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Review Article

The classification, genetic diagnosis and modelling of monogenic autoinflammatory disorders

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Monogenic autoinflammatory disorders are an increasingly heterogeneous group of conditions characterised by innate immune dysregulation. Improved genetic sequencing in recent years has led not only to the discovery of a plethora of conditions considered to be 'autoinflammatory', but also the broadening of the clinical and immunological phenotypic spectra seen in these disorders. This review outlines the classification strategies that have been employed for monogenic autoinflammatory disorders to date, including the primary innate immune pathway or the dominant cytokine implicated in disease pathogenesis, and highlights some of the advantages of these models. Furthermore, the use of the term 'autoinflammatory' is discussed in relation to disorders that cross the innate and adaptive immune divide. The utilisation of next-generation sequencing (NGS) in this population is examined, as are potential *in vivo* and *in vitro* methods of modelling to determine pathogenicity of novel genetic findings. Finally, areas where our understanding can be improved are highlighted, such as phenotypic variability and genotype–phenotype correlations, with the aim of identifying areas of future research.

Introduction

The phrase '*autoinflammatory disease*' was proposed as an alternative to '*autoimmune disease*' by McDermott et al. [1] in the paper identifying the genetic cause of Tumour Necrosis Factor (TNF) Receptor Associated Periodic Syndrome (TRAPS). This was considered a suitably representative term at the time, as individuals with inherited periodic fever syndromes had innate immune dysregulation but lacked high titres of autoantibodies and self-reactive T cells [1]. 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 [2,3].

Since this time, over 30 conditions have been added to the list of monogenic autoinflammatory disorders. 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 seen. 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 autoinflammatory disorders maintained by the International Society for Systemic Autoinflammatory Diseases (ISSAID) (Figure 1) [4,5].

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Classification

The term inflammasomopathy was introduced in the first review of autoinflammatory disorders categorising conditions based on the pathway implicated in disease pathogenesis [6], including disorders affecting inflammasomes, the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) pathway, the complement system, protein folding, 'cytokine signalling' and those resulting in or from macrophage

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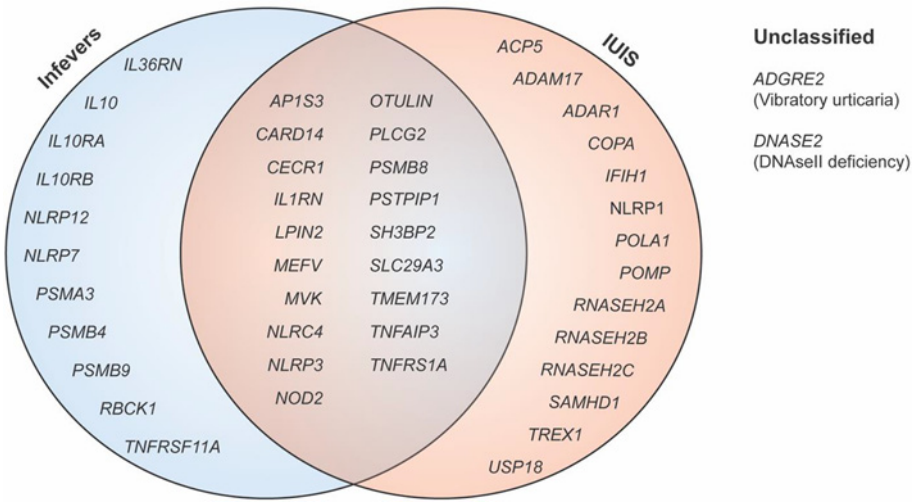

Figure 1. Genes involved in monogenic autoinflammatory disorders

Genes involved in monogenic autoinflammatory disorders according to ISSAID as listed in the Infevers database, compared with the IUIS.

activation. Since this time, a number of reviews have adopted a briefer version, with both inflammasome or interleukin (IL)-1β mediated disorders and NF-κB pathway-associated disorders universally included, but the other categories far less frequently [7]. The term 'interferonopathies' was first used to describe a group of monogenic disorders characterised by increased type I interferon (IFN) signalling in 2011 [8] but these disorders were only grouped with autoinflammatory disorders by the IUIS in 2017 [4]. No rationale for this change was provided, preventing a unified strategy of classification to be adopted those researching and managing these conditions.

The pathway model

The pathway model has the advantage of highlighting possible targets for treatment downstream of an abnormal protein, as with Janus kinase (JAK) inhibitors for individuals with stimulator of IFN genes (STING)-associated vasculopathy with onset in infancy (SAVI), as well as possible candidate genes for autoinflammatory disorders, such as that encoding a member of the linear ubiquitin assembly complex (LUBAC), *SHARPIN*. There are, however, limitations to this classification. A clear example is the case of mutations in *TNFRSF1A* causing TRAPS. While TNF receptor 1 (TNFR1) is a key receptor in the NF-κB pathway, the disease is not necessarily caused by increased signalling through this pathway alone [1,9-13]. A number of pathogenic mechanisms have been explored, such as defective shedding of TNFR1 [1], retention of TNFR1 in cytoplasmic aggregates with reduced surface expression [11], and abnormal apoptosis and signalling [12].

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 NOD-like receptor (NLR) pyrin domain containing 3 (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 haploinsufficiency of A20 (HA20) [14]. Furthermore, although not yet shown in human cells, transforming growth factor (TGF)-β activated kinase-1 (TAK1) has been shown to regulate NLRP3, with spontaneous NLRP3 activation documented in TAK1-deficient murine macrophages [15]. 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 [16,17], but whether these two pathways are actually uncoupled in the case of an overactive STING *in vivo* is unclear. 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 IFN regulatory transcription factor (IRF) 3 in the inflammation associated with SAVI [18]. Furthermore, the role of NF-κB as a member of the IFN-β enhanceosome [19], a multicomponent complex that optimises transcriptional activation of IFN-β, suggests that the pathways are closely connected.

The cytokine model

An alternative classification strategy is based on the primary cytokine dysregulated, either increased or decreased, in individuals with autoinflammatory disorders [20]. This is of potential therapeutic benefit as the primary cytokine driving disease can be therapeutically targeted. An example of this is the treatment of individuals with cryopyrin-associated periodic syndrome (CAPS). In the original manuscript linking Neonatal Onset Multisystem Inflammatory Disease (NOMID) to mutations in *NLRP3*, cell lysates from unstimulated monocytes of a case had high pro-IL-1β expression as determined by Western blot, and increased *IL-1*β mRNA in unstimulated peripheral blood mononuclear cells (PBMCs) when compared with healthy controls [21]. The empiric treatment of two cases with recombinant IL-1 receptor antagonist and the rapid resolution of symptoms within hours, and inflammatory markers within days, highlighted the role of IL-1β in the disease pathogenesis [22]. Having said this, the detection of IL-1β in serum of cases is difficult, with both cases 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 individuals 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 [23]. From this it is clear that even without elevated serum levels, a therapeutic response to IL-1β neutralising therapy suggests that this cytokine is important [23,24]. The response of individuals with colchicine-resistant FMF [25], mevalonate kinase deficiency (MKD) [26], and TRAPS [27] to the neutralising 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 individuals with TRAPS [28]. The gene expression profile of TRAPS moved towards the healthy control profile with canakinumab treatment [27]. More recently, the randomised, double-blind, placebo-controlled study of canakinumab in the above groups demonstrated efficacy in controlling and preventing disease flares [29]. An interesting addition to the literature was a retrospective analysis by Savic et al., of individuals with undifferentiated systemic autoinflammatory disorders who were treated with anakinra [30]. A total of 11 cases were identified over a 3-year period, and nine responded completely to treatment with anakinra within 4–6 weeks of commencement. Although individuals 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, subjects could undergo a broader approach with whole exome sequencing (WES) or whole genome sequencing (WGS) and novel genes involved in the IL-1β pathway may be revealed.

Evaluation of the major cytokine/s involved in monogenic autoinflammatory disorders may point to distinctions between conditions within the same pathway. The gain of function mutations in inflammasome forming proteins that lead to disease can be presumed to cause an increase in IL-1β processing and release. Mutations in *NLRC4* that result in an autoinflammatory phenotype are associated with markedly increased serum free IL-18 levels in cases when compared with healthy controls and individuals with CAPS [31,3233].

There are also conditions that may involve pathways distinct from those used to categorise autoinflammatory disorders in the literature. Through the study of autosomal recessive generalised pustular psoriasis (GPP) in a number of multiplex families, Marrakchi et al. [34] identified homozygous missense mutations in *IL36RN*, which encodes the IL-36 receptor antagonist (IL-36Ra), causing deficiency in IL-36Ra (DITRA). IL-36 is a member of the IL-1 family of cytokines and, like IL-1β, acts via its receptor IL-36R and, in concert with IL-1 receptor accessory protein (IL1RAcP), signals to NF-κB through myeloid differentiation primary response 88 (MyD88). The binding of IL-36Ra to IL-36R prevents the association of IL1RAcP and downstream signalling. While there have been four case reports of the successful treatment of DITRA with anakinra therapy [35-38], therapeutic benefit has also resulted from TNF inhibition [39-41], IL-17 inhibition with secukinumab [42] and IL-12/IL-23 inhibition with ustekinumab [43-45]. This suggests that these agents may be targeting cytokines that are downstream of IL-36 [38]. The possibility of developing a therapeutic agent that is specific for IL-36 has been explored. Mbow et al. characterised a mouse anti-human antibody (MAB92) with high affinity to the IL-36 receptor that blocks signalling through this pathway [46]. 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 individuals 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) [47,48]. IL-10 has regulatory effects on the inflammatory response which are mediated through signal transducer and activator of transcription (STAT) 3, with IL-10-deficient mice developing chronic enterocolitis [49-51]. PBMCs from cases with loss of function of these proteins had higher proinflammatory cytokine responses to lipopolysaccharide (LPS) stimulation, including IL-6, TNF and IL-1β, when compared with healthy controls [48]. Although multiple agents have

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been trialled in these cases including corticosteroids, azathioprine, methotrexate, cyclosporine A and anti-TNF therapy, only mild clinical benefit has resulted [52]. A number of cases have undergone allogenic haemopoetic stem cell transplantation (HSCT) with marked improvement in their inflammatory bowel disease [52,53]. While recombinant human IL-10 replacement (rhuIL-10) in individuals 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 individuals with Crohn's disease [54]. Furthermore, this option would not be effective in individuals with mutations in *IL10RA* or *IL10RB*. 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 little is known about the steps that lead to this alteration. Proteasome-associated autoinflammatory syndrome (PRAAS) is an autosomal recessive autoinflammatory disorder that encompasses conditions previously considered distinct entities: Nakajo-Nishimura syndrome (NKJO), joint contractures, muscular atrophy, microcytic anaemia, and panniculitis-induced lipodystrophy syndrome (JMPS), 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 [55-57]. Individuals with homozygous loss of function 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 [55-57]. Homozygous mutations were associated with poor proteasome assembly as well as reduced chymotrypsin-like activity and accumulation of ubiquitinated proteins in either Epstein–Barr virus (EBV)-transformed B cells or immortalised lymphoblastoid cell lines from cases [55-57]. In these early papers, increased serum IL-6 was noted in all cases, but the role of IFN was only identified later [58]. Liu et al. noted an almost 80-fold increase in IFN-γ-inducible protein 10 (IP-10) in cases compared with healthy controls and individuals with CAPS, prompting whole blood microarray analysis to determine the gene signature of these cases. The IFN pathway was the most differentially regulated pathway in individuals with PRAAS, further supported by stronger STAT1 phosphorylation in response to IFN-γ stimulation of monocytes when compared with healthy controls. These authors also highlighted cases with the clinical phenotype of PRAAS without *PSMB8* mutations, later explored by Goldbach-Mansky et al. [59]. 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 cases with the clinical diagnosis of PRAAS. One individual harboured a compound heterozygous mutation in *PSMB4*, and another 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 cases 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.

Clarifying the dominant cytokine or pathway implicated in disease may lead to the development of targeted therapeutic strategies for autoinflammatory conditions, as reviewed in recent publications [60,61]. Recent work has looked at individuals with interferonopathies treated with JAK1/2 inhibitor baricitinib. Initial work was performed by Goldbach-Mansky et al. to determine a dosing regimen for paediatric cases with interferonopathies [62]. This work was then extended with treatment of a number of individuals with interferonopathies including PRAAS and SAVI and longitudinal assessment of response and adverse reactions [63]. Most patients were able to reduce their prednisolone requirements and five out of ten PRAAS cases achieved remission. Adverse events included upper respiratory infections, gastroenteritis and BK viremia. Given that only 18 patients were recruited over a span of 6 years, more work is needed to establish the clinical efficacy and adverse reaction profile in this selected population. The availability of this drug on compassionate grounds through NCT01724580 suggests that more information will become available in the future with ongoing recruitment and follow-up of these cases.

Similarly, elucidating elevated serum free IL-18 levels in an individual with autoinflammation with infantile enterocolitis, an NLRC4-associated autoinflammatory disease, lead to the successful therapeutic trial of a recombinant IL-18 binding protein (rhIL-18) [64]. Following from this, there is a Phase 3 randomised, double-blind, placebo-controlled trial of rhIL-18 in NLRC4-associated autoinflammatory diseases (NCT03113760) as well as an open-label extension (NCT03512314) underway. 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 as well as possible epigenetic factors, that results in one cytokine dominating over another.

Autoinflammation, autoimmunity and immune deficiency

The boundaries of what is classified as an autoinflammatory disorder 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 Aicardi–Goutieres syndrome (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 [65]. The genetic causes of AGS are numerous, and all involve the processing of nucleic acid, either self or foreign, in the cytoplasm. Loss of function mutations in genes encoding deoxyribonuclease three prime repair exonuclease 1 (TREX1) [66], deoxynucleoside triphosphate triphosphohydrolase SAM and HD domain containing protein 1 (SAMHD1) [67], ribonuclease components ribonuclease H2 (RNASEH2) A (RNASEH2A), RNASEH2B or RNASEH2C [68], or adenosine deaminase acting on RNA 1 (ADAR1) [69] have been identified in individuals with AGS. The link between AGS and autoimmunity was initially made when Aicardi and Goutières speculated that the phenotype of two individuals 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 [70]. The phenotypic link has subsequently been highlighted by a number of groups [71,72], although the number of subjects in a large cohort of 374 mutations confirmed that AGS with clinically diagnosed lupus was low [73]. An abnormal serum autoantibody profile was seen in a minority of individuals with AGS in one cohort study [74], however another detected persistent antinuclear antibodies (ANA) or autoantibodies against extractable nuclear antigens (ENA), dsDNA and cardiolipin in the majority of their cases with mutation confirmed AGS [72]. Subsequent work using multiplex autoantibody microarrays identified unique autoantibodies in cases with AGS [75]. 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. Homozygous loss of function mutations in *HOIL1* or *HOIP* have been described in cases with evidence of systemic inflammation, susceptibility to pyogenic infections, and amylopectinosis [76,77]. The original description by Boisson et al. highlighted the importance of haem-oxidised IRP2 ubiquitin ligase 1 (HOIL1) in maintaining the stability of the LUBAC, involved in the ubiquitination of components in the NF-κB pathway, as well as promoting the association of inhibitor of NF-κB kinase subunit γ (IKKγ) with TNF or IL-1 receptor signalling complexes [76]. The authors noted cell-type specific defects associated with HOIL1 deficiency. Fibroblasts and EBV-immortalised B cells from subjects displayed impaired canonical NF-κB pathway activation in response to TNF or IL-1β as well as partial impairment of the response to toll-like receptor (TLR) stimuli. The inflammatory phenotype was determined to originate from monocytes, with 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 cases overlap with those seen in an individual with homozygous loss of function mutations in *HOIP* [77]. The publication of a series of ten cases from eight families with polyglucosan storage myopathy harbouring either homozygous or compound heterozygous mutations in *HOIL1* [78] 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.

Classifying these as autoinflammatory diseases inherently fails to acknowledge the associated immunodeficiency, and *vice versa*. A similar problem concerns the conditions caused by mutations in *PLCG2* encoding phospholipase c γ-2 (PLCγ2), PLCγ2-associated antibody deficiency and immune dysregulation (PLAID) and autoinflammation and PLAID (APLAID) [79,80]. PLCγ2 was linked to autoimmune and autoinflammatory diseases initially through an N-ethyl-N-nitrosourea (ENU) mutagenesis screen [81]. A heterozygous point mutation in *PLCG2* 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 [79]. 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 natural killer (NK) cells demonstrated reduced calcium flux and reduced phosphorylation of mitogen-activated protein kinase (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 pathway 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

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report, the individuals 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 [80]. Of the two cases described, neither had cold-induced symptoms. Increased baseline PLCγ2 activity was noted in an overexpression COS-7 cell model. Chae et al. [82] 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 and Tsukada [83], resulted in increased NLRP3 activation and IL-1β release when assessing PBMCs from cases compared with healthy controls. It would be interesting to determine whether the same increase in NLRP3-driven IL-1β is seen when PBMCs from PLAID subjects are examined, as their inflammatory phenotype was not as profound, and was temperature dependent. Furthermore, the partial response to IL-1β-targeted therapy [80] 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 to this autoinflammatory disease. 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 loss of function mutations in *CECR1* were found in individuals with polyarteritis nodosa [84] as well as early onset stroke, vasculopathy and febrile episodes [85]. Although immunodeficiency and autoimmunity were not a major feature, IgM deficiency was noted in a number of cases [85]. Treatment of ten individuals with TNF targeting therapy by Levy-Lahad et al. led to significant clinical improvement, highlighting the role of this cytokine in disease pathogenesis [84]. The response to TNF directed therapy has since been reproduced [86,87]. In a subsequent study of 48 cases with polyarteritis nodosa associated with livedo reticularis and/or strokes, Gattorno et al. performed Sanger sequencing of *CECR1* and determined that 15 cases harboured homozygous or compound heterozygous mutations [88]. Since the time of the original description, there has been an expansion of the clinical phenotype of cases with DADA2, from cytopaenias and pure red cell aplasia [89], to lymphoproliferative disease [90-92], and combined immune deficiency, as well as common variable immune deficiency (CVID) [93]. Indeed, a cohort study of 181 cases with antibody deficiency diagnosed 11 individuals with mutation and enzyme activity confirmed DADA2 [94]. An interesting finding in this group was that anti-TNF therapy resulted in an improvement in IgM levels in one case, and there was an inverse correlation between c-reactive protein (CRP) and IgG in another. Further complicating the potential mechanisms of this disease, individuals with DADA2 have also been reported to have an IFN gene signature [95,96]. Researchers investigated individuals with features overlapping with AGS-5 (caused by mutations in *SAMHD1)*. In each report, cases 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 individuals with DADA2 to this therapy, it would be interesting to determine whether the IFN gene signature is abrogated with the use of anti-TNF therapy.

Furthermore, a number of conditions classified as disorders of predominantly antibody deficiency by the IUIS have marked autoinflammatory features. The syndrome of sideroblastic anemia with B-cell immunodeficiency, periodic fevers and developmental delay (SIFD) was first described by Wiseman et al. in 2013 [97], with 11 out of the 12 cases described experiencing periodic fevers. It was subsequently determined to be caused by homozygous or compound heterozygous mutations in *TRNT1* [98], encoding the CCA-adding enzyme tRNA nucleotidyltransferase [99]. Aksentijevich et al. investigated the inflammatory phenotype of these cases, noting markedly elevated acute phase reactants and inflammatory cytokines in cases with active disease [100]. The authors documented reduced expression of mature cytosolic tRNA, as well as increased reactive oxygen species when corrected for live cells in fibroblasts after 72 h compared with healthy controls. Using an siRNA knockdown THP-1 cell model, *TRNT1*-knockdown cells demonstrated increased IL-1β production at baseline and in response to LPS which was reversed with the small molecule NLRP3 inhibitor MCC950, suggesting an NLRP3-dependent inflammatory phenotype.

Autoinflammatory disease classification summary

From the above discussion, it is apparent that there are significant barriers to a simple definition or classification criteria for what are considered monogenic autoinflammatory disorders. As research progresses, the inflammatory component of disorders previously considered to be primarily of immune deficiency or autoimmunity will become more apparent. Provided in Figure 2 is a summary of conditions listed as autoinflammatory disorders 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.

Table 1 Monogenic autoinflammatory disorder summary table

Condition	Gene/s	Protein	MOI	GOF/LOF	Pathway	Cytokine group	System involved	Human cell model	Potential murine model	Reference/s
ADAM17 deficiency	*ADAM17*	ADAM17	AR	LOF	Unknown	Unknown	Skin GIT	**PBMC**: ↓TNF-α response to LPS, PMA + anti-CD3/anti-CD28 antibodies	Nil	[148]
AGS1	*TREX1*	TREX1	AR or AD	LOF	IFN	T1IFN	CNS	**Human neural stem cell-derived astrocytes, primary astrocytes, brain-derived endothelial cells**: shRNA knockdown → IFN gene signature + ↑ proinflammatory cytokines	*Trex1*$^{-/-}$ mice	[66,149-151]
AGS2	*RNASEH2B*	RNASEH2B	AR	LOF	IFN	T1IFN	CNS		*Rnaseh2b knockout first* [*KOF*] mice	[68,74]
AGS3	*RNASEH2C*	RNASEH2C	AR	LOF	IFN	T1IFN	CNS		*Rnaseh2c*$^{-/-}$ mice	[68,74,152,153,154]
AGS4	*RNASEH2A*	RNASEH2A	AR	LOF	IFN	T1IFN	CNS	**Human neural stem cell-derived astrocytes, primary astrocytes, brain-derived endothelial cells**: shRNA knockdown minimal change in ISG/IFN cytokine profile	*Rnaseh2a*$^{G37S/G37S}$	[68,74,149]
AGS5	*SAMHD1*	SAMHD1	AR	LOF	IFN	T1IFN	CNS	**HeLa cells**: transfection of mutant SAMHD1 showed abnormal localisation**Human neural stem cell-derived astrocytes primary astrocytes, brain-derived endothelial cells**: shRNA knockdown → IFN gene signature + ↑ proinflammatory cytokines	*Samhd1*$^{-/-}$ mice	[67,73,149,155-157]
AGS6	*ADAR1*	ADAR1	AR	LOF	IFN	T1IFN	CNS	**HEK293Tcells**: IFN 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 knockdown minimal change in ISG/IFN cytokine profile	*Adar*$^{1/-}$ *SCL*-Cre-ER^{T} * mice	[149,158]
AGS7	*IFIH1*	MDA5	AD	GOF	IFN	T1IFN	CNS	**HEK293T**: IFN reporter assay	*Ifih1*$^{gs/+}$ mice	[159-161]
AIADK	*NLRP1*	NLRP1	AD	GOF	Inflam	IL-18 ?IL-1β	Multiple		*Nlrp1a*^{Q593P} mice*	[162,153]
AIFEC	*NLRC4*	NLRC4	AD	GOF	Inflam	IL-18	Multiple	**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	[147,32, 163-165]
AILJK	*COPA*	COPA	AD	Dominant negative	?NF-κB ?IFN		Multiple incl lungs, kidney	**CD4 T cells**: skewing to Th17 response **BLCL**: ↓ autophagy, ↑ transcription IL-1 β, IL-6, IL-23 **HEK293T cells**: siRNA knockdown → ↑ER stress	Nil	[166,167]
APLAID	*PLCG2*	PLCγ2	AD	GOF	Unknown ?Inflam ?NF-κB	Unknown ?IL-1β	Multiple	**PBMC**: ↑response to NLRP3 activation **B cells**: ↑ERK phosphorylation	Multiple*	[80,82]
Blau syndrome	*NOD2*	NOD2	AD	GOF	NF-κB	Multiple TNFα	Multiple	**HEK293T cells**: NF-κB luciferase assay. ↑ activity with transfection of mutants **PBMC**: single patient w novel variant ↓ NF-κB response	*Nod2*^{2939ic} mice	[168-170]
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*$^{A350V neoR/+}$ *Nlrp3*$^{L351P neoR/+}$ mice *Nlrp3*^{R258W} mice	[21,154, 171-173]
Cherubism	*SH3BP2*	SH3BP2	AD	? GOF ? dominant negative	? NF-κB ? NFATc1	TNF-α	Bone		*Sh3bp2* $^{P416R/+}$ mice	[174,175]
DADA2	*CECR1*	ADA2	AR	LOF	Unknown	?T1IFN ?TNFα	Multiple incl vascular	**PBMC**: ↑ B cell death when cultured without stimulation **Monocytes**: differentiate into M1>M2	Nil	[84,85,92,93]
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*	[176,177,178,179,180,18140-42]
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	[182]

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Table 1 Monogenic autoinflammatory disorder summary table (Continued)

Condition	Gene/s	Protein	MOI	GOF/LOF	Pathway	Cytokine group	System involved	Human cell model	Potential murine model	Reference/s
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	[47,48,51,183]
FCAS2	*NLRP12*	NLRP12	AD	LOF	NF-κB ? Inflam	TNFα, IL-6, IL-1β	Skin Multiple	**HEK293T cells**: NF-κB luciferase assay **PBMC**: ↑ spontaneous TNF-α, IL-6, IL-1β c/w WT	*NLRP12*$^{-/-}$ mice	[184-186]
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	[178, 179, 187, 188]
H syndrome	*SLC29A3*	SLC29A3	AR	LOF	Unknown	Unknown	Multiple		*ENT3*$^{-/-}$ mice	[189,190]
HA20	*TNFAIP3*	A20	AR	LOF	NF-κB Inflam		Multiple	**HEK293T cells**: NF-κB 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*	[14]
HIDS	*MVK*	MVK	AR	LOF	Inflam	IL-1β	Multiple	**PBMC**: ↑ IL-1β, IL-6 and TNFα at rest and with stimulation **EBV-LCL**: accumulation unprenylated Rab proteins (temperature dependent)	*Mvk*$^{-/+}$ mice	[191-195]
HOIL1 deficiency	*RBCK1*	HOIL1	AR	LOF	NF-κB	Multiple	Multiple	**Fibroblasts, B cells**: ↓NF-κB 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)	[76]
HOIP deficiency	*RNF31*	HOIP	AR	LOF	NF-κB	Multiple	Multiple	**Fibroblasts**: ↓IKK phosphorylation, IL-6 production in response to TNF or IL-1β stimulation **B cells**: ↓ CD80 up-regulation 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	[77]
HYDM1	*NLRP7*	NLRP7	AR	Unknown	? Inflam ? NF-κB	Unknown	Placenta	**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)	[180, 181, 196-199]
Majeed syndrome	*LPIN2*	Lipin 2	AR	LOF	Inflam	IL-1β	Bone Skin Multiple	**HEK293T cells**: PAP activity assay. Hepa1-6 cells: PPARα luciferase assay.	*Lpin2*$^{-/-}$ mice	[200-203]
MSPC	*NLRP1*	NLRP1	AD	GOF	Inflam	IL-1β	Skin	**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*	[153,204]
ORAS	*OTULIN*	Otulin	AR	LOF	NF-κB	TNF	Multiple	**HEK293T cells**: transfection of mutant ↑NF-κB pathway c/w WT; NF-κB luciferase assay showed ↓inhibitory effect of mutant Otulin c/w WT **T cells**: Normal proliferation and NF-κB response to TCR stimulation **B cells**: Normal proliferation and NF-κB 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	[205,206]
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	[207,208]
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 show interaction between PSTPIP1 and pyrin **Macrophages**: ↓invasion and podosome formation **T cells**: ↓numbers, ↓proliferation response to mitogen. Normal migration **PBMC**: ↓ IL-1Ra, ↑IL-1β, IL-6, TNFα and GMCF in response to multiple stimuli. siRNA knockdown of *NLRP3* ↓IL-1β in response to LPS	Rosa-*PSTPIP1* A230T$^{STOP\ del/+}$ mice	[209-213]

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Table 1 Monogenic autoinflammatory disorder summary table (Continued)

Condition	Gene/s	Protein	MOI	GOF/LOF	Pathway	Cytokine group	System involved	Human cell model	Potential murine model	Reference/s
PLAID	*PLCG2*	PLCγ2	AD	GOF	Unknown	Unknown	Multiple	**COS7, A20 cells**: transfection model. Mutants- ↑phospholipase activity at subphysiological 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*	[79]
PRAAS	*PSMB8, PSMB9, PSMB4, PSMA3, POMP*	PSMB8, PSMB9, PSMB4, PSMA3, POMP	AR	LOF	?NF-κB ?IFN	T1IFN	Multiple	**HeLa cells**: Transfection studies show poor formation of proteasome with mutant c/w WT **Primary fibroblasts**: ↑ precursor complexes in patients. siRNA knockdown 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	[59,214]
PRP	*CARD14*	CARD14	AD	GOF	NF-κB		Skin	**HEK293T cells**: NF-κB luciferase assay **Immortalised primary keratinocytes:** Expression + NF-κB activity	Nil	[215,216]
Pustular psoriasis	*AP1S3*	AP1S3	AR	LOF	?NF-κB	IL36 IL-1	Skin	**Primary keratinocytes and dermal fibroblasts**: abnormal autophagy, accumulation of p62. Abnormal TLR2/6 signalling	Nil	[217,218]
SAVI	*TMEM173*	STING	AD	GOF	IFN	T1IFN	Multiple incl lungs, vessels	**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	[16-18]
SPENCD	*ACP5*	ACP5	AR	LOF	IFN	T1IFN	Multiple	**Primary human macrophages**: colocalisation studies**Plasmacytoid dendritic cells**: co-localisation studies. TLR9 stimulation in shRNA *ACP5* knockdown studies → ↑ transcription ISGs **HEK293T cells**: cotransfection TRAP and osteopontin followed by immunoprecipitation **THP1 cells**: shRNA *ACP5* knockdown studies → ↑ phosphorylation of osteopontin	*Acp5*$^{-/-}$ mice	[219-222]
TRAPS	*TNFRS1A*	TNFR1	AD	Unknown	NF-κB	?IL-1β	Multiple	**PBMC**: ↑surface expression TNFR1 + ↓shedding (conflicting data) **Monocytes**: ↑surface expression TNFR1 and ↓shedding; Abnormal autophagy →↑IL-1β + NF-κB 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 (13) *Tnfrsf1a*$^{C33Y/+}$ mice (232) *Tnfrsf1a*^{p55delINS} mice (10)	[1,9-11,13,223-232]
USP18 deficiency	*USP18*	USP18	AR	LOF	IFN	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*	[233]
XLPDR	*POLA1*	POLA1	XLR	LOF	IFN > NF-κB	T1IFN	Multiple	**Primary dermal fibroblasts**: ↑IFN + NF-κB response to stimulation with poly(da:dt) or TNF; ↑IRF and NF-κB pathway activation; ↓RNA:DNA levels; Lentiviral transduction of WT rescued phenotype **Fibroblast and HeLa cell line**: siRNA *POLA1* knockdown → ↑ IFN + NF-κB response to stimulation with poly(da:dt) or TNF	Nil	[234]

Abbreviations: AD, autosomal dominant; AIADK, autoinflammation with arthritis and dyskeratosis; AID, autoinflammatory disorder; AIFEC, autoinflammation with infantile enterocolitis; AILJK, autoimmune interstitial lung, joint and kidney disease; AR, autosomal recessive; BLCL, EBV-transformed B-lymphoblastoid cell lines; c/w, compared with; CD, cluster of differentiation; COPA, coatomer subunit α; DIRA, deficiency of IL-1 receptor antagonist; ER, endoplasmic reticulum; FCAS2, familial cold autoinflammatory syndrome 2; GIT, gastrointestinal tract; GOF, gain of function; HIDS, hyper IgD syndrome, HYDM1, hydatidiform molar pregnancy; Inflam, inflammasome; ISG, IFN-stimulated gene; LOF, loss of function; MOI, mode of inheritance; MSPC, multiple self-healing palmoplantar carcinoma; NFATc1, nuclear factor of activated T cell, cytoplasmic 1; ORAS, otulin-related autoinflammatory syndrome; PAAND, pyrin-associated autoinflammation with neutrophilic dermatosis; POLA, DNA polymerase α catalytic subunit; PRP, pityriasis rubra pilaris; SMS, Singleton–Merten syndrome; SPENCD, spondyloenchondrodysplasia; T1IFN, type 1 IFN; XLPDR, x-linked pigmentary disorder, reticulate, with systemic manifestations.

*Murine model prior to the description of monogenic condition.

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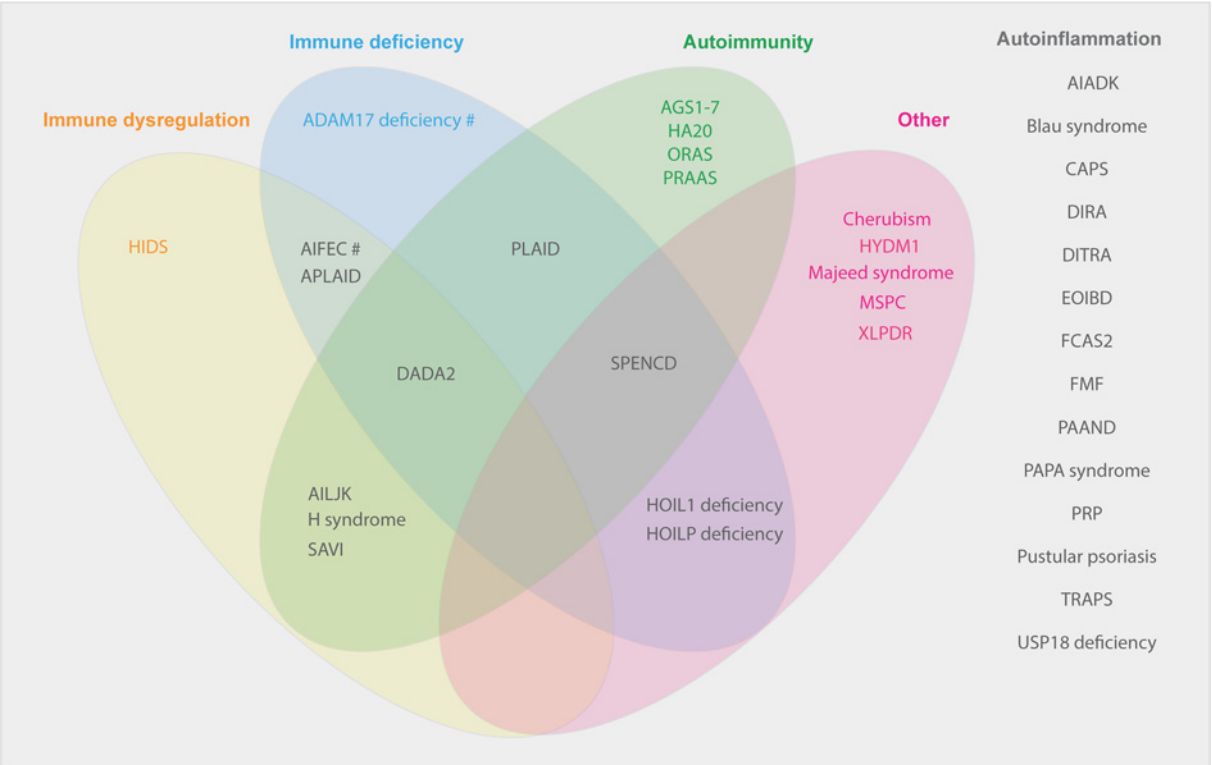

Figure 2. Phenotypic spectrum of monogenic autoinflammatory disorders

Abbreviations: AIADK, autoinflammation with arthritis and dyskeratosis; AID, autoinflammatory disorder; AIFEC, autoinflammation with infantile enterocolitis; AILJK, autoimmune interstitial lung, joint and kidney disease; DIRA, deficiency of IL-1 receptor antagonist; FCAS2, familial cold autoinflammatory syndrome 2; HIDS, hyper IgD 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; PRP, pityriasis rubra pilaris, SPENCD, spondyloenchondrodysplasia; XLPDR x-linked pigmentary disorder, reticulate, with systemic manifestations. #Susceptibility.

Genetic sequencing of autoinflammatory disorders

The phenotypic heterogeneity of what is considered a monogenic autoinflammatory disorder, 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 recognised (Figure 3). 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 [76].

NGS

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 analyse 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 [102]. 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 have 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 20000 or 4000000 variants

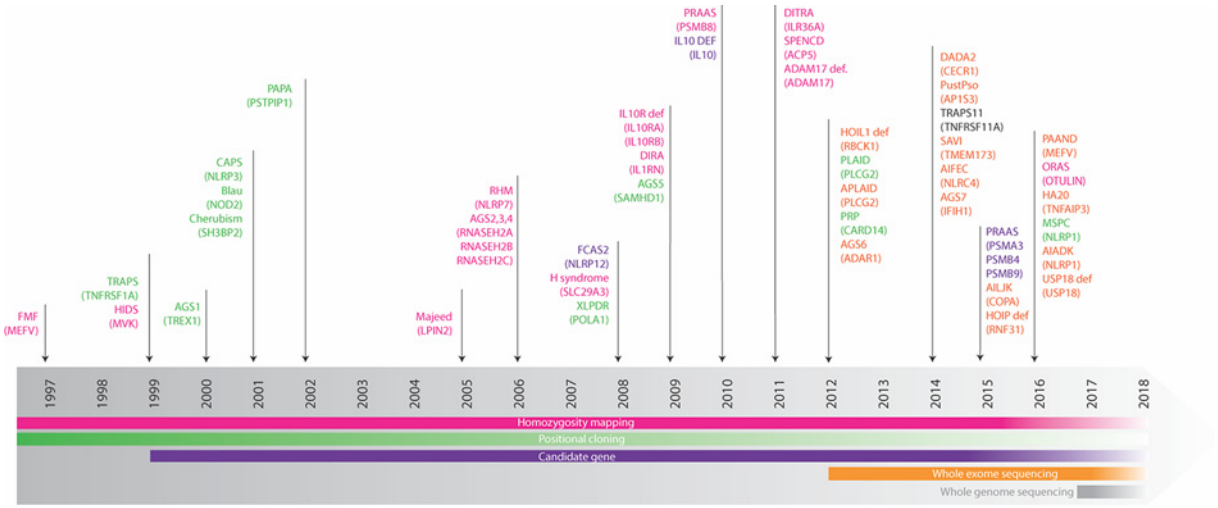

Figure 3. Timeline of monogenic autoinflammatory disorder discovery and genetic sequencing technique used

Abbreviations: AIADK, autoinflammation with arthritis and dyskeratosis; AIFEC, autoinflammation with infantile enterocolitis; AILJK, autoimmune interstitial lung, joint and kidney disease; DIRA, deficiency of IL-1 receptor antagonist; Dysreg, dysregulation (including lymphoproliferation); FCAS2, familial cold autoinflammatory syndrome 2; HIDS, hyper IgD 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; RAAS, proteasome associated autoinflammatory syndrome; PRP, pityriasis rubra pilaris; SPENCD, spondyloenchondrodysplasia; XLPDR, x-linked pigmentary disorder, reticulate, with systemic manifestations. Adapted and updated from [101] with permission granted by *Springer Nature*, licence number: 4371831027106.

respectively, an appropriate filtering strategy must be employed to determine which of these variants are potentially pathogenic [103,104].

NGS has been employed in the diagnostic evaluation of individuals with autoinflammatory disorders. Ceccherini et al. 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 cases with genetically confirmed autoinflammatory disorders [105]. The expected mutations were correctly called in most cases, although there was a failure to detect p.Val377Ile *MVK* in a number of cases 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 individual. Taking a different approach, Nakayama et al. [106] prospectively recruited individuals with a clinical diagnosis of an autoinflammatory disorder prior to any genetic testing. Using an MiSeq platform developed in house, they sequenced 9 genes (*IL1RN*, *MEFV*, *MVK*, *NLRP12*, *NLRP3*, *NOD2*, *PSMB8*, *PSTPIP1* and *TNFRSF1A*) in 108 cases. 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 further addition to the literature was by Omoyinmi et al. [107] with their development of a vasculitis and inflammation panel targeting up to 166 genes. Initially, 16 samples with known pathogenic mutations were analysed and the best performing pipeline carried over to the assessment of individuals with unknown diagnosis. Pathogenic mutations were detected in 12% of cases, 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

Somatic mosaicism in *NLRP3* causing disease was first described in 2005 in an individual with a p.Tyr507Cys variant occurring at a frequency of 16.7% detected using Sanger sequencing [108]. Somatic mosaicism in *NLRP3* has since been reported by multiple groups with a mutation frequency as low as 2.7% noted [109-116]. Importantly, a recent study highlighted that NGS was able to detect somatic *NLRP3* mutations in eight individuals symptomatic of CAPS who had previously tested negative for mutations in *NLRP3* sequencing using Sanger techniques [116]. Retrospective review of the Sanger chromatogram identified small peaks in only three of the eight cases, each with an allele frequency

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of greater than 10%, suggesting that Sanger sequencing is not a sensitive technique for detecting low frequency somatic mosaicism.

Autoinflammatory genetics summary

While the use of NGS panels for the diagnosis of autoinflammatory disorders in the clinical setting is increasing, the key limitation from a research perspective is the inability to discover new disease-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 [117-119]. While WGS has the benefit of capturing introns and intergenic regions, and detecting copy number variants [104], 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.

Modelling monogenic autoinflammatory disorders

Modelling genetic findings experimentally is of great importance in determining the clinical significance of a novel variant. In recent years, many groups have taken advantage of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 gene editing techniques. CRISPR/Cas9 gene editing utilises features of an adaptive immune response seen in bacteria and archaea.

CRISPR approaches for functional validation

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 [120]. *In vivo* editing with CRISPR/Cas9 has allowed for genome editing of fertilised mouse eggs [121,122]. 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 double-strand break (DSB). The subsequent repair of this break is mediated by non-homologous end joining (NHEJ) or homology directed repair (HDR). The former often results in frameshift 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% [122,123].

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% [124-127], but HDR efficiency of only 3–8% [126]. 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 [128]. 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) [129,130]. 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 [131,132]. 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 tRNA adenosine deaminase to edit DNA, allowing for A●T to G●C conversion [133]. The significance of this development in the potential for disease modelling and future gene editing was highlighted by the correction of the *c.845G>A HFE* mutation implicated in hereditary haemochromatosis in an immortalised-lymphoblastoid cell line [133]. 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.

Autoinflammatory disease models using mice

In vivo murine models have provided great insight into disease pathology given genetic homologies between humans and mice and the ability to create transgenic, knockout and knockin mice. For example, the initial murine model of CAPS published in 2009 by Hoffman et al. recapitulated the IL-1β-mediated inflammation [134]. Furthermore, a number of teams have used murine models to explore the skeletal consequences of CAPS [135,136]. 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 [137]. There are, however, shortcomings here. The recent attempt to model LPS-responsive and beige-like anchor protein (LRBA) deficiency using $Lrba^{-/-}$ mice, which in humans cause a range of manifestations including autoimmunity, hypogammaglobulinaemia, organomegaly and chronic diarrhoea [138], failed to recapitulate the clinical or immunological phenotype [139,140]. Furthermore, 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* may thus be similarly problematic. Additionally, humanised mouse models, using immunodeficient mice engrafted with human haematopoietic cells, are useful in the study of haematopoiesis, but have been limited in the investigation of the innate immune system due to quantificative and functional deficiencies of a number of cells including monocytes and macrophages [141].

Autoinflammatory subject derived induced pluripotent stem cells

An alternative method of modelling autoinflammatory disorders is the use of subject-derived induced pluripotent stem cells (iPSCs). This method of reprogramming somatic cells to pluripotency [142] allows for indefinite propagation as well as differentiation to a variety of human cell types that would previously have been unobtainable [143-145]. Saito et al. utilised this method in the investigation of autoinflammatory disorders involving NLRP3 [146] and NLRC4 [147]. Two individuals with somatic mutations in *NLRP3* had both wild-type (WT) and mutant NLRP3 iPSC lines generated [146]. 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 an individual suspected of CAPS but without pathogenic mutations in *NLRP3* [147]. 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 case samples demands expertise, as well as specific ethical considerations. It also requires access to case samples which may be difficult in the case of critically ill individuals who succumb to disease prior to genetic evaluation.

Having said this, the use of these cells has the potential to overcome a significant limitation in the modelling of autoinflammatory disorders to date. Using either the genetic manipulation of healthy iPSCs or subject-derived iPSCs, it is possible to explore the effect of a genetic mutation on a variety of cell types. For example, in our recent paper exploring a novel *NLRC4* variant, THP-1 monocyte like cells were used to model and the variant was determined to be pathogenic [31]. iPSCs would have permitted exploration of the effect of this variant on NK cells and T cells, allowing for addressing questions that remain unanswered including the pathogenesis of macrophage activation syndrome in this population. Likewise, the use of iPSCs differentiated to keratinocytes would allow interrogation of mechanisms of the dermatological manifestations in specific autoinflammatory disorders. As summarised in Table 1, most monogenic autoinflammatory disorders described to date have not had immunological assessment of multiple cell types.

Future directions and questions

Questions remain in the field about the phenotype–genotype correlation as well as phenotypic variability of individuals with a specific genetic variant. This includes disorders of 'variable penetrance'. There are alternative explanations for differing phenotypes among cases 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 [235,236]. The broader term of epistasis refers to possible interactions between genes [237]. 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 [238,239].

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Another possible explanation for the phenotypic heterogeneity among cases 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 [240]. 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 [241]. This finding highlights that epigenetic factors could account for phenotypic variations. There have been two publications assessing DNA methylation in individuals with monogenic autoinflammatory disorders, but in each case the diseased population was compared with a healthy control [242,243]. Vento-Tormo et al. [243] assessed the DNA methylation status of genes encoding various components of the inflammasome in monocytes of cases with CAPS and compared this with healthy controls. 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 normalised when individuals 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 cases, 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 [244]. However, drawing conclusions from only a few individuals with different genetic backgrounds may not be feasible.

Exploring factors that can account for this phenotypic variability may provide insight into the pathways involved in disease. Furthermore, the comprehensive genetic evaluation and investigation of various immune and non-immune cells of individuals with these conditions will likely enlighten the field to the intimate link between the innate and adaptive immune system as well as the role of 'innate immune' proteins in non-immune cells.

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Author contribution

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Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Abbreviations

AGS, Aicardi–Goutieres syndrome; APLAID, autoinflammation and PLCγ2-associated antibody deficiency and immune dysregulation; CAPS, cryopyrin-associated periodic syndrome; CARD, caspase activation and recruitment domain; CRISPR, clustered regularly interspaced short palindromic repeat; CVID, common variable immune deficiency; DADA2, deficiency of adenosine deaminase 2; DITRA, deficiency in IL-36Ra; DSB, double-strand break; EBV, Epstein–Barr virus; FMF, familial mediterranean fever; HDR, homology directed repair; HHMI, Howard Hughes Medical Institute; HOIL1, haem-oxidised IRP2 ubiquitin ligase 1; HSCT, haemopoetic stem cell transplantation; IFN, interferon; IL, interleukin; IL-36Ra, IL-36 receptor antagonist; IL1RAcP, IL-1 receptor accessory protein; iPSC, induced pluripotent stem cell; IUIS, International Union of Immunological Societies; JAK, Janus kinase; LPS, lipopolysaccharide; LUBAC, linear ubiquitin assembly complex; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cell; NGS, next-generation sequencing; NHEJ, non-homologous end joining; NHMRC, National Heath and Medical Research Council; NK, natural killer; NLR, NOD-like receptor; NOD, nucleotide-binding oligomerization domaim; PBMC, peripheral blood mononuclear cell; PLAID, PLCγ2-associated antibody deficiency and immune dysregulation; PLCγ2, phospholipase c γ-2; PRAAS, proteasome-associated autoinflammatory syndrome; rAAV, recombinant adeno-associated virus; rhuIL-10, recombinant human IL-10; RNASEH2, ribonuclease H2; SAMHD1, SAM and HD domain containing protein 1; SAVI, STING-associated vasculopathy with onset in infancy; sgRNA, single guide RNA; ssOligo, single-strand oligonucleotide; STAT, signal transducer and activator of transcription; STING, stimulator of IFN gene; TAK1, transforming growth factor-β activated kinase-1; TNF, tumour necrosis factor; TNFR1, TNF receptor 1; TRAPS, TNF receptor associated periodic syndrome; WES, whole exome sequencing; WGS, whole genome sequencing; WT, wild-type.

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