding to pyrin. **Cos-7L cells**: inflammasome reconstitution assay. Mutant PSTPIP1 ↑IL-1β processing. **THP1 cells**: immunoprecipitation to shown interaction between PSTPIP1 and pyrin **Macrophages**: ↓invasion and podosome formation. **T cells**: ↓numbers, ↓proliferation response to mitogen. Normal migration	Rosa-*PSTPIP1* A230T$^{STOP\ del/+}$ mice	(85-87) (88, 89)

									PBMC: ↓ IL-1Ra, ↑IL-1β, IL-6, TNFα and GMCF in response to multiple stimuli. siRNA knock down of *NLRP3* ↓IL-1β in response to LPS.		
PLAID	*PLCG2*	PLCγ2	AD	GOF	Unknown	Unknown	Multiple	ID AI	**COS7, A20 cells**: transfection model. Mutants- ↑phospholipase activity at sub-physiological temperatures. **LAD2 mast cells**: transfection of mutant → spontaneous degranulation at 20°C **B cells and NK cells**: ↓ERK phosphorylation in response to stimulation **T cells**: normal response to CD3 cross-linking.	Multiple*	(90)
PRAAS	*PSMB8, PSMB9, PSMB4, PSMA3, POMP*	PSMB8, PSMB9, PSMB4, PSMA3, POMP	AR	LOF	?NF-kB ?Interferon	T1IFN	Multiple		**HeLa cells**: transfection studies show poor formation of proteasome with mutant c/w WT. **Primary fibroblasts**: ↑ precursor complexes in patients. siRNA knock down in control cells → IFN induction and proteasome dysfunction. **Lymphoblastoid cell line**: ↑ precursor complexes, ↓ proteasome formation **EBV transformed B cells**: generally, ↓ chymotryptic like activity. **Primary keratinocytes**: Ubiquitin aggregation.	*Lmp7*$^{-/-}$ mice	(92, 93)
PRP	*CARD14*	CARD14	AD	GOF	NF-kB		Skin		**HEK293T cells**: NF-kB luciferase assay **Immortalised primary keratinocytes:** Expression + NF-kB activity.	Nil	(94, 95)
PUSTULAR PSORIASIS	*AP1S3*	AP1S3	AR	LOF	?NF-kB	IL36 IL-1	Skin		**Primary keratinocytes and dermal fibroblasts**: abnormal autophagy, accumulation of p62. Abnormal TLR2/6 signalling.	Nil	(19, 20)
SAVI	*TMEM173*	STING	AD	GOF	Interferon	T1IFN	Multiple incl lungs, vessels	AI	**HEK293T cells**: *IFNB1* reporter assay. Immunoblot analysis of STING pathway. **CD4, CD8 T cells, CD19 B cells**: constitutive STAT1 phosphorylation. **PBMC and dermal fibroblasts**: ↑ *IFNB1* transcription at rest. No change with cGAMP exposure. Transcription of *TNF* and *IL-6* ↑ at baseline and with cGAMP treatment.	*Sting*$^{N153S/+}$ mice	(96-98)
SPENCD	*ACP5*	ACP5	AR	LOF	Interferon	T1IFN	Multiple	AI ID	**Primary human macrophages**: colocalisation studies. **Plasmacytoid dendritic cells**: colocalisation studies. TLR9 stimulation in shRNA *ACP5* knock down studies → ↑ transcription ISGs.	*Acp5*$^{-/-}$ mice	(101-104)

									HEK293T cells: cotransfection TRAP and osteopontin followed by immunoprecipitation. **THP1 cells**: shRNA *ACP5* knock down studies → ↑ phosphorylation of osteopontin.		
TRAPS	*TNFRS1A*	TNFR1	AD	Unknown	NF-kB	?IL-1β	Multiple		**PBMC**: ↑surface expression TNFR1 + ↓shedding (conflicting data). **Monocytes**: ↑surface expression TNFR1 and ↓shedding. Abnormal autophagy →↑IL-1β + NF-kB activation. **Dermal fibroblasts**: mutant TNFR1 ↓ receptor shedding. **Neutrophils**: mutant TNFR1 abnormal retention in cytoplasm. **HEK293T cells**: minor differences in receptor shedding when TNFR1 WT or mutant overexpressed. Cytoplasmic retention and reduced surface expression of mutant.	*Tnfrsf1a*$^{T50M/+}$ mice (105) *Tnfrsf1a*$^{C33Y/+}$ mice (106) *Tnfrsf1a*^{p55deltNS} mice (107)	(105-119)
USP18 DEFICIENCY	*USP18*	USP18	AR	LOF	Interferon	T1IFN	CNS Liver		**Primary dermal fibroblasts**: ↑ transcription ISG after IFN stimulation. Persistent STAT2 phosphorylation. No sig difference in IL-6 response to IL-1β or poly(I:C). ↑ISGylation.	*Usp18-/-* mice*	(120)
XLPDR	*POLA1*	POLA1	XLR	LOF	Interferon > NF-kB	T1IFN	Multiple	ID	**Primary dermal fibroblasts**: ↑IFN + NF-kB response to stimulation with poly(da:dt) or TNF. ↑IRF and NF-kB pathway activation. ↓RNA:DNA levels. Lentiviral transduction of WT rescued phenotype. **Fibroblast and HeLa cell line**: siRNA *POLA1* knock down → ↑ IFN + NF-kB response to stimulation with poly(da:dt) or TNF.	Nil	(91)

Table 1.1 Monogenic autoinflammatory disorder summary table. Abbreviations: AD autosomal dominant, AGS Aicardi-Goutieres syndrome, AI autoimmune, AIADK autoinflammation with arthritis and dyskeratosis, AID autoinflammatory disorder, AIFEC autoinflammation with infantile enterocolitis, AILJK autoimmune interstitial lung, joint, and kidney disease, APLAID autoinflammation and PLCG2-associated antibody deficiency and immune dysregulation, AR autosomal recessive, BLCL Epstein-Barr virus-transformed B-lymphoblastoid cell lines, c/w compared with, CAPS cryopyrin associated periodic syndromes, CD cluster of differentiation, CNS central nervous system, COPA coatomer subunit alpha, DADA2 deficiency in ADA2, DIRA deficiency of IL-1 receptor antagonist, DITRA deficiency of IL-36 receptor antagnosit, Dysreg dysregulation (including lymphoproliferation), EOIBD early onset inflammatory bowel disease, ER endoplasmic reticulum, FCAS2 familial cold autoinflammatory syndrome 2, FMF familial Mediterranean fever, GIT gastrointestinal tract, GOF gain of function, HA20 haploinsufficiency of A20, HIDS hyperIgD syndrome, HYDM1 hydatidiform molar pregnancy, ID immune deficiency, IFN interferon, Inflam inflammasome, ISG

interferon stimulated genes, LOF loss of function, LPS lipopolysaccharide, MOI mode of inheritance, MSPC multiple self-healing palmoplantar carcinoma, NF-kB nuclear factor kappa B, NFATc1 nuclear factor of activated T-cells, cytoplasmic 1, O other, ORAS otulin related autoinflammatory syndrome, PAAND pyrin associated autoinflammation with neutrophilic dermatosis, PAPA pyogenic arthritis, pyoderma gangrenosum and acne, PBMC peripheral blood mononuclear cells, PLAID PLCG2 associated antibody deficiency and immune dysregulation, PMA phorbol myristate acetate, POLA DNA polymerase alpha catalytic subunit, PRAAS proteasome associated autoinflammatory syndrome, PRP pityriasis rubra pilaris, SAVI STING associated vasculopathy with onset in infancy, shRNA short hairpin RNA, siRNA short interfering RNA, SMS Singleton-Merten Syndrome, SPENCD spondyloenchondrodysplasia, T1IFN type 1 interferon, TLR toll like receptor, TRAPS TNF receptor associated periodic syndrome, WT wild type, XLPDR x-linked pigmentary disorder, reticulate, with systemic manifestations.
* Murine model prior to the description of monogenic condition
# susceptibility

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8.3 Appendix 3: NATA accredited NGS panel testing gene list

Gene	Protein
CARD14	CARD14
CECR1	ADA2
IL10	IL10
IL10RA	IL10RA
IL10RB	IL10RB
IL1RN	IL1Ra
IL36RN	IL36Ra
LPIN2	LPIN2
MEFV	Pyrin
MVK	MVK
NLRP12	NLRP12
NLRP3	NLRP3
NLRP7	NLRP7
NOD2	NOD2
PLCG2	PLCγ2
PSMB8	PSMB8
PSTPIP1	PSTPIP1
SH3BP2	SH3BP2
SLC29A3	SLC29A3
TMEM173	STING
TNFRSF1A	TNFR1

8.4 Appendix 4: AADRY protocol

Project Number: HREC/15/MonH/31

Title: Australian Autoinflammatory Diseases RegistrY (AADRY): A national approach to the genetic and immunological evaluation of patients with suspected autoinflammatory syndromes

Principle Investigators:
Coordinating: Dr Fiona Moghaddas (WEHI, Melbourne Health)
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Sites and investigators:

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Monash Health	Dr Angela Cox, Dr Peter Gowdie, Dr Samar Ojaimi, A/Prof Sue Piper, Dr Matthew Hunter
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Walter + Eliza Hall Institute	Dr Vanessa Bryant

South Australia	
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Royal Adelaide Hospital	Dr Pravin Hissaria
Flinders Medical Centre	Dr Tiffany Hughes, Dr Henning Johannsen, Dr Leigh Mackey, Dr Christine Ziegler

Queensland	
Princes Alexandra Hospital	Dr David Gillis
Royal Brisbane & Women's Hospital	Dr Paul Kubler
Lady Cilento Children's Hospital	Dr Navid Adib, Dr Ben Whitehead, Dr David Coman

NSW	
Sydney Children's Hospital Network	Dr Paul Gray, Prof Dianne Campbell, Dr Sam Mehr, Dr Melanie Wong, Dr Damien McKay, A/Prof Davinder Singh-Grewal, Dr Jeffrey Chaitow
Liverpool Hospital	A/Prof Geoffrey Cains, Dr Dunja Vekic, Dr Jane Woods, Dr Damien McKay
Royal Prince Alfred Hospital	Dr Roger Garsia

WA	
Perth Children's Hospital	Dr. Senq Lee, A/Prof. Gareth Baynam, Dr. Emily Boulter, Dr. Kevin Murray
Fiona Stanley Hospital	Dr. Michael O'Sullivan
Path West Fiona Stanley Hospital	Dr. Michael O'Sullivan

Background

Monogenic autoinflammatory diseases, also known as periodic fever syndromes, are an uncommon collection of conditions characterized by abnormalities in the innate immune system, often characterized by dysregulation of signaling proteins in the body called interleukins or cytokines. Well-characterized examples include Familial Mediterranean Fever (FMF), Cryopyrin Associated Periodic Syndromes (CAPS), HyperIgD Syndrome (HIDS) and Tumour Necrosis Factor (TNF) receptor associated periodic fever syndrome (TRAPS). Patients with these conditions often present with recurrent febrile episodes with no underlying infective or malignant process found

on detailed examination and investigation. Each autoinflammatory condition has unique features and may be inherited in an autosomal recessive or autosomal dominant manner, or occurs de novo. Patients with FMF tend to have abdominal and chest pain, whereas patients with CAPS may have a spectrum of presentations, ranging from rashes on exposure to cold, to intellectual impairment, chronic aseptic meningitis and bone deformities. The treatment of these conditions is also quite variable. Good response to colchicine is seen in patients with FMF, but patients with CAPS fail to show any improvement with this therapy.

Research into the cause of monogenic autoinflammatory diseases has resulted in a dramatic shift in the prognosis of patients with specific diagnoses. Prior to the knowledge that CAPS was a disorder of interleukin 1-β (IL-1 β) dysregulation, patients were treated with non-specific immunosuppressive medications with relatively poor response. A common outcome of poorly controlled chronic or recurrent acute inflammation was amyloidosis and the complication of this included renal and heart failure. Once the basis was shown to be mutations in *NLRP3*, the gene encoding cryopyrin, the dominant role of IL-1 in CAPS was established and targeted treatment with IL-1 antagonists was established.

As it stands, there are approximately 25 monogenic autoinflammatory conditions known and 25 genes involved (http://fmf.igh.cnrs.fr/ISSAID/infevers/). A European registry has documented over 1000 patients with a monogenic autoinflammatory disease (http://www.printo.it/eurofever/eurofever_results.asp). There is no data from Australia on the incidence or prevalence of patients with an established genetic diagnosis of autoinflammatory diseases, and no information on the number of patients with suspected disease that have received negative genetic test results.

There are a number of limitations to the diagnosis of monogentic autoinflammatory diseases. These are rare conditions with a degree of phenotypic variability. The actual incidence and prevalence in Australia is unknown as there have been no investigative efforts to determine this. There is no one specialty dedicated to the care of these patients, and it is possible that no one center would look after more than a handful of patients. Furthermore, there are NATA accredited laboratories in Australia offering genetic testing for mutations known to cause only four conditions (FMF, CAPS, HIDS, TRAPS). These are listed below (http://genetictesting.rcpa.edu.au).

Diagnosis	Gene	Laboratory
TRAPS	*TNFRSF1A*	• Department of Molecular Genetics The Children's Hospital at Westmead NSW • Molecular Genetics Laboratory Douglass Hanly Moir Pathology NSW
HIDS	*MVK*	• Immunology Laboratory, Children's Hospital at Westmead, NSW
CAPS	*NLRP3*	• Department of Molecular Genetics, The Children's Hospital at Westmead, NSW
FMF	*MEFV*	• Department of Clinical Immunology PathWest Laboratory Medicine, Royal Perth Hospital WA • Department of Molecular Genetics The Children's Hospital at Westmead NSW • Molecular Genetics Laboratory Douglass Hanly Moir Pathology NSW • Molecular Genetics Unit (at Women's & Children's Hospital) SA Pathology, SA

There are currently no unified approach to the further investigation of patients who test negative for mutations in these genes and/or those who have an atypical phenotype.

Whole exome sequencing is increasingly being used in the investigative algorithm of patients with undiagnosed illnesses. The improvement in technology has made the possibility of whole exome sequencing more cost effective and hence more feasible. The process detects functional variants located in exons[1]. Despite accounting for 1-5% of the human genome, 85% of mutations with effects on disease related traits are in these protein-coding genes[2]. The process is useful when the cause of the mutation is unknown [3].

The use of exome sequencing as part of the investigative approach of patients without a confirmed diagnosis has been shown in both familial [4] and denovo [2] conditions. With functional testing, whole exome sequencing may yield clinically useful information[2]. There have been a few reviews listing the disorders that have been discovered or diagnosed with whole exome sequencing [1, 5].

With reference to autoinflammatory diseases, there have been recent publications outlining new conditions determined by whole exome sequencing. By way of example, this approach allowed for the description of a condition linked to deficiency adenosine deaminase 2 (DADA2) as well as linking mutations in *CECR1* (encoding ADA2) to familial cases of polyarteritis nodosa[6, 7]. Both PLC γ 2-associated antibody deficiency and immune dysregulation (PLAID) and Autoinflammation and PLC γ 2-associated antibody deficiency and immune dysregulation (APLAID) were discovered through the use of whole exome sequencing [8-10]. Although the exact pathophysiology is yet to be determined, progress has been made in linking the genetic mutation with pathways involved in the immune response and hence phenotype and potentially targeted therapy.

Overview of project

This project aims to establish a national registry of patients with autoinflammatory syndromes (Australian Autoinflammatory Diseases RegistrY- AADRY) with a total of three groups of participants being recruited:

1. Patients with suspected autoinflammatory diseases (genetically undefined)
2. Biologic relatives of patients in group 1 (predominantly parents)
3. Patients with genetically defined autoinflammatory diseases (prevalent and incident cases)

AADRY will collect epidemiological and clinical data on patients with suspected and confirmed autoinflammatory diseases allowing for determination of number of patients with and without a genetic diagnosis for their autoinflammatory phenotype (group 1 and 3). We may also determine communities with high rates of diagnoses so that resources may be appropriately allocated. Results may show that there may be a need for information in certain languages to be made available. Furthermore, current treatment trends and a list of specialists caring for these patients will be established. There is little collaboration between clinicians caring for patients with suspected autoinflammatory diseases. Clinicians from various specialties, including but not limited to rheumatology and immunology, care for these complicated patients and the registry may provide insight into where education of trainees is best directed.

The next part of this project aims to sequence the whole exome of patients with suspected autoinflammatory diseases as part of a research tool in an attempt to evaluate for a diagnosis (group 1 participants). Prior to participation, patients are likely to have been tested for available genetic mutations as deemed appropriate as part of diagnostic workup by the treating clinician. Whole exome sequencing would involve testing both the patient and where possible biologic parents/blood relatives of the patient (group 2) to see if there are any new mutations in genes that may have a role in the immune system and hence the patient's symptoms. Subsequent to this, we will perform in vitro testing to determine functional significance of any mutations that are thought to be relevant.

The third part of this project is to establish a tissue bank of patients in group 1. Tissue will be stored for the purpose of this study and future unspecified studies.

Specific Aims

Our research project has the following aims:

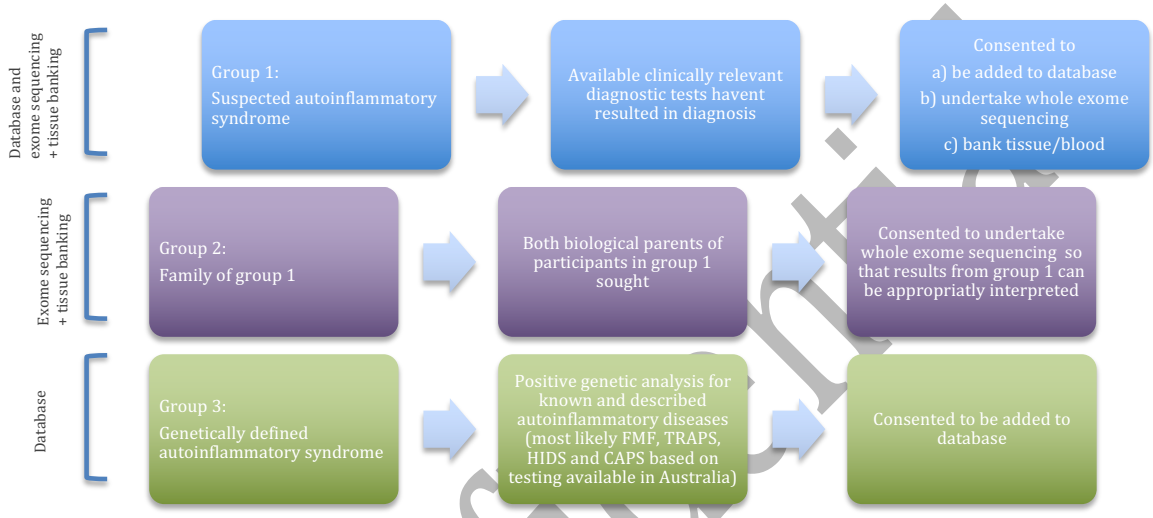
1. Establish an Australian Autoinflammatory Diseases Registry to achieve the following
    a. Epidemiological and clinical data on patients with suspected autoinflammatory diseases.
    b. Epidemiological and clinical data on patients with established diagnoses of autoinflammatory diseases.
        i. This will be achieved through recruitment and survey completion.
2. Perform exome sequencing on patients with suspected autoinflammatory diseases.
    a. We aim to investigate patients with a suspected autoinflammatory disease in an attempt to establish a diagnosis. Prior to participation, patients would have been tested for available genetic mutations as deemed appropriate for diagnostic workup by the treating clinician. Whole exome sequencing would also involve testing biologic parents/blood relatives of the

patient to see if there are any **new mutations** (de novo) in genes that may have a role in the immune system and hence the patient's symptoms, as well as familial mutations.

b. Perform in vitro tests to determine functional significance of any mutations that are thought to be relevant.

3. Establish a tissue bank of patients with suspected autoinflammatory diseases.
   a. Tissue will be stored for the purpose of this study and future unspecified studies. This research may include further work in the pathophysiology of autoinflammatory syndromes.

Clinical Methods



Ascertainment:

Group 1: Proband → Suspected autoinflammatory diseases.

- Participants will be invited to participate in the study by their treating clinician, who will also be an investigator on the study, through their attendance at clinical service. It is expected that recruitment would occur during routine care and the patient would not be required to attend additional appointments. This project will be promoted through Australasian Society of Clinical Immunology and Allergy (ASCIA) and Australian Rheumatology Association (ARA), the national immunology and rheumatology societies respectively.
- The registry will be accessible to clinicians who are part of the project via an online database (REDcap). This is a well established database platform.
- Patients not under the routine care of a specialist unit at an affiliated hospital will be flagged as potential subjects by their GP or specialist in private practice. They will then be consented by the coordinating investigator after information and consent forms mailed to the patient. These patients will have their data entered into REDCap by the coordinating investigator.
- Conferences will be attended. Coordinating investigators will discuss autoinflammatory diseases, the aims of the project, method of recruitment and interim results (deidentified). A flyer will be available with information (see Clinician Flyer, Version 1, 23/03/2017). Minor changes to the presentation and flyer will be made to tailor to audience, but major changes will be submitted to the ethics committee for review.
- Interviews with the media, including but not limited to radio and television, will be used to provide information about the project. Exact details will be tailored to audience, but will be based on Patient/Family Flyer (see Patient/Family Flyer, Version 1, 23/03/2017)
- A website will be created with broad overview of project, method of recruitment and de-identified interim results of numbers of patients recruited and clinical diagnosis. There will be a section for clinicians and a section for patients/families. This will provide information on method of recruitment, support services and

links to external sources of evidence based information for families.

Group 2: Family of patients suspected of having an autoinflammatory disease
- Blood relatives of those in Group 1 will be sought. We will request probands' permission to contact the family directly for participation. In order to determine de novo mutations, trios (proband + 2 parents) are required for interpretation.

Group 3: Patients with established diagnoses.
- Patients with established diagnoses will be sought from:
    - Laboratory records and hospital records (via test and diagnosis coding respectively)
    - Rheumatology and immunology departments
    - ASCIA and ARA (Appendix 1)
- Where possible, treating clinicians who are members of the project will be asked to consent patients for data to be entered into registry.
- With the patient's consent, medical records and laboratory results may be accessed to complete the survey.
- Patients under the care of a GP or specialist in private practice will be referred as a potential participant, and consent will be performed via the coordinating investigator. These patients will have their data entered into REDCap by the coordinating investigator.
- Conferences with a clinical audience will be attended. Coordinating investigators will discuss autoinflammatory diseases, the aims of the project, method of recruitment and interim results (deidentified). A flyer will be available with information (see Clinician Flyer, Version 1, 23/03/2017). Minor changes to the presentation and flyer will be made to tailor to audience, but major changes will be submitted to the ethics committee for review.
- Interviews with the media, including but not limited to radio and television, will be used to provide information about the project. Exact details will be tailored to audience, but will be based on Patient/Family Flyer (see Patient/Family Flyer, Version 1, 23/03/2017)
- A website will be created with broad overview of project, method of recruitment and de-identified interim results of numbers of patients recruited and clinical diagnosis. There will be a section for clinicians and a section for patients/families. This will provide information on method of recruitment, support services and links to external sources of evidence based information for families.

Informed consent:
Consent will be obtained for all groups in this project. The study will be explained to the patient or person responsible at the time of their appointment or inpatient episode by an investigator who will be responsible for obtaining patient's informed consent to participate. As we have the potential to recruit patients of all ages, if a young person understands relevant information but considered to be relatively immature in their ability to consent, both child and parent consent will be obtained.

Furthermore, we have the potential to enroll patients who do not speak English as their first language. Consent will be obtained with an interpreter.

Patients who are flagged as potential participants by their GP or specialist in private practice will receive information via mail. They will also receive a phone call from the coordinating investigator to ensure that any questions/concerns can be addressed. They will be consented over the phone by the coordinating investigator. The signed consent form will be emailed where possible and mailed to the coordinating investigator.

Inclusion/exclusion criteria:
Patients or their guardian must be able to provide informed, voluntary, written consent as outlined in the Patient Information and Consent Form. Patients will be excluded from the study if they (or their guardian) decline to consent to the study or have a condition which interferes with their ability to provide informed consent.

Group 1: Patients of all ages will be included. As the clinical phenotype of patients will be varied, no exclusion criteria have been established. The expectation is that patients will have undergone clinically relevant testing to investigate for infection, immunodeficiency, malignancy and autoimmune disease as well as known periodic fever syndromes as deemed appropriate and necessary by the treating clinician.

Group 2: Blood relatives of those in Group A will be recruited, including biological parents. and siblings

Group 3: Patients in this group will have a *genetically confirmed* autoinflammatory disease. As this group is health information collection, there are no specific exclusion criteria.

Baseline evaluation:
Baseline data will be collected for the registry through templates as shown at the end of this document. This will allow for consistency in data collection and summary, as well as possible pattern recognition. These data entry points are based on clinical criteria for periodic fever syndromes with details to allow for possible detection of patterns between patients/patient groups.

Transport of specimens and logistics:
Blood samples from patients will be couriered to The Walter & Eliza Hall Institute of Medical Research (WEHI). Mail kits will be returned to (WEHI). Both will be addressed to the Coordinating investigator. The cost will be covered by the Inflammation Department at WEHI.

Follow-up
Group 1: Patients will be followed up as per their routine appointments and any results will be relayed via the recruiting clinician. Contact will be made when a child turns 18 for ongoing storage of tissue in tissue bank.

Group 2: These patients will not be followed up routinely. If there are results from whole exome sequencing that are of importance, referral with appropriate counseling services will be provided. Contact will be made when a child turns 18 for ongoing storage of tissue in tissue bank.

Group 3: These patients will not be followed up routinely.

Laboratory studies and methods
Group 1: These patients will undergo whole exome sequencing from a blood sample obtained via venipuncture. Where possible, this will be taken at the time of collection of another sample that may be required for routine care. DNA will be extracted either on site or at the Walter and Eliza Hall Institute. DNA will then be sequenced by a third party. Raw sequence data will be analysed by a bioinformatician using a common analytical pipeline for genome assembly, alignment and variant calling. Filters will be applied such that only variants in genes associated with innate immune system and autoinflammation will be prioritised. A research report listing the annotated variants prioritized according to annotation and likely clinical significance will be produced.

If a mutation is found that is thought to be potentially disease causing, there will be a number of laboratory methods required to help elaborate this. This will include trying to make that same mutation in a different (laboratory created, not patient derived) cell so that the response to a number of tests can be seen. We hope to store blood/cells to perform functional studies on patients sample if these laboratory tests show a possible disease causing mutation. The volume of blood required is conservative, with approx. 1-2 teaspoons for children and approximately 20mL for adults (for DNA and also tissue banking purposes).

Group 2: Whole exome sequencing will be performed from DNA collected from saliva via home kit. Comparing a patient's whole exome sequence with their parents' will allow for determination of de novo mutations.

Sample size and statistical considerations
As we are trying to elucidate the pathogenesis of suspected autoinflammatory diseases in individuals, there is no minimum number of recruits. These conditions are uncommon but as there has not been an avenue to explore further in Australia, there may be a number of patients who have been suspected of having an autoinflammatory disease for some time. We predict roughly 20 patients per year in Group 1 and would hope to recruit at least two blood relatives for Group 2.

Ethical implications
Participant time
Participants will be recruited during the consultation time with their clinician. They are unlikely to require more than one venesection, and all effort will be made to have this done at the time that another blood test is

required, for example bloods taken for evidence of ongoing inflammation such as ESR (erythrocyte sedimentation rate) and CRP (C-reactive protein).

Their blood relatives (if present) will be consented at the time of consultation with Group 1. If not present, phone contact will be made and PICF will be sent out. They will be called with regards to their consent and a mail out saliva kit sent if they consent. They would spend less than a few minutes collecting saliva.

Patients with a known diagnosis will have their questionnaire completed with their clinician at the time of consultation if attending specialist care. The coordinating investigator will send a pdf copy of data entry points to the GP or specialist in private practice so that REDCap access is not required. The coordinating investigator will enter the data in to the REDCap database. This will ensure that de-identified patient information is not accessed by associate investigators from other sites. The coordinating investigator may contact the patient to clarify data entry points if the survey is incomplete.

Pain/discomfort from procedures
Group 1: Blood volumes will be conservative and will be collected by experienced phlebotomists at the hospital they are attending. Children may opt for an anaesthetic patch to be administered prior to blood collection. There may be some bruising at the venipuncture site. The volume required is approximately 5ml for children and 10ml for adults.

Group 2: Saliva sample kits are painless

Benefits to overall patient care
Group 1: Patients in this group are likely to have exhausted currently available diagnostic/investigative options to obtain a diagnosis. Although only a possible outcome, an established diagnosis may be psychologically helpful for patient and family, it may also lead to an understanding of the pathophysiology of disease and potentially guide treatment options. Patients with undifferentiated autoinflammatory diseases are often treated with broad/general immunosuppressive agents with short and long-term side effects. This project may help narrow the spectrum of medications required to maintain quality and quantity of life. It is possible that the study will not result in change in management.

Group 2: Although not directly benefited by this, their sample will assist in interpretation of genetic information for Group 1.

Group 3: As this is purely epidemiological and clinical data, the patient is unlikely to benefit from this particular project.

Privacy and confidentiality
For those who have had genetic testing performed, data will be re-identifiable so that genetic information/pertinent results can be relayed. All information stored securely in locked filing cabinet at WEHI, itself secured. Data will be stored in password protected computer database and biospecimens stored in freezer in locked lab.

The database will be set up using the Redcap platform. There is graded security, with the coordinating investigator having a code and able to access identified data so that important results can be relayed to treating clinician. The other investigators will be able to access de-identified data. Each investigator will be able to access the data that they themselves have entered. GPs and specialists in private practice will not have access to the database.

Summary statistics (de-identified trends in number of people in the registry gender age, diagnosis etc) will be made available via publications.

Future researchers may access database with appropriate ethics. This information will be de-identified and is outlined in consent forms for participants.

Human genetics
This project aims to determine novel familial or de novo mutations that may be causing or contributing to a

patient's presentation. Familial or hereditary mutations may be passed on in an autosomal dominant, recessive or X-linked fashion, whereas denovo mutations present for the first time in a family member a result of a mutation in a germ cell of one of the parents or the fertilized egg.

The limitations of genomic sequencing will be explained to patients at recruitment and again when results are provided. The possible results are that:

- No likely mutation associated with the condition is found. However, this may be due to technical limitations of the test
- Variant of unknown significance is found in a gene relevant to the condition affecting the patient. This would require further investigation
- A likely pathogenic mutation is found

Internationally, there is no consensus on best practice regarding incidental findings arising from genomic research. Although there will be a robust analysis pipeline looking for variants of significance to autoinflammation and the innate immune system, unexpected incidental results may be found. Patients (or parent/guardian) will be made aware of findings that are considered related to the disease being investigated. If there is an incidental finding of a mutation that is thought to be unrelated to their presentation but potentially clinically significant, the results will be relayed to the patient via the treating clinician, and will be referred to a genetic counseling service through the public system (http://www.genetics.edu.au/Genetics-Services/genetic-counselling-services). The coordinating investigator will work with the treating clinician to ensure follow up is provided.
Although it is possible to determine non-paternity with whole exome sequencing, these results will not be noted or revealed to the patient/family. In the rare event that a heritable incidental finding is made this may need to be disclosed. These instances will be treated on a case-by-case basis and will require consultation with genetic counselors before such disclosure occurs.

As per the relevant guidelines, consent will be obtained from the participants for family involvement where relevant, but if research discloses that a family member may be at risk of illness for which there is treatment available or pending, information may be conveyed to family without participant consent with HREC approval.

Expected outcomes
We hope to determine disease-causing mutations in patients with suspected autoinflammatory diseases. Realistically, we will find in some patients one of the 20 or so already described but with no testing available in Australia. We may find a new mutation that leads to a particular phenotype of autoinflammatory disease, or find a group of patients with a similar yet unusual presentation. It is expected that any potentially diseases causing novel variant will be evaluated in vitro to determine the possible effects on the immune system.

We will also have a database that provides insight into autoinflammatory diseases in Australia, and in doing so will have increased awareness of these conditions and encouraged collaboration between clinicians and scientists looking after and researching these patients.

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8.5 Appendix 5: AADRY PICFs

Participant Information and Consent Form for patients

Full Project Title:

Australian Autoinflammatory Diseases RegistrY (AADRY): A national approach to the genetic and immunological evaluation of patients with suspected autoinflammatory syndromes

HREC No: **HREC/15/MonH/31**

Principal Researchers: Dr Fiona Moghaddas, Dr Seth Masters, Professor Ian Wicks

1. Introduction

You are invited to take part in this research project, as you are currently being investigated for or diagnosed with an autoinflammatory disease. This Participant Information and Consent Form tells you about the research project and explains the procedures involved. Knowing what is involved will help you decide if you want to take part in the research.

Please read this information carefully. Ask questions about anything that you don't understand or want to know more about. Before deciding whether or not to take part, you might want to talk about it with a relative, friend or healthcare worker.

Participation in this research is voluntary. If you don't wish to take part, you don't have to. You will receive the best possible care whether you take part or not.

If you decide you want to take part in the research project, you will be asked to sign the consent section. By signing it you are telling us that you:

- understand what you have read;
- consent to take part in the research project;
- consent to participate in the research processes that are described;
- consent to the use of your personal and health information as described

You will be given a copy of this Participant Information and Consent Form to keep.

2. What is the purpose of this research project?

Autoinflammatory diseases, also known as periodic fever syndromes, are an uncommon group of diseases that may present with recurrent fever and skin rashes. Patients with these conditions often have an over active immune system, with elevated levels of cytokines (signalling proteins) telling the body to respond to danger such as infection when there is no infection. There are four well-known autoinflammatory diseases that have an underlying genetic cause that we can test for in Australia. In recent years, more conditions with features of an overactive immune system have been described. These newer conditions were diagnosed through genetic testing.

This project aims to look at the genes in your cells to see if there is a change that may explain why your immune system is not functioning normally. Genes are made up of DNA and RNA. These are molecules that carry the genetic information in our cells. Genes are inherited from our parents. Genes provide the information that determines our physical features such as hair and eye colour, and how our bodies function. Differences in our genes help explain why we are all different. Sometimes genes can be altered. The alterations to these genes can sometimes cause a specific disease or make a person more likely to develop a specific disease. The research we wish to conduct could identify alterations in genes that may be responsible for or contributing to your condition. In order to do this, we want to look at the genetic material in both your as well as your blood relatives' cells. If we find a mutation that we think may be causing your disease, we would go on to do testing in the laboratory to see if it causes changes in the signalling protein levels.

We would also like to create a database of all patients with autoinflammatory diseases in Australia, including patients without a confirmed diagnosis. This may help us find patients around the country who have the same symptoms as you now or in the future, and see if there are similarities in your test results.

3. What does participation in this research project involve?

Participation in this project will involve you providing a blood sample. This may be collected at the same time that you are scheduled to have blood tests to either diagnose your medical condition or for the ongoing clinical management of your condition. The amount of blood taken (approximately two teaspoons) will have no negative effects on your health. A trained phlebotomist will take the blood sample from a vein in your arm.

In the case where you have already had whole exome sequencing performed but haven't had the results analysed, you can still consent to be enrolled and your data will be obtained through your clinician.

We will also access your medical history to obtain information about your illness. Your doctor will provide us with details about your suspected autoinflammatory condition. This information will be stored in a national database. If there are details that need to be clarified, the coordinating investigator will contact you to discuss this further. This will be done at your convenience.

To study whether your immune system disease is passed on in families, we would like to invite some of your blood relatives to participate in this study. If you agree for us to contact your blood relatives, we will ask you to provide their contact details.

4. What will happen to my test samples?

We will store samples of your blood, blood cells, tissue, DNA, RNA or protein extracts out of your blood cells for this research purpose. The samples will be marked only with a code number allocated to you for the study that is linked to your identity by a master key to the code. Your name or other identifying information will not be recorded on the blood samples. The blood samples will be stored and analysed in the Inflammation Department at the Walter and Eliza Hall Institute.

The lymphocytes will be collected from your blood and studied in the laboratory. DNA will be obtained from cells and used to identify potentially disease-related genes. If your blood relatives participate in this study, the genetic analysis we conduct may reveal non-paternity or non-maternity (be able to tell that someone is not the biological parent of a child). This information will not be disclosed to you or your family. However, in the rare circumstance where a heritable incidental finding is made, this may need to be disclosed.

We would like to store your samples, or what is left of the samples after this research project has finished, in a Tissue Bank for its use in future research projects that are in the same area of research ie. analysis of the immune system in immunological diseases. The name of the Tissue Bank is the Australian Autoinflammatory Diseases RegistrY (AADRY) and it will be stored in a secure freezer in the Inflammation Division at the Walter + Eliza Hall Institute of Medical Research. Dr Seth Masters is the custodian of the Tissue Bank.

We cannot say exactly what projects your samples will be used for in the future, but they may include the study of the genetic material inside your cells while others may include the study of the proteins produced by those cells. Rapid advances in technology make it impossible to predict what new tests or studies may be possible in the future. Any future research projects will have to be approved by a Human Research Ethics Committee before your samples can be used. However, your samples will always be provided to the researchers in a coded form to ensure your confidentiality. This may include collaborators and researchers interstate and overseas. You will not be asked for further consent for your samples to be used in such future research, nor will you receive any results from any future research.

If consent for future research use is declined your samples will be disposed of following completion of this research project.

5. What are the possible benefits?

You will have access to a research investigative method that is not in routine clinical practice, and may receive information on or a diagnosis for your suspected autoinflammatory disease. However, we cannot guarantee or promise that this will occur.

6. What are the possible risks?

Having a blood sample taken may cause some discomfort or bruising. Sometimes, the blood vessel may swell, or blood may clot in the blood vessel, or the spot from which tissue is taken could become inflamed. Rarely, there could be a minor infection or bleeding. If this happens, it can be easily treated.

We are looking for mutations in genes that are involved in the immune system in some way. It is unlikely that the testing on your samples will produce any other information (incidental findings) that will have an effect on your future health or treatment of the future health of your family. However, if this is the case your doctor will contact you. If you choose to be informed of this result, arrangements will be made for you to receive this information and any counselling that may be required. This information may also be of potential importance to the future health of your blood relatives. However, it is important to understand that genetic research may only show an increased risk of developing a disease or disorder and not that it actually will develop.

You should be aware that if you receive results of your genetic testing that is significant with regards to your health or future treatment you may be obliged to inform your health or life insurer. Such information may affect your ability to maintain or obtain health or life insurance in the future. Disclosure of participation in a genetic research project is not required except in those projects where a personal result has been given.

7. Do I have to take part in this research project?

Participation in any research project is voluntary. If you do not wish to take part, you don't have to. If you decide to take part and later change your mind, you are free to withdraw from the project at any stage.

Your decision whether to take part or not to take part, or to take part and then withdraw, will not affect your routine treatment or your relationship with those treating you.

8. What if I withdraw from this research project?

If you decide to withdraw, please notify a member of the research team before you withdraw. If you do choose to withdraw from the study the investigators will continue to use information gained from tests that have already been done on your blood sample. This is done to ensure that the results of the study are true and not biased. However, no further testing of your sample will be done nor information collected from the date of withdrawal.

9. Could this research project be stopped unexpectedly?

The research project could be stopped unexpectedly. However, this would not affect your medical treatment.

10. How will I be informed of the results of this research project?

At the end of this study when the results are known you will be given a written plain English summary of the outcomes of the research upon request.

11. What else do I need to know?

- **What will happen to information about me?**

Any information obtained in connection with this research project that can identify you will remain confidential and will not be disclosed unless required by law. The information collected (data) will be identified using a code number, which will be accessible only to the study team. The key to the code will not be given to other researchers in the future. Data will be recorded in a computer database. Any paper data that is generated will be stored in locked filing cabinets and on password-protected databases (for electronic records). Although unlikely, any results that impact on your health or future treatment will be added to your medical record.

Your data will be stored in a database in the Department of Inflammation at the Walter + Elisa Hall Institute in Melbourne. It will be made available to other researchers conducting approved research in research similar to this project only. We intend on keeping the information indefinitely but it will be retained for a minimum of 7 years following completion of research project.

We intend to publish the results of this research. In any publication, information will be provided in such a way that you cannot be identified.

Your medical record and any information obtained during the study are subject to inspection, for the purpose of verifying study procedures and the data, by the Monash Health Human Research Ethics Committee or as required by law.

- **How can I access my information?**

In accordance with relevant Australian and/or Victorian privacy and other relevant laws, you have the right to access the information collected and stored by the researchers about you. You also have the right to request that any information, with which you disagree, be corrected. Please contact one of the researchers named at the end of this document if you would like to access your information.

- **What happens if I am injured as a result of participating in this research project?**

If you suffer any injuries or complications as a result of this research project, you should contact the study team as soon as possible and you will be assisted with arranging appropriate medical treatment. If you are eligible for Medicare, you can receive any medical treatment required to treat the injury or complication, free of charge, as a public patient in any Australian public hospital.

- **Is this research project approved?**

The ethical aspects of this research project have been approved by the Monash Health Human Research Ethics Committee.

This project will be carried out according to the *National Statement on Ethical Conduct in Human Research* (2007) produced by the National Health and Medical Research Council of Australia. This statement has been developed to protect the interests of people who agree to participate in human research studies.

12. Consent

I have read, or have had read to me in a language that I understand, this document and I understand the purposes, procedures and risks of this research project as described within it.

I give permission for my doctors, other health professionals, hospitals or laboratories outside this hospital to release information to the Walter and Eliza Hall Institute of Medical Research concerning my disease and treatment that is needed for this project. I understand that such information will remain confidential.

I have had an opportunity to ask questions and I am satisfied with the answers I have received.

I freely agree to participate in this research project as described.

I understand that I will be given a signed copy of this document to keep.

I consent to the storage and use of my health information in the form of a registry:

☐ Initial:……Date:……

I consent to the storage and use of my blood, tissue samples and whole exome sequence results taken from me for use in:

☐ this specific research project only Initial:……Date:……

OR

☐ this research project and other research that is closely related to this research project

Initial:……Date:……

I wish to be informed of any significant genetic results generated by this research:

☐ Yes Initial:……Date:……

OR

☐ No Initial:……Date:……

Participant's name (printed) ……

Signature **Date**

Witness (Required when participant cannot read this document except when English is not their preferred language.)

Name of witness ……

Signature Date

Interpreter (Required when English is not the participant's preferred language)

Name of interpreter ……

Signature Date

Declaration by researcher*: I have given a verbal explanation of the research project, its procedures and risks and I believe that the participant has understood that explanation.

Researcher's name (printed) ……

Signature Date

** A senior member of the research team must provide the explanation and provision of information concerning the research project.*

Note: All parties signing the consent section must date their own signature.

13. Who can I contact?

Who you may need to contact will depend on the nature of your query, therefore, please note the following:

For further information or appointments:

If you want any further information concerning this project you can contact the principal researcher Dr Fiona Moghaddas or the clinical coordinator Ms. Jenni Harris on (03) 9345 2555.

For complaints:

If you have any complaints about any aspect of the project, the way it is being conducted or any questions about being a research participant in general, then you may contact:

Name:

Position:

Telephone:

Reviewing HREC contact details:

Name: Ms Deborah Dell

Position: Manager, Human Research Ethics Committee

Telephone: 9594 4611

Participant Information and Consent Form for Family

Full Project Title:

Australian Autoinflammatory Diseases RegistrY (AADRY): A national approach to the genetic and immunological evaluation of patients with suspected autoinflammatory syndromes

HREC No: **HREC/15/MonH/31**

Principal Researchers: Dr Fiona Moghaddas, Dr Seth Masters, Professor Ian Wicks

1. Introduction

You are invited to take part in this research project as a family member is currently being investigated for or is diagnosed with an autoinflammatory disease. This Participant Information and Consent Form tells you about the research project and explains the procedures involved. Knowing what is involved will help you decide if you want to take part in the research.

Please read this information carefully. Ask questions about anything that you don't understand or want to know more about. Before deciding whether or not to take part, you might want to talk about it with a relative, friend or healthcare worker.

Participation in this research is voluntary. If you don't wish to take part, you don't have to. You will receive the best possible care whether you take part or not.

If you decide you want to take part in the research project, you will be asked to sign the consent section. By signing it you are telling us that you:

- understand what you have read;
- consent to take part in the research project;
- consent to participate in the research processes that are described;
- consent to the use of your personal and health information as described

You will be given a copy of this Participant Information and Consent Form to keep.

2. What is the purpose of this research project?

Autoinflammatory diseases, also known as periodic fever syndromes, are an uncommon group of diseases that may present with recurrent fever and skin rashes. Patients with these conditions often have an over active immune system, with elevated levels of cytokines (signalling proteins) telling the body to respond to danger such as infection when there is no infection. There are four well-known autoinflammatory diseases that have an underlying genetic cause that we can test for in Australia. In recent years, more conditions with features of an overactive immune system have been described. These newer conditions were diagnosed through genetic testing.

Genes are made up of DNA and RNA. These are molecules that carry the genetic information in our cells. Genes are inherited from our parents. Genes provide the information that determines our physical features such as hair and eye colour, and how our bodies function. Differences in our genes help explain why we are all different. Sometimes genes can be altered. The alterations to these genes can sometimes cause a specific disease or make a person more likely to develop a specific disease. The research we wish to conduct could identify alterations in genes that may be responsible for your relative's condition. In order to do this, we want to look at the genetic material in both your as well as your relative's cells. If we find a mutation that we think may be causing or contributing to their disease, we would go on to do testing in the laboratory to see if it causes changes in the signalling protein levels.

3. What does participation in this research project involve?

Participation in this project will involve you donating 5 ml (one tablespoon) of saliva for research purposes. Your saliva contains skin cells from the inside of your mouth. Skin cells, like the rest of the cells in your body, contain a complete set of your DNA. Since it is so easy to collect saliva, this is a good source of DNA to use for genetic

testing.

4. What will happen to my test samples?

We will store samples of your DNA, RNA or protein extracts out of your cells for this research purpose. The samples will be marked only with a code number allocated to you for the study that is linked to your identity by a master key to the code. Your name or other identifying information will not be recorded on the blood samples. The blood samples will be stored and analysed in the Inflammation Department at the Walter and Eliza Hall Institute.

The genetic analysis we conduct may reveal non-paternity or non-maternity (be able to tell that someone is not the biological parent of a child). This information will not be disclosed to you or your family. However, in the rare circumstance where a heritable incidental finding is made, this may need to be disclosed.

We would like to store what is left of the samples after this research project has finished, in a Tissue Bank for its use in future research projects that are in the same area of research ie. analysis of the immune system in immunological diseases.

The name of the Tissue Bank is the Australian Autoinflammatory Diseases RegistrY and it will be stored in a secure freezer in the Inflammation Division at the Walter + Eliza Hall Institute of Medical Research. Dr Seth Masters is the custodian of the Tissue Bank.

We cannot say exactly what projects your samples will be used for in the future, but they may include the study of the genetic material inside your cells while others may include the study of the proteins produced by those cells. Rapid advances in technology make it impossible to predict what new tests or studies may be possible in the future. Any future research projects will have to be approved by a Human Research Ethics Committee before your samples can be used. However, your samples will always be provided to the researchers in a coded form to ensure your confidentiality. This may include collaborators and researchers interstate and overseas. You will not be asked for further consent for your samples to be used in such future research, nor will you receive any results from any future research.

If consent for future research use is declined your samples will be disposed of following completion of this research project.

5. What are the possible benefits?

We cannot guarantee or promise that there will be any benefit to you from your participation in this study. However, your participation may help your blood relative, or to further medical knowledge and may improve diagnosis and treatment of immune-based diseases in the future.

6. What are the possible risks?

We are looking for mutations in genes that are involved in the immune system in some way. It is unlikely that the testing on your samples will produce any other information (incidental findings) that will have an effect on your future health or treatment of the future health of your family. However, if this is the case your doctor will contact you. If you choose to be informed of this result, arrangements will be made for you to receive this information and any counselling that may be required. This information may also be of potential importance to the future health of your blood relatives. However, it is important to understand that genetic research may only show an increased risk of developing a disease or disorder and not that it actually will develop.

You should be aware that if you receive results of your genetic testing that is significant with regards to your health or future treatment you may be obliged to inform your health or life insurer. Such information may affect your ability to maintain or obtain health or life insurance in the future. Disclosure of participation in a genetic research project is not required except in those projects where a personal result has been given.

7. Do I have to take part in this research project?

Participation in any research project is voluntary. If you do not wish to take part you don't have to. If you decide to take part and later change your mind, you are free to withdraw from the project at any stage.

Your decision whether to take part or not to take part, or to take part and then withdraw, will not affect your or your relative's routine treatment or relationship with the treating team.

8. What if I withdraw from this research project?

If you decide to withdraw, please notify a member of the research team before you withdraw. If you do choose to withdraw from the study the investigators will continue to use information gained from tests that have already been done on your sample. This is done to ensure that the results of the study are true and not biased. However, no further testing of your sample will be done nor information collected from the date of withdrawal.

9. Could this research project be stopped unexpectedly?

The research project could be stopped unexpectedly. However, this would not affect your medical treatment.

10. How will I be informed of the results of this research project?

At the end of this study when the results are known you will be given a written plain English summary of the outcomes of the research upon request.

11. What else do I need to know?

- **What will happen to information about me?**

Any information obtained in connection with this research project that can identify you will remain confidential and will not be disclosed unless required by law. The information collected (data) will be identified using a code number, which will be accessible only to the study team. The key to the code will not be given to other researchers in the future. Data will be recorded on paper and in a computer database. Data will be stored in locked filing cabinets (for paper records) and on password-protected databases (for electronic records). Although unlikely, any results that impact on your health or future treatment will be added to your medical record.

Your data will be stored in a database in the Department of Inflammation at the Walter + Elisa Hall Institute in Melbourne. It will be made available to other researchers conducting approved research in research similar to this project only.

We intend to publish the results of this research. In any publication, information will be provided in such a way that you cannot be identified.

Your medical record and any information obtained during the study are subject to inspection, for the purpose of verifying study procedures and the data, by the Melbourne Health Human Research Ethics Committee or as required by law.

- **How can I access my information?**

In accordance with relevant Australian and/or Victorian privacy and other relevant laws, you have the right to access the information collected and stored by the researchers about you. You also have the right to request that any information, with which you disagree, be corrected. Please contact one of the researchers named at the end of this document if you would like to access your information.

- **What happens if I am injured as a result of participating in this research project?**

If you suffer an injury as a result of participating in this research project, hospital care and treatment will be provided by the public health service at no extra cost to you if you elect to be treated as a public patient.

- **Is this research project approved?**

The ethical aspects of this research project have been approved by the Monash Health Human Research Ethics Committee.

This project will be carried out according to the *National Statement on Ethical Conduct in Human Research* (2007) produced by the National Health and Medical Research Council of Australia. This statement has been developed to protect the interests of people who agree to participate in human research studies.

12. Consent

I have read, or have had read to me in a language that I understand, this document and I understand the purposes, procedures and risks of this research project as described within it.

I give permission for my doctors, other health professionals, hospitals or laboratories outside this hospital to release information to the Walter and Eliza Hall Institute of Medical Research concerning my health that is needed for this project. I understand that such information will remain confidential.

I have had an opportunity to ask questions and I am satisfied with the answers I have received.

I freely agree to participate in this research project as described.

I understand that I will be given a signed copy of this document to keep.

I consent to the storage and use of tissue samples taken from me for use in:

☐ this specific research project only Initial:……..Date:……

OR

☐ this research project and other research that is closely related to this research project Initial:……Date:……

I wish to be informed of any significant genetic results generated by this research:

☐ Yes Initial:……Date:……

OR

☐ No Initial:……Date:……

Participant's name (printed) ……

Signature **Date**

Witness (Required when participant cannot read this document except when English is not their preferred language.)

Name of witness ……

Signature Date

Interpreter (Required when English is not the participant's preferred language)

Name of interpreter ……

Signature Date

Declaration by researcher*: I have given a verbal explanation of the research project, its procedures and risks and I believe that the participant has understood that explanation.

Researcher's name (printed) ……

Signature Date

** A senior member of the research team must provide the explanation and provision of information concerning the research project.*

Note: All parties signing the consent section must date their own signature.

13. Who can I contact?

Who you may need to contact will depend on the nature of your query, therefore, please note the following:

For further information or appointments:

If you want any further information concerning this project you can contact the principal researcher Dr Fiona Moghaddas or the clinical coordinator Ms. Jenni Harris on (03) 9345 2555.

For complaints:

If you have any complaints about any aspect of the project, the way it is being conducted or any questions about being a research participant in general, then you may contact:

Name:

Position:

Telephone:

Reviewing HREC contact details:

Name: Ms Deborah Dell

Position: Manager, Human Research Ethics Committee

Telephone: 9594 4611

8.6 Appendix 6: AADRY data entry forms

Demographics

Study ID

Consent

	Yes	No
Database		
Exome sequencing		
Tissue banking		

Date subject signed consent

Contact information

First name

Last name

Street, City, State, Postcode

Phone number

Email

Date of birth

Age (years)

Gender

Country of birth

Ethnicity

Comments

Clinical disposition

Age of symptom onset (months)	
Comorbidities	
Clinical disposition	Genetically confirmed
	Clinically confirmed
	Clinically suspected

Previous genetic testing

Gene	Normal	Heterozygous mutation	Homozygous mutation	Not done
NLRP3				
MEFV				
MVK				
TNFRSF1A				
Other				

Laboratory	
Other genes tested	

Family details

Consanguinity	Yes	No	Not known	

Relation	Affected	Not affected	Not applicable	Not known
Mother				
Father				
Sibling(s)				
Other				

Details	

Febrile episodes

Temperature > 38°C	Yes	No		
Average duration (days)	<2	2-5	5-10	>10
Number of episodes per year	<2	2-6	6-12	>12
Fever during summer	Yes	No		
Disease course	Continuous	Recurrent	Continuous and recurrent	

Clinical manifestations

Mucocutaneous manifestations Yes No Not known

	Always	**Often**	**Sometimes**	**Never**
Oral aphthosis				
Genital aphthosis				
Oral herpetic like lesions				
Exudative pharyngitis				
Maculopapular rash				
Urticarial rash				
Pseudofolliculitis				
Other				

Details

Musculoskeletal manifestations Yes No Not known

	Always	**Often**	**Sometimes**	**Never**
Arthralgia				
Myalgia				
Arthritis				
Other				

Details

Ocular manifestations Yes No Not known

	Always	**Often**	**Sometimes**	**Never**
Periorbital oedema				
Periorbital pain				
Conjunctivitis				
Other				

Details

Gastrointestinal manifestations Yes No Not known

	Always	**Often**	**Sometimes**	**Never**
Abdominal pain				
Diarrhea				
Constipation				
Vomiting				
Other				

Details

Cardiorespiratory manifestations Yes No Not known

	Always	**Often**	**Sometimes**	**Never**
Thoracic pain				
Pleurisy				
Pericarditis				
Other				

Details

Haematological manifestations Yes No Not known

	Always	**Often**	**Sometimes**	**Never**
Lymphadenopathy				
Pain at lymph node				
Splenomegaly				
Other				

Details

Other manifestations Yes No Not known

	Always	**Often**	**Sometimes**	**Never**
Headache				
Fatigue				
AA amyloidosis				
Other				

Details

Investigations

Test	Normal	Abnormal	Unknown
Acute phase reactants (baseline)			
Acute phase reactants (during episode)			
Routine blood investigations			
FBE			
UEC			
LFT			
Coag profile			
Other			

Details

Immunological investigations

Test	Normal	Abnormal	Unknown
Neutrophil function			
T cell function/workup			
B cell function/workup			
Other			

Details

Imaging

Details

Treatment

Current therapy

Date commenced

Previous therapy 1

Previous therapy 2

Previous therapy 3

Medication	Full response	Partial response	No response	Unknown
Current therapy				
Previous therapy 1				
Previous therapy 2				
Previous therapy 3				

8.7 Appendix 7: ACMG variant classification

Table 1: Classification of PTV and Null variants

Evidence (NOTE: Each evidence block as signified by continuous color can only have ONE yes checked)	Cat.	Yes	No	Not available	Not applicable
1.1.1 Protein truncating (PTV) or Null Variant in a gene where LOF is known mechanism of pathogenicity (e.g. through functional assay or if truncating variants have been shown to segregate in multiple families) - E.g. frameshift, initiator codon disruption, premature stop codon (nonsense), canonical splice site mutation - IF Canonical splice site (intronic 1-2bp upstream/downstream of exon-intron boundary), functional data required on protein or RNA level - IF initiator codon (ATG) mutation, no known alternative transcription start sites allowing functional protein production AND next available initiator codon (if any) results in frameshift - Affects critical functional domains - Novel Stop codon is not in the last exon or the last 50bp of the second to last exon - Region harboring the variant is not known to be subject to alternative splicing/missing in alternative RefSeq transcripts	PVS				
1.1.2 Affects canonical splice site (within 2bp of exon-intron boundary) WITHOUT functional data supporting splice effect on protein or RNA level	PS				
1.1.3 PTV/Null Variant where alternative splicing of the exon occurs BUT there is strong evidence for the exon to be PRESENT in the transcript relevant for the disease of interest (e.g. cardiac specific transcrpt in cardiomyopathy) AND where LOF is known mechanism of pathogenicity	PS				
1.1.4 PTV/Null Variant where alternative splicing of the exon occurs AND there is strong evidence for the exon to be ABSENT in the transcript relevant for the disease of interest (e.g. cardiac specific transcrpt in cardiomyopathy)	BS				
1.1.5 PTV/Null Variant where alternative splicing is proposed to occur BUT supporting evidence for alternative splicing of the exon is not well established AND in a gene where LOF is known mechanism of pathogenicity	PM				
1.1.6 Novel Protein truncating (PTV) or Null Variant in the last exon or the last 50bp of the second to last exon in a gene with less than 4 exons AND where LOF is known mechanism of pathogenicity	PM				
1.2.1 MAF >=0.05 in at least one sufficiently large subpopulation	BA				
1.2.2 MAF >=0.03 and <0.05 in at least one sufficiently large subpopulation OR MAF is out of keeping with known disease frequency. CAUTION: if similar truncating mutations are common in the same gene (~>0.005, e.g. TITAN, APC), to be used as supporting evidence (BP) only.	BS				
1.2.3 IF dominant disorder MAF >0.01 in at least one sufficiently large subpopulation	BS				
1.3.1 In-silico predicted effect on splicing by at least two tools (NNSplice, HSF3) in gene where splice defects are known pathogenic mechanism and nucleotide position is conserved (NOT FOR CANONICAL splice site intronic 1-2bp upstream/downstream of exon-intron boundary)	PP				
1.3.2 In-silico predicted effect on splicing by at least two tools (NNSplice, HSF3) in gene where LOF is known mechanism of pathogenicity and alternative splicing would cause a frame-shift mutation (NOT FOR CANONICAL splice site intronic 1-2bp upstream/downstream of exon-intron boundary)	PM				
1.3.3 In-silico predicted effect on splicing by at least two tools (NNSplice, HSF3) where affected exon is missing in alternative transcripts (NOT FOR CANONICAL splice site intronic 1-2bp upstream/downstream of exon-intron boundary)	BP				
1.4.1 Variant affects at least one well established (essential) functional domain	PM				
1.5.1 In-frame insertion or deletion in repetitive region without known function or not conserved	BP				
1.5.2 In-frame insertion or deletion in non-repetitive region that is conserved in at least mammals and birds	PM				
1.6.1 Same variant type in affected codon/region has previously been shown to be pathogenic (IF in-frame Insertion/Deletion mutation, affecting same region; IF initiator codon mutation, different nucleotide change has been shown to be pathogenic; IF Premature stop with NMD: haploinsufficiency or complete loss of protein established pathogenic mechanism (beware of exceptions such as collagen disorders); IF Premature stop w/o NMD: premature stop downstream of variant is known pathogenic)	PS				
1.7.1 Heterozygous variant detected in *trans* with a second, known pathogenic, heterozygous variant in a recessive disease (Variant phasing may need to be confirmed by testing of parents)	PM				
1.7.2 Heterozygous variant observed in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in *cis* with a pathogenic variant in any inheritance pattern (Variant phasing may need to be confirmed by testing of parents)	BP				
1.8.1 Functional consequence of variant is not consistent with expected spectrum of pathogenic variants in the affected gene	BP				
1.9.1 Existing classification of pathogenicity (Variant has previously been classified by experts) NOTE: Can be seen as PVS if expert panel is known to have considered extensive additional information that is not available to the lab (i.e. InSight database, some BRCA gene classifications)	PS				
1.9.2 Existing classification of neutrality (Variant has previously been classified by experts) NOTE: Can be seen as BA if expert panel is known to have considered extensive additional information that is not available to the lab (i.e. InSight database, some BRCA gene classifications)	BS				
1.9.3 Previous description of pathogenicity (Variant has previously been described as pathogenic in databases or literature in independent patients)	PP				
1.9.4 Previous description of neutrality (Variant has previously been described as benign in databases or literature in independent patients)	BP				
1.10.1 Strong functional evidence supporting ABNORMAL protein function of the variant (eg. endogenous tissue)	PS				
1.10.2 Moderate functional evidence supporting ABNORMAL protein function of the variant (eg. exogenous cell line)	PM				
1.10.3 Strong functional evidence supporting NORMAL protein function of the variant (eg. endogenous tissue)	BS				
1.10.4 Moderate functional evidence supporting NORMAL protein function of the variant (eg. exogenous cell line)	BP				
1.11.1 Significant Segregation: Variant segregates with the same phenotype as the patient in 3 or more independent families or 1 large family to at least a third degree relative (LOD >= 3)	PS				
1.11.2 Moderate Segregation: Variant segregates with the same phenotype as the patient in 2 independent families only (LOD >=1.5 and <3)	PM				
1.11.3 Low Segregation: Variant segregates with the same phenotype as the patient in 1 family only OR homozygous variant in a recessive condition where both parents are confirmed to be carriers	PP				
1.11.4 Non-segregation has been clearly demonstrated	BS				
1.12.1 De Novo Variant (Parental status confirmed for both mother and father)	PS				
1.12.2 De Novo Variant (Parental status not tested but assumed)	PM				
1.13.1 Variant in a gene that is known to be causative for a well defined syndrome with unambigous clinical presentation. Gene has to have low tolerance for variation (EXAC Constraint LOF pLI >=0.9) and inheritance pattern of the variant has to be consistent with suspected disease.	PP				

Table 2: Classification of Missense Variants

Evidence (NOTE: Each evidence block as signified by continuous color can only have ONE yes checked)	Cat.	Yes	No	Not available	Not applicable
2.1.1 MAF for dominant indication <0.0002 or absent in databases with sufficient coverage	PS				
2.1.2 MAF for dominant indication >=0.0002 and <0.001	PM				
2.1.3 MAF for recessive indication <0.01 (use caution with minority ethnicities)	PM				
2.1.4 MAF for recessive indication <0.03 and >=0.01 (use caution with minority ethnicities)	PP				
2.1.5 MAF >=0.05 in at least one sufficiently large subpopulation	BA				
2.1.6 MAF >=0.03 and <0.05 in at least one sufficiently large subpopulation OR MAF is out of keeping with known disease frequency	BS				
2.1.7 IF dominant disorder MAF >=0.01 in at least one sufficiently large subpopulation	BS				
2.1.8 MAF is out of keeping with known disease frequency OR Homozygous mutations are present in ExAc at a higher frequency than expected for the disease (e.g. homozygous variant present in ExAc when investigating lethal recessive early onset childhood condition)	BS				
2.2.1 Variant region conserved in at least mammals and birds with Grantham score > 100	PM				
2.2.2 Variant region conserved in at least mammals and birds with Grantham score > 65	PP				
2.2.3 Variant region not conserved in at least mammals and birds with Grantham score < 65	BP				
2.3.1 In-silico consistently supports pathogenicity (SIFT, PolyPhen2, Mutation Taster, CADD)	PP				
2.3.2 In-silico consistently supports neutrality (SIFT, PolyPhen2, Mutation Taster, CADD)	BP				
2.4.1 Variant is located in well established, critical functional region without benign variation	PP				
2.5.1 Variant is located in exon with strong evidence for alternative splicing where the exon is known NOT to be present in tissue relevant transcript for the disease of interest	BS				
2.6.1 Heterozygous variant detected in *trans* with a second, known pathogenic, heterozygous variant in a recessive disease (Variant phasing may need to be confirmed by testing of parents)	PM				
2.6.2 Heterozygous variant observed in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in *cis* with a pathogenic variant in any inheritance pattern (Variant phasing may need to be confirmed by testing of parents)	BP				
2.7.1 Novel missense change in a codon where a different amino acid change has previously been described as pathogenic	PP				
2.8.1 Functional consequence of variant is not consistent with expected spectrum of pathogenic variants in the affected gene. NOTE: Can be seen as BS depending on level of evidence	BP				
2.9.1 Existing classification of pathogenicity (Variant has previously been classified by experts) NOTE: Can be seen as PVS if expert panel is known to have considered extensive additional information that is not available to the lab (i.e. InSight database, some BRCA gene classifications)	PS				
2.9.2 Existing classification of neutrality (Variant has previously been classified by experts) NOTE: Can be seen as BA if expert panel is known to have considered extensive additional information that is not available to the lab (i.e. InSight database, some BRCA gene classifications)	BS				
2.9.3 Previous description of pathogenicity (Variant has previously been described as pathogenic in databases or literature in independent patients)	PP				
2.9.4 Previous description of neutrality (Variant has previously been described as benign in databases or literature in independent patients)	BP				
2.10.1 Strong functional evidence supporting ABNORMAL protein function of the variant (eg. endogenous tissue)	PS				
2.10.2 Moderate functional evidence supporting ABNORMAL protein function of the variant (eg. exogenous cell line)	PM				
2.10.3 Strong functional evidence supporting NORMAL protein function of the variant (eg. endogenous tissue)	BS				
2.10.4 Moderate functional evidence supporting NORMAL protein function of the variant (eg. exogenous cell line)	BP				
2.11.1 Significant Segregation: Variant segregates with the same phenotype as the patient in 3 or more families or 1 large family to at least a third degree relative (LOD >= 3)	PS				
2.11.2 Moderate Segregation: Variant segregates with the same phenotype as the patient in 2 families only (LOD >=1.5 and <3)	PM				
2.11.3 Low Segregation: Variant segregates with the same phenotype as the patient in 1 family only OR homozygous variant in a recessive condition where both parents are confirmed to be carriers	PP				
2.11.4 Non-segregation has been clearly demonstrated	BS				
2.12.1 De Novo Variant (Parental status confirmed for both mother and father)	PS				
2.12.2 De Novo Variant (Parental status not tested but assumed)	PP				
2.13.1 Variant in a gene that is known to be causative for a well defined syndrome with unambigous clinical presentation. Gene has to have low tolerance for variation (ExAC Constraint Missense z >= 2) and inheritance pattern of the variant has to be consistent with suspected disease.	PP				

Count Evidence Levels (Categories):

Category	Count
PVS	
PS	
PM	
PP	
BA	
BS	
BP	

Worksheet classification:

Reason for change of Classification weight

NOTE: The classification formulas below represent a MINIMUM requirement for a given class. The curator has to decide if conflicting evidence is sufficient to justify VUS classification. E.g. 1BS + 3BP + 1PM = 2 - Likely benign, 1BS + 2BP + 1PS + 2PP = 3b - VUS

5- Pathogenic:	4 - Likely Pathogenic:	3a - VUS (potentially pathogenic)	3b - VUS	3c - VUS (potentially benign)	2 - Likely Benign	1 - Benign:
2 PVS	1 PVS and >= 1 PM	Not unambigously classifiable with predominantly pathogenic evidence	Not unambigously classifiable	Not unambigously classifiable with predominantly benign evidence	1 BS + >= 1 BP	>= 1 BA
1 PVS and > 1 PS	1 PVS and >= 4 PP				>= 2 BP	>= 2 BS
1 PVS and >= 2 PM	1 PS and >2 PM					
1 PVS and 1 PM and >= 2 PP	1 PS and 1 PM and > 2 PP					
>= 2 PS	1 PS and >= 3 PP					
1 PS and >=3 PM	>= 3 PM					
1 PS and 2 PM and >= 2 PP	2 PM and >= 2 PP					
1 PS and 1 PM and >= 4 PP	1 PM and >= 4 PP					

Category	Description
PVS	Pathogenic very strong
PS	Pathogenic strong
PM	Pathogenic moderate
PP	Pathogenic supporting
BS	Benign strong
BP	Benign supporting
BA	Benign stand alone

8.8 Appendix 8: Curation of gene list variants

						Details				
	Gene	Variant	Classification	Ensemble ID	Zygosity	OMIM reference	Zygosity	Disease mechanism	ExAC constraint	.%ExAC_RVIS score
1	ACP5	R272C	3a	ENST00000592828	HET	171640	AR	Loss of function	Y	77.35
2	ADAM17	V673I	3c	ENST00000310823	HET	603639	AR	Loss of function	Y	18.9
3	ADAM17	Q30R	3c	ENST00000310823	HET	603639	AR	Loss of function	Y	18.9
4	AP1S3	F4C	3a	ENST00000396654	HET	616106	AD	Association only	Y	81.21
5	AP1S3	R33W	3a	ENST00000396654	HET	616107	AD	Association only	Y	81.21
6	AP1S3	T32I	1	ENST00000396654	HET	616108	AD	Association only	Y	81.21
7	CARD14	P506L	1	ENST00000573882	HET	173200, 602723	AD	Gain of function	Y	95.48
8	CARD14	R682W	3c	ENST00000573882	HET	173200, 602723	AD	Gain of function	Y	95.48
9	CECR1	V349I	3c	ENST00000399839	HET	615688	AR	Loss of function	Y	47.17
10	IFIH1	D112G	3a	ENST00000263642	HET	606951	AD	Gain of function	N	92.98
11	IL10	G15R	2	ENST00000423557	HET		AR	Loss of function	Y	68.97
12	IL10RB	V148M	3c		HET	612567	AR	Loss of function	Y	73.99
13	IL36RN	N47S	3b	ENST00000393200	HET	614204	AR	Loss of function	Y	84.8
14	LPIN2	D891N	3c	ENST00000261596	HET	609628	AR	Unknown	Y	16.22
15	LPIN2	E601K	2	ENST00000261596	HET	609628	AR	Unknown	Y	16.22
16	NLRP1	G106R	3c	ENST00000572272	HET	606636	AD	Gain of function	Y	92.05
17	NLRP1	V939M	3c	ENST00000572272	HET	606636	AD	Gain of function	Y	92.05
18	NLRP1	T656M	3a	ENST00000572272	HET	606636	AD	Gain of function	Y	92.05
19	NLRP12	R971G	3c	ENST00000324134	HET	611762	AD	Gain of function	Y	69.53
20	NLRP7	K511R	1	ENST00000588756	HET	231090	AR	Gain of function	Y	99.25
21	NLRP7	L311I	3b	ENST00000588756	HET	231090	AR	Gain of function	Y	99.25
22	NLRP7	Q310H	3b	ENST00000588756	HET	231090	AR	Gain of function	Y	99.25
23	NLRP7	Q310R	3b	ENST00000588756	HET	231090	AR	Gain of function	Y	99.25
24	NLRP7	R156Q	2	ENST00000588756	HET	231090	AR	Gain of function	Y	99.25
25	NOD2	G908R	3b	ENST00000300589	HET	186580	AD	Gain of function	Y	97.81
26	NOD2	R684W	3c	ENST00000300589	HET	186580	AD	Gain of function	Y	97.81
27	NOD2	R703C	3b	ENST00000300589	HET	186580	AD	Gain of function	Y	97.81
28	NOD2	S431L	3a	ENST00000300589	HET	186580	AD	Gain of function	Y	97.81
29	NOD2	V793M	3c	ENST00000300589	HET	186580	AD	Gain of function	Y	97.81
30	NOD2	L1007Pfs*2	3c	ENST00000300589	HET	186580	AD	Gain of function	Y	97.81
31	PLCG2	I671V	3b	ENST00000359376	HET	614878, 614468	AD	Both	Y	16.17
32	PLCG2	M28L	3c	ENST00000359376	HET	614878, 614468	AD	Both	Y	16.17
33	PLCG2	P522R	3c	ENST00000359376	HET	614878, 614468	AD	Both	Y	16.17
34	PSMB4	E197V	3a	ENST00000290541	HET		AR	Loss of function	Y	22.53
35	PSMB8	G8R	3b	ENST00000374882	HET	256040	AR	Loss of function	Y	55.04
36	PSMB9	V32I	3c	ENST00000374859	HET	177045	AR	Loss of function	Y	76.18
37	PSMB9	R173C	3b	ENST00000374859	HET	177045	AR	Loss of function	Y	76.18
38	RNASEH2A	D205E	3c	ENST00000221486	HET	606034	AR	Loss of function	Y	54.18
39	SAMHD1	V112I	3c	ENST00000262878	HET	606754	AR	Loss of function	Y	15.42
40	SAMHD1	D585N	3a	ENST00000262878	HET	606754	AR	Loss of function	Y	15.42
41	SAMHD1	P22S	3c	ENST00000262878	HET	606754	AR	Loss of function	Y	15.42
42	SH3BP2	A212V	3b	ENST00000503393	HET	118400	AD	Dom neg or gain of function	Y	60.18
43	TNFAIP3	I207L	3c	ENST00000237289	HET	616744	AD	Loss of function from truncation	Y	25.7
44	TNFAIP3	T108A	3b	ENST00000237289	HET	616744	AD	Loss of function from truncation	Y	25.7
45	TNFAIP3	T647P	2	ENST00000237289	HET	616744	AD	Loss of function from truncation	Y	25.7
46	TNFRSF1A	P75L	3c	ENST00000162749	HET	142680	AD	Abnormal processing	Y	41.99

	Insilico analysis								
Diff SNP in same codon	PolyPhen2	Interpret	SIFT	SIFT interpret	Mutation Taster	CADD	FATHMM	Weighted FATHMM	Unweighted FATHMM
Y	0.166999996	BENIGN	Deleterious	0.039999999	Polymorphism	11.48	Inconsistent	-0.48	-4.11
Y	0.990999997	PROBABLY_DAMAGING	Deleterious	0	Disease causing	22.5	Tolerated	1.73	-2.14
N	0.001	BENIGN	Tolerated	0.670000017	Polymorphism	11.73	Tolerated	2.13	0.13
N	1	Probably damaging	Damaging	0	Disease causing	29.5	Damaging		-3.71
N	1	Probably damaging	Damaging	0	Disease causing	33	Damaging		-5.68
N	0	Benign	Tolerated	1	Polymorphism	16.95	Tolerated		3.11
N	0	Benign			Polymorphism	4.58	Tolerated	1.84	0.11
N	1	Probably damaging			Disease causing	22.4	Inconsistent	3.25	-10.28
N	0.066	Benign	Tolerated	0.21	Polymorphism	3.36	Inconsistent	-3.62	-1.18
N	0.995999992	Probably damaging	Deleterious	0	Disease causing	22.3	Tolerated	3.37	-2.63
N	0.023	Benign	Tolerated	0.38	Polymorphism	9.46	Inconsistent	-0.62	-4.15
N	0.65	Possibly damaging	Tolerated	0.23	Polymorphism	13.03	Tolerated	1.4	
N	0.998	Probably damaging	Tolerated	0.08	Polymorphism	22	Tolerated	-0.93	1.3
N	0	Benign	Tolerated	0.14	Disease causing	22	Inconsistent	-1.52	-0.41
N	0.313	Benign	Tolerated	0.66	Disease causing	15.09	Tolerated	-1.41	-0.68
Y	0.096000001	BENIGN	Tolerated	0.150000006	Polymorphism	6.739	Tolerated	-0.53	-1.79
Y	0.973999977	PROBABLY_DAMAGING	Tolerated	0.100000001	Polymorphism	22.8	Tolerated	2.48	-2.61
Y	0	BENIGN	Deleterious	0.02	Polymorphism	15.4	Tolerated	-0.55	-2.68
Y	0.842	Possibly damaging	Tolerated	0.06	Polymorphism	17.1	Tolerated	0.64	-1.54
N	0.006	Benign			Polymorphism	0	Tolerated	-0.75	-1.03
N	0.68	Possibly damaging			Polymorphism	0.33	Tolerated	-1.16	-0.62
Y	0.971	Probably damaging			Polymorphism	0	Tolerated	-1.42	-2.12
Y	0.002	Benign			Polymorphism	0	Tolerated	-1.42	-0.77
Y	0.449	Benign			Polymorphism	15.33	Tolerated	-0.7	-0.84
Y	1	Probably damaging	Damaging	0.02	Disease causing	29.9	Tolerated	0.57	-2.02
N	0.01	Benign	Damaging	0.03	Polymorphism	16.15	Inconsistent	-0.47	-4.94
Y	0.999	Probably damaging	Damaging	0.02	Polymorphism	17.53	Inconsistent	-0.52	-4.57
N	0.886	Possibly damaging	Damaging	0.02	Polymorphism	12.01	Tolerated	-1.19	-2.44
Y	0.85	Possibly damaging	Tolerated	0.11	Disease causing	16.2	Tolerated	0.59	-2.11
Y					Disease causing				
Y	0	Benign	Tolerated	1	Disease causing	13.48	Inconsistent	-2.54	1.55
N	0.855	Possibly damaging	Tolerated	0.23	Disease causing	23.8	Tolerated	0.18	0.42
Y	0	Benign	Tolerated	0.09	Polymorphism	12.18	Tolerated	-0.15	-0.07
Y	0.605	Possibly damaging	Damaging	0.01	Disease causing	21.8	Inconsistent	1.8	-3.08
N	0.437	Benign	Tolerated	0.08	Disease causing	22.4	Tolerated	1.68	-2.13
Y	0.118000001	BENIGN	Deleterious	0.039999999	Disease causing	16.41	Tolerated	0.52	-2.58
Y	0.009	BENIGN	Tolerated	0.050000001	Disease causing	22.4	Inconsistent	1.31	-3.6
N	0.008	BENIGN	Tolerated	0.469999999	Disease causing	20.4	Inconsistent	-2.03	0.04
N	0	BENIGN	Tolerated	0.449999988	Polymorphism	0.002	Inconsistent	-3.55	0.41
N	0.081	BENIGN	Deleterious	0.02	Disease causing	11.36	Inconsistent	-3.82	-1.85
Y	0.222000003	BENIGN	Tolerated	0.119999997	Polymorphism	12.91	Inconsistent	-3.70	-1.07
Y	0.792	Possibly damaging			Polymorphism	22.3	Tolerated	-1.3	-2.57
Y	0.132	Benign	Damaging	0.01	Disease causing	15.07	Inconsistent	0.96	-4.77
N	0.001	Benign	Tolerated	1	Polymorphism	8.81	Tolerated	1.7	1.45
N	0	Benign	Tolerated	0.2	Polymorphism	13.37	Tolerated	1.9	-2.1
	0.999	Probably damaging	Tolerated	0.07	Polymorphism	10.82	Inconsistent	-3.28	-2.65

Population frequency						Conservation analysis			
1000G	dbSNP ref	dbSNP	ExAC	ExAC coverage	ExAC GnomAD	Homologene	Phylop	AGVGD	Grantham score
	rs147025508	0.006	0.002974	76.98	0.003	Low	0.688	C65	180
	rs61754177	0.018	0.015323	53.38	0.008447	High	5.748	C25	29
	rs775793715	0	4.9E-05	53.38	0.00002324	Low	-0.696	C45	43
0.003	rs116107386	0.016	0.007826	38.01	0.007952	Very high	4.819	C65	205
0.0022	rs78536455	0.007	0.00345	38.01	0.00767	High	1.755	C65	101
0.0024	rs138292988	0.016	0.00798	38.01	0.003547	Moderate	0.403	C65	89
0.0156	rs61751630	0.32	0.0165	39.67	0.01346	Low	0.298	C65	98
0.0048	rs117918077	0.022	0.01103	39.67	0.0114	High	5.508	C65	101
0.0008	rs74317375	0.004	0.00217	64.03	0.002144	Low	-0.84	C25	29
						High	3.624	C65	94
0.0002	rs145922845	0.004	0.001888	58.51	0.001834	Moderate	-0.335	C65	26
	rs45545138	0.002	0.0011	68.17	0.001082	Moderate	-0.351	C15	21
0.0132	rs28938777	0.009	0.0046	51.68	0.004474	Low	1.373	C45	46
0.0004	rs200648652	0.001	0.007899	62.34	0.0003428	Moderate	1.341	C15	23
0.0052	rs61735393	0.016	0.0003228	62.34	0.008543	High	2.579	C55	56
0.001	rs72827640	0.003	0.002878	63.13	0.002941	Low	-0.404	C65	125
0.0116	rs61754791	0.015	0.009146	63.13	0.01452	Low	0.293	C15	21
	rs769370941	0	2.6E-05	63.13	0.00006132	Low	0.486	C65	81
NA	rs753280591	0.000008252	0.000008252	68.22	0.00002165	Low	1.702	C65	43
0.0248	rs61743949	0.028	0.0141	67.92	0.01487	Moderate	-1.171	C25	26
0.0052	rs79513034	0.027	0.01346	67.92	0.01442	Low	-0.191	C0	5
0.0012	rs145973556	0.003	0.001618	67.92	0.001754	Low	1.601	C15	24
0.0052	rs77812009	0.027	0.01346	67.92	0.01442	Low	-0.317	C35	43
0.0016	rs61746625	0.014	0.007102	67.92	0.007351	Low	-1.429	C35	43
0.0046	rs2066845	0.02	0.0099	65.37	0.01085	High	3.42	C65	125
0.0002	rs5743276	0.001	0.0004	65.37	0.0003952	Low	0.885	C65	101
0.001	rs5743277		0.0033	65.37	0.003245	High	0.524	C65	180
0.0002	rs104895431	0.002	0.0008	65.37	0.0009437	High	0.949	C65	145
0.0002	rs104895444	0.002	0.0011	65.37	0.001123	High	2.737	C15	21
	rs5743293	0.021	0.01306	65.37	0.01533				
0.005	rs150833842	0.012	0.0061	58.14	0.005005	Very high	2.433	C25	29
0.0084	rs61749044	0.022	0.0111	58.14	0.0106	Very high	4.543	C0	15
0.0028	rs72824905	0.01	0.0051	58.14	0.005141	High	1.861	C65	103
0.0004	rs570825621		0.00003	84.67	0.00002165	Very high	4.548	C65	121
0.0168	rs114772012	0.037	0.019	65.81	0.02003	Very high	1.253	C65	125
0.0218	rs241419,CM065409	0.016	0.015553	56.53	0.01658	High	5.098	C25	29
0.0008	rs17213861	0.001	0.003553	56.53	0.003092	Low	1.17	C65	180
0.0084	rs62619782	0.011	0.010925	81.52	0.01101	Moderate	-0.263	C65	45
	rs144353824	0	0.000281	79.44	0.0003176	Low	-0.524	C25	29
	rs202165710,COSM368312	0	4.9E-05	79.44	0.00006871	High	3.621	C15	23
	rs772335453	0	8E-06	79.44	0.00001219	Low	3.116	C65	74
0.004	rs35313240	0.011	0.0056	46.76	0.005152	Moderate	3.186	C55	64
	rs141807543		0.0002	72.06	0.0001263	Moderate	1.242	C0	5
	rs376205580		0.0002	72.06	0.000123	Low	-0.02	C55	58
0.0008	rs142253225	0.004	0.0019	72.06	0.001789	Low	-0.311	C35	38
0.0282	rs4149637		0.0127	39.78	0.007643	Moderate	1.631	C65	76

Protein details				
Domain	SubRVIS percentiles	Previously Reported	ClinVar	Databases
MPP_ACP5 (163621).	92.06787		Y- 2 benign, 1 likely benign	N
No domain aligned (-)	88.81001		N	N
No domain aligned (-)	86.11403		N	N
No domain aligned (-)	99.08235		Y- risk factor	Y
No domain aligned (-)	99.08235		Y- likely benign, risk factor	Y
No domain aligned (-)	99.08235		N	N
No domain aligned (-)	22.34814		N	N
SH3_ZO (212793)	38.09064		Y- benign	N
ADGF (238646)	7.742231		Y- Conflicting interpretations of pathogenicity	Y
No domain aligned (-)	23.70144		N	N
IL10 (250087)	28.68066		N	N
No domain aligned	8.327562		N	N
IL1 (128430)	25.47195		N	Y
No domain alignment	16.283148		Y- 2 independent of uncertain significance	N
No domain alignment	5.712004		Y- 6 subissions. Benign/likely benign	Y- personal communication, psoriasis
No domain aligned (-)	98.53556		N	N
LRR_RI (238064)	29.06269		N	N
No domain aligned (-)	90.14656		N	N
LRR_RI (238064)	37.723688		N	N
No domain alignment	15.09741		Y-benign 1 submision	Y
NACHT	97.24816		N	Y
NACHT	97.24816		N	Y
NACHT	97.24816		N	Y
No domain alignment	80.02446		N	Y
LRR_RI (238064)	59.91544		Y-Likely benign(2);Uncertain significance(1)	Y
No domain aligned (-)	94.7862		Y- Likely benign (2)	Y- no alteration of nf-kb
No domain aligned (-)	94.7862		Y- Likely benign(3);Uncertain significance(1)	Y- Non alteration of the NF-kB response
NACHT (253353)	22.44923		Y- Likely benign (2)	Y- Activation of the NF-kB response
No domain aligned (-)	94.7862		Y- Likely benign (1)	Y- Non alteration of the NF-kB response
LRR_RI (238064)	59.91544		Y- Likely benign, risk factor	Y- Diminution of the PG-induced potential of NF-kB activation
SH2_C-SH2_PLC_gamma_like (198186)	19.50582		N	N
PH_PLC_gamma (241516)	33.057796		N	N
No domain aligned (-)	95.383816		N	N
proteasome_beta_type_4 (239729)	22.13702		N	N
No domain aligned (-)	92.30022		N	Y- but patient with pash
proteasome_beta_type_6 (239731)	95.28049		N	N
proteasome_beta_type_6 (239731)	95.28049		N	N
RNase_HII_eukaryota_like (260002)	60.88950			
No domain aligned (-)	41.192109		Y- Likely benign (2)	N
No domain aligned (-)	2.935591		N	
No domain aligned (-)	86.114028		N	
No domain aligned (-)	3.584035		Y- Benign (1) Likely benign(1)	N
OTU (251233)	93.69987		Y- no significance provided. Reported in cancer association study	N
OTU (251233)	93.69987		Y- no significance provided. Reported in cancer association study	N
No domain aligned (-)	89.30821		Y- no significance provided. Reported in cancer association study	N
TNFR (238109)	91.71433		Y- Likely benign (3), Benign (1)	Y

8.9 Appendix 9: Curation of de novo variants

					Details		
	Gene	Variant	Ensemble ID	Zygosity	OMIM reference	Disease mechanism	ExAC constraint
1	BTBD7	C245fs*Ter1132	ENST00000334746	R0.591:V0.409	NA	NA	N
2	C16orf78	R78Q	ENST00000299191	R0.481:V0.519	NA	NA	Y
3	CACNA2D2	E1058Ter	ENST00000424201	R0.417:V0.583	NA	NA	N
4	CAMTA2	S491P	ENST00000414043	R0.895:V0.105	NA	NA	0
5	CCRN4L	R62P	ENST00000280614	R0.727:V0.273	NA	NA	N
6	CLASP2	S378Ter	ENST00000468888	R0.500:V0.500	NA	NA	N
7	DDX31	R835K	ENST00000372159	R0.765:V0.235	NA	NA	N
8	DDX31	Q838K	ENST00000372159	R0.812:V0.188	NA	NA	N
9	DDX47	E413_insTTTTT	ENST00000358007	R0.882:V0	NA	NA	N
10	DLG2	R888W	ENST00000376104	R0.526:V0.474	NA	NA	N
11	EPPK1	V2291M	ENST00000525985	R0.899:V0.101	Cloned from patient with subepidermal blistering. Antigen in serum	NA	Y
12	FAM8A1	S153L	ENST00000259963	R0.800:V0.200	NA	NA	N
13	FAM8A1	L135P	ENST00000259963	R0.847:V0.153	NA	NA	N
14	FAM8A1	Q149R	ENST00000259963	R0.786:V0.214	NA	NA	N
15	FAM8A1	A140T	ENST00000259963	R0.804:V0.176	NA	NA	N
16	FAM8A1	G155S	ENST00000259963	R0.784:V0.216	NA	NA	Y
17	HLA-A	I218V	ENST00000396634	R0.816:V0.184	NA	NA	N
18	HLA-B	A182T	ENST00000412585	R0.816:V0.184	Susceptibility to drug sensitivity	NA	Y
19	HLA-DRB5	R54Q	ENST00000374975	R0.462:V0.538	NA	NA	Y
20	IFNA14	T179K	ENST00000380222	R0.800:V0.200	NA	NA	N
21	IFNA17	T179K	ENST00000413767	R0.750:V0.250	NA	NA	N
22	INTS8	S755R	ENST00000523731	R0.500:V0.500	NA	NA	Y
23	KDM2B	T28Sfs*4	ENST00000377071	R0.674:V0.326	NA	NA	Y
24	MAGI1	Q421_T422insL	ENST00000402939	R0.609:V0.391	NA	NA	Y
25	MUC16	I7261M	ENST00000397910	R0.500:V0.500	NA	NA	N
26	MUC16	S7025L	ENST00000397910	R0.511:V0.489	NA	NA	N
27	MYCBP2	R3271H	ENST00000544440	R0.783:V0.205	NA	NA	N
28	NBPF10	R3484S	ENST00000342960	R0.857:V0.143	NA	NA	N
29	NCOA6	R1652Q	ENST00000374796	R0.892:V0.108			Y
30	PCDHB11	L496P	ENST00000354757	R0.750:V0.250	NA	NA	0
31	PCDHB11	G532S	ENST00000354757	R0.643:V0.357	NA	NA	0
32	PNPLA5	S82N	ENST00000216177	R0.556:V0.444	NA	NA	N
33	PSKH2	Q47E	ENST00000276616	R0.694:V0.306	NA	NA	0
34	SELO	S184P	ENST00000380903	R0.389:V0.611	NA	NA	N
35	SHARPIN	H315N	ENST00000398712	R0.510:V0.490	NA	NA	0
36	SIRPA	T50S	ENST00000358771	R0.860:V0.140	NA	NA	Y
37	SIX1	R119C	ENST00000247182	R0.489:V0.511	Autosomal dominant deafness (605192) E125K in one paper, Y129C, del133E and R110W in another. Also associated with branchiootic syndrome 3 (608389).	AD, ? Loss of function	N
38	SRRM4	R582P	ENST00000267260	R0.333:V0.667	NA	NA	
39	TTLL3	R130Q		R0.492:V0.508	NA	NA	N
40	UBE2QL1	L10P	ENST00000399816	R0.510:V0.490	NA	NA	N
41	VAV1	R276T	ENST00000602142	R0.533:V0.467	NA	NA	
42	ZBTB41	D21_delTCTT	ENST00000367405	R0.424:V0.576	NA	NA	N
43	ZNF626	A349D	ENST00000601440	R0.773:V0.227	NA	NA	N
44	ZNF814	A158V	ENST00000435989	R0.188:V0.812	NA	NA	N

			Insilico analysis						
.%ExAC_RVIS score	Diff SNP in same codon	PolyPhen2	Interpret	SIFT	SIFT interpret	Mutation Taster	CADD	FATHMM	
7.44	N					Disease causing			
72.96		0.001	BENIGN		1.00 TOLERATED	Polymorphism	0.006	Tolerated	
4.98	N	NA	NA	NA	NA	Disease causing	NA	NA	
17.5	N	0.987	Probably Damaging		0.01 Damaging	Polymorphism	25.300	Inconclusive	
17.28	N	1	Probably damaging		0.33 Tolerated	Polymorphism	7.930	Tolerated	
NA	N	NA				Disease causing			
73.12	N	0.001	Benign		0.11 Tolerated	Polymorphism	8.870	Tolerated	
73.12	N	0	Benign		0.35 Tolerated	Polymorphism	3.230	Tolerated	
50.84	Y	NA	NA	NA	NA	?	NA	NA	
2.19	Y	1	Probably Damaging		0.01 Damaging	Disease causing	34.000	Inconclusive	
NA	N	0.999	Probably damaging		0.02 Damaging	polymorphism	18.470	Tolerated	
21	N	0.062	Benign		0.17 Tolerated	Polymorphism	10.910	Tolerated	
21	N	1	Probably Damaging		0.30 Tolerated	Disease causing	18.380	Damaging	
21	N	0.046	Benign		0.63 Tolerated	Polymorphism	8.395	Tolerated	
21	N	0.005	Benign		0.26 Tolerated	Disease causing	17.890	Damaging	
21	N	0.005	Benign		0.29 Tolerated	Polymorphism	9.263	Tolerated	
99.93	N	0	BENIGN		0.52 Tolerated	Polymorphism	0.605	Tolerated	
99.88		0.016	BENIGN		0.14 Tolerated	Polymorphism	9.294	Tolerated	
NA	Y	0.035	Benign		0.02 *Damaging	Polymorphism	16.050	Tolerated	
67.95	N	0.004	Benign		0.02 Damaging	Polymorphism	4.840	Tolerated	
93.75	N	0.02	Benign		0.02 Damaging	Polymorphism	0.220	Tolerated	
7.26	Y	0.703	Possibly damaging		0.06 Tolerated	Disease causing	22.600	Tolerated	
2.02						Disease causing			
2.73	Y					Polymorphism			
99.99	Y	0.006	Benign			Polymorphism	0.000	Inconclusive	
99.99	N	0.006	Benign			Polymorphism	1.550	Inconclusive	
NA	Y	0.998	Probably damaging		0.16 Tolerated	Disease causing	22.300	Tolerated	
NA	Y	0.061000001	BENIGN		0.09 Tolerated	Polymorphism		Tolerated	
5.7	N	1	Probably damaging		0.19 Tolerated	Disease causing	27.200	Tolerated	
46.74	N	0	BENIGN		1.00 Tolerated	Polymorphism	0.001	Inconclusive	
46.74	Y	0.141	Benign		3.08 Tolerated	polymorphism	0.006	Tolerated	
94.55	N	0.659	Possibly damaging		0.52 Tolerated	Disease causing	20.900	Tolerated	
93.01	Y (5 het Exac)	0.139	Benign		0.99 Tolerated	Polymorphism	21.000	Tolerated	
65.96	N	1	Probably damaging		0.00 Damaging	Disease causing	22.400	Inconclusive	
87	Y	0	Benign		0.57 Tolerated	polymorphism	1.700	Tolerated	
75.66	Y	0	Benign		0.95 Tolerated	Polymorphism	0.000	Tolerated	
29.97	N	1	Damaging		0.00 Damaging	Disease causing	21.500	Damaging	
58.83		0	UNKNOWN		0.00	Polymorphism	15.680		
79.05	N	0.003	Benign		0.03 Tolerated	Polymorphism	10.770	Tolerated	
NA	N	0.927	Possibly damaging		0.04 Damaging	Disease causing	13.900	Tolerated	
9.95	N	0.997	Probably damaging		0.22 Tolerated	Disease causing	22.800	Tolerated	
11.7	Y	NA	NA	NA	NA	Disease causing		NA	
NA	N	0.35	Benign				0.837	Tolerated	
65.66	N	0	Benign		0.42 Tolerated	Polymorphism		Tolerated	

		Population frequency						
Weighted FATHMM	Unweighted FATHMM	1000G	dbSNP ref	dbSNP	ExAC	ExAC coverage	ExAC GnomAD	Homologene
			N	0	0	66.56	0	
1.03	-0.21	0.001	rs143965492	0.003	0.005702	51.03	0.00002501	Low
NA	NA	0	N	0	0	48.55	0	NA
-3.5	2.5	0	N	0	0	66.26	0	?Low
1.4	-2.79		rs943299857	0.00003	0	60.29	0.00026	Low
					0	37.49		
4.4	-0.38	0	N	0	0	58.79	0	Low
4.35	-0.87	0	N	0	0	58.79	0	?Low
NA	NA	0	N	0	0	66.49	0	
0.88	-6.66	0	N	0	0	59.45	0.000008126	Very high
-0.4	-1.45	0.0008	rs201579633		0.0018	47.46	0	Low
	-2.37		N	0		43.94	0	Low
	-6.87	0	rs1004559299	no frequency data	0	43.94	0	High
	-1.12	0	rs761785475		0.00002	43.94	0	Low
	-3.8		rs995187092	no frequency data		43.94	0	Very high
	-1.82		rs201905369		0.00001	43.94	0.00001181	Low
2.59	0.78		rs9260179	0.006	0.002845	54.87	0.04417	Low
5.66	-1.82		rs1050683	0.002	0.002065	48.6		?High
7.77	-2.26	37 het	rs112906420		0.0002198	31.3	0	?Low (only mammals in homologene)
3.93	-0.71	0	N	0	0	92.25	0	Low
3.93	-0.71	0	rs375364040	0.000008	0	83.86	0	Low
1.45	-1.64	0.0018	rs150502842	0.008	0.003969	60.29	0.000004528	High
		0.0012	rs575373073,COSM4384801	0	0.003172	52.68	0.002876	
		0.0034	rs142043619	0.089	0.00003349	70.58	0.3212	
4.19	-3.63	0	N	0	0	72.92	0	Low
4.21	-3.55	0	N	0	0	72.92	0	Low
1.58	-1.71	0	N	0	0	62.34	0	Low
3.33	0.13	0.0064	rs201192694	0.006	0.006383	17.56		NA
1.81	-1.84	0	rs371013508	0.00003	0.00001647	74.47	0.00001443	
1.17	-6.23	0	rs377311561	0	0	77.79	0.00009342	Low
4.71	0.73	Y	rs138686663	0.0041	0	77.79	0	Moderate
-1.11	-0.57	0		0	0	40.65	0	Low
1.15	0.21	0	N	0	0	59.66	0	? High
0.24	-6.3	0	N	0	0	42.13	0	?Very high (missing in wolf)
1.98	-0.35	0	N	0	0	50.48	0	Moderate
-0.04	-1.13	0.2111	rs17855609	0.002	0.008724	60.51	0	Low
-2.52	-6.06	0	N	0	0	62.34	0	Very high
			rs140426282,COSM4760652,COSM4760653,COSM4760654	0.005	0.008986	29.58	0.01153	High
2.72	-0.74	0	rs747479295	0.00008	0	61.33	0	? Moderate
1.17	0.08	0		0	0	7.039	0	?Low (fly and mosquito change)
0.02	0.75	0	N	0	0	60.85	0	High
NA	NA	0	N	0	0	54.96	0	
1.26	-0.74	0	rs200037994	0.001	0.0007	62.51	0	NA
3.48	1.53	0	rs201805246	0.007	0.0037	35.64	0	Low

Conservation analysis			Protein details		
Phylop	AGVGD	Grantham score	Domain	SubRVIS percentiles	
3.186					
-0.409	C35	43			
3.561	NA	NA	No domain aligned (-)	25.39208	
2.731	C65	74	No domain aligned (-)	8	
0.908	C65	103	endonuclease/Exonuclease/phosphatase family	86.922768	
6.248		6		NA	
-0.783	C25	26	No domain aligned (-)	90.21	
0.72	C45	53	No domain aligned (-)	90.21	
NA	NA	NA	No domain aligned (-)	9.255825	
4.588	C65	101	Guanylate kinase-like domain ends at 855, ? No features beyond this. According to subRVIS is in GuKc (214504)	12.861084	
1.157	C15	21	Plectin 37 domain	NA	
2.258	C55	145			
2.189	C65	98			
0.821	C35	43			
0.413	C55	59			
1.467	C55	56			
-1.653	C25	29	IgC_MHC_I_alpha3 (143322)	81.41909	
0.031	C55	58	MHC_1 domain	99.78	
0.677	C35	43	In extracellular domain	99.8	
-1.529	C65	78	Interferon	4.430754	
1.07	C65	58	Interferon	92.66605	
2.285	C65	110		1.378991	
1.324					
			No domain aligned (-)	18.39604	
-0.648	C0	10	Part of ser-rich compositionaly biased region	99.99	
-0.097	C65	145	Part of ser-rich compositionaly biased region	99.99	
4.381	C25	29	No domain aligned (-)		
-0.318	C65	110			
5.571	C35	43	?EP300/CRSP3-binding region. Lost	0.0457988	
-0.518	C65	98	In 5th cadherin domain	79.3	
-1.243	C55	56	In 5th cadherin domain	79.3	
0.434	C45	46	In PNPLA domain	94.62814	
2.66	C25	29	No domain aligned (-)	30.92313	
4.158	C65	74		1.387368	
2.912	C65	68.35	5 aa from interaction with SHANK1 region.	44.2	
-1.235	C55	58	In Ig like V type domain	60.34438	
2.404	C65	180		23.70144	
0.616	C65	103			
-0.724	C35	43		85.2287706	
4.192	C65	98	UBCc	55.85666	
2.507	C65	71	DH domain, RhoGEF domain	38.09064	
	C65	126	C2H2-type 7	41.19211	
-0.805	C55	64	No domain aligned (-)	17.24213	

8.10 Appendix 10: Curation of autosomal recessive variatns

					Details			
	Gene	Variant	Ensemble ID	Zygosity	OMIM reference	Disease mechanism	ExAC constraint	.%ExAC_RVIS score
1	ALDH1A3	R15G	ENST00000329841	R0.0:V1.000	615113	AR	Y	11.68
2	DMKN	G270delG	ENST00000339686	R0:V1.000	NA	NA	N	71.43
3	GPR137C	H120R	ENST00000321662	R0.0:V1.000	NA	NA	Y	24.07
4	CCND3	P134S	ENST00000372991	R0.0:V1.000	NA	NA	Y	60
5	BEND6	H167D	ENST00000370746	R0.019:V0.981	NA	NA	Y	66.27
6	EFHC1	I401T	ENST00000371068	R0.0:V1.000	NA	NA	Y	88.11
7	PHLDA1	Q195delQ	ENST00000266671	R0.100:V0.900	NA	NA	N	53.17
8	MUC6	P1906S	ENST00000421673	R0.011:V0.979	NA	NA	Y	99.98
9	FAM231B	F72del*fs	ENST00000601199	R0.077:V0.923	NA	NA	Unknown	28.98
10	CEP128	V354A	ENST00000555265	R0.0:V1.000	NA	NA	Y	99.98
11	GEMIN5	R1016C	ENST00000285873	R0.0:V1.000	NA	NA	Y	88.13
12	EPOR	P488S	ENST00000222139	R0.0:V1.000	NA	NA	Y	47.63

					Insilico analysis			
Diff SNP in same codon	PolyPhen2	Interpret	SIFT	SIFT interpret	Mutation Taster	CADD	FATHMM	Weighted FATHMM
N	0	BENIGN	0.180	TOLERATED	Polymorphism	14.73999977	Tolerated	-0.95
Y					Polymorphism			
Y	0.003	BENIGN	0.520	TOLERATED	Polymorphism	12.89999962	Tolerated	1.01
Y	0.13	BENIGN	0.250	TOLERATED	Disease causing	18.46		
Y	0	BENIGN	0.320	TOLERATED	Polymorphism	13.6		
Y	0.34	BENIGN	0.040	DELETERIOUS	Disease causing	23.7		
N					Polymorphism			
Y	0.603	Possibly damaging	0.420	Tolerated	Polymorphism	4.330999851	Tolerated	2.25
Unknown								
Y	0.009	BENIGN	0.280	TOLERATED	Polymorphism	4.834		
N	0.999	Probably damaging	0.010	Damaging		34	Inconclusive	-0.58
N	0.01	Benign	0.160	Tolerated	Polymorphism	17.29000092	Tolerated	0.97

	Population frequency						Conservation	
Unweighted FATHMM	1000G	dbSNP ref	dbSNP	ExAC	ExAC coverage	ExAC GnomAD	Homologene	Phylop
-0.97	0.002	rs1130737	0.007	0.003388	53.08	0.004	Low	-0.105
		rs763222290	0	0	52.84	0	Low	-1.774
0.21	0	rs769056681	0	2.5E-05	45.19	0.00003297	Moderate	0.451
	0.009	rs3218089	0.005	0.002351	47.65	0.002567	High	3.99
	0	rs751178504	0	0.000008	62.6	0	Low	0.69
	0	rs769124873	0	0.00000824	34.59	0.000008128	Moderate	3.306
	0.0094	rs71716769	0.002	0	28.91	0.000007699	Low	0.46
-0.43	0.0057	rs200257435	0.012	0.0057	49.52	0.00204	Low	0.113
	0	rs71574191	0	0		0		
	0.0048	rs116610923		0.008717	63.77	0.009549	Moderate	0.066
-3.27	0.0028	rs61749643	0.009	0.0045	67.39	0.004522	High	2.921
-0.29	0.0048	rs142094773	0.012	0.005841	40.7	0.007441	High	1.319

ı analysis		Protein details	
AGVGD	Grantham score	Domain	SubRVIS percentiles
C65	125	No domain alignment	68.98
		No domain alignment	94.32151
C25	29	No domain alignment	3.957686
C65	74	Cyclin_N (249617)	78.72031
C65	81	No domain alignment	5.518755
C65	89	No domain alignment	35.61806
		PH domain	19.50582
C65	38	repeat #2	99.445946
	64	No domain alignment	0.8254954
C65	180		92.62025
C65	74	Close to JAK2 modification sites	92.10809