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Title:

Autoinflammatory Diseases – update on classification diagnosis and management

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Abstract

The spectrum of systemic autoinflammatory disorders broadens continually. In part, this is due to the more widespread application of massive parallel sequencing, helping with novel gene discovery in this and other areas of rare diseases. Some of the conditions that have been described fit neatly into a conventional idea of autoinflammation. Others, such as Interferon-mediated autoinflammatory diseases, are broadening the concept which we consider to be autoinflammatory disorders. There is also a widening of the clinical phenotypes associated with certain genetic mutations, as genetic testing is used more regularly and increasing numbers of patients are screened. It is also increasingly evident that both autoinflammatory and autoimmune problems are frequently seen as complications of primary immunodeficiency disorders.

The aim of this review is to provide an update on some recently discovered conditions, and to discuss how these disorders help to define the concept of autoinflammation. The review will also cover recent discoveries in the biology of innate-immune mediated inflammation, and describe how this has provided the biological rationale for using anti-IL-1 therapies in the treatment of many such conditions. Finally, we discuss the importance of recognising somatic mutations as causes of autoinflammatory clinical phenotypes, and provide practical advice on how this could be tackled in everyday clinical practice.

Introduction

The field of autoinflammatory disorders has expanded significantly in the last decade. In addition to the prototypic conditions such as hereditary fever syndromes, which have defined this field, there are number of new conditions which are included in this spectrum. Furthermore, recent advances in basic sciences have provided new insights into biology of the innate-immune mediated inflammatory responses. The aim of this review is to highlight some of the recently described disorders which have helped to expand the concept of autoinflammation. In addition, we will discuss new findings related to the function of pyrin that provide important insights into the biology of inflammatory responses. Finally, we will discuss new approaches toward investigating patients who present with autoinflammatory-like disorders.

Expanding concept of autoinflammation

IL-1 mediated autoinflammatory syndromes

Familial Mediterranean Fever (FMF) is the first systemic autoinflammatory disorder (SAID) to be genetically defined. Biallelic mutations in the *MEFV* (MEditerranean FeVer) gene, encoding pyrin, were identified as a cause of FMF by two groups, working independently in 1997 [1 2]. However, it was not until 1999 that the concept of autoinflammation was first proposed [3]. This followed the discovery of the genetic basis for another SAID, TNF receptor-associated periodic fever syndrome (TRAPS), where heterozygous mutations in the *TNFRSF1* gene were shown to cause the disease [3]. Both of these conditions are characterised by seemingly unprovoked episodic sterile inflammation manifesting as, but not limited to, unexplained pyrexias, skin rashes, serositis, arthralgia and myalgia. These episodes will usually resolve spontaneously and are not associated with production of autoantibodies or autoreactive T cells. In both conditions the cause relates to genetically determined dysfunction of the innate immune system. Pyrin is predominantly expressed in neutrophils, which are the main effector cells associated with inflammation in FMF, whilst the TNF signalling pathway is critical in early stages of the innate immune response. The term autoinflammation was therefore coined to capture these unique clinicopathological features of innate immune-mediated inflammation, which differentiate these disorders from disturbances in adaptive immune function, associated with established autoimmune disorders. The genetic basis of two other SAIDs, hyperimmunoglobulinaemia D with periodic fever syndrome (HIDS), due to biallelic mutations in the mevalonate kinase (*MVK*) [4 5], and cryopyrin-associated periodic fever syndromes (CAPS) due to heterozygous mutations in *NLRP3* [6 7], were identified shortly after the term autoinflammation was introduced. Both of these conditions are typical autoinflammatory disorders. In CAPS, gain of function mutations in *NLRP3* result in inappropriate activation of the NLRP3 inflammasome and dysregulated IL-1β release [8], whilst in HIDS, the link between deficiency of mevalonate kinase activity and the pathological innate-immune inflammatory response was less well understood until very recently. However, it now appears that the biochemical and inflammatory pathways are closely linked, and that, in the case of HIDS, a defect of geranylgeranylation results in inappropriate IL-1β release [9].

Although from an immunopathogenic perspective these conditions initially seemed to comprise a heterogeneous group of disorders, they all share a common feature, which is responsiveness to IL-1 blockade. Both anakinra (recombinant anti-IL1 receptor antagonist) and canakinumab (anti-IL1β monoclonal antibody) are now licensed for treatment of CAPS [10]. The early reports from a phase III trial of canakinumab in TRAPS, HIDS and Colchicine Resistant (CR) FMF are very favourable for all three conditions [11]. In addition, there are numerous case series reported in the literature describing successful use of anakinra and canakinumab for treatment of these disorders. Now that the basic biology of these conditions is much better understood, it is no surprise

that IL-1 blockade is an effective treatment modality. However, even before this was fully realised, the favourable clinical response to IL-1 blockade became one of the defining features of autoinflammatory conditions. The IL-1 superfamily of cytokines comprises of 11 members, and, in addition to IL-1β, there are other members of this family, which have been implicated in the pathogenesis of SAIDs. Deficiency of naturally IL-1 Receptor antagonist (Ra) or DIRA was described in 2009 [12] and deficiency of IL-36Ra or DITRA, as a cause of generalised pustular psoriasis with systemic inflammation, was identified in 2011 [13].

IL-18 mediated autoinflammation and susceptibility to MAS

Despite the fact that the NLRP3 inflammasome processes both pro-IL-1β and pro-IL-18 (another potent proinflammatory cytokine from the IL-1 superfamily) into their active forms, targeted IL-1β blockade alone remains extremely effective in patients with CAPS. However, recently a new autoinflammatory disorder was identified, which is characterised by excessive IL-18 release, due to gain-of-function mutations in *NLRC4*. Two mutations, p.V337S and p.V341A, located in the NACHT domain, arising *de novo* in two unrelated families, were shown to cause an autoinflammatory phenotype associated with enterocolitis and macrophage activation syndrome (MAS) [14 15]. In total, four patients were described, three from one family and one other sporadic case. All patients presented with recurrent fevers and early onset enterocolitis; one of these patients died in infancy and three out of four developed MAS, two in infancy and one later in life. NLRC4 is an innate immune sensor and, similarly to NLRP3, when activated it associates with ASC (apoptosis associated speck-like protein containing a CARD) and leads to the assembly of a caspase-1 activating inflammasome. However, mutated forms of the NLRC4 inflammasome, which in transfection experiments were shown to have increased tendency towards oligomerisation and, therefore, activation, appear to process IL-18 in preference over IL-1β. Although the serum levels of both cytokines were elevated in all patients, the IL-18 levels were several folds higher then IL-1β, unlike the findings in patients with Chronic Infantile Neurologic, Cutaneous, Articular syndrome (CINCA) also known as neonatal onset multisystem inflammatory disease (NOMID). Additional studies also showed increased spontaneous and induced macrophage activation and pyroptosis as the main source of elevated IL-18. These cases illustrate that an intrinsic monocyte defect associated with excessive IL-18 release can result in MAS. This pathogenic mechanism may be important in clinical scenarios, where a cytotoxic defect is not thought to be causative of MAS, and, therefore, IL-18 could be a potential therapeutic target in conditions such as systemic juvenile idiopathic arthritis (sJIA). Interestingly, another activating *NLRC4* mutation p.H443P has been identified as a cause of familial cold autoinflammatory syndrome (FCAS) in a large Japanese family [16]. None of these patients developed MAS, despite the fact that this mutation was also located in the NACHT domain; however, it is unknown if their IL-18 levels were significantly elevated, since this was not measured. As more patients with *NLRC4* mutations are identified in the future it is possible that we will see a spectrum of clinical phenotypes associated with this gene, which is reminiscent of the history of CAPS.

A20 haploinsufficiency

Another novel immunopathogenic mechanism that leads to the development of SAID has recently been identified by Zhou e*t al*[17]. The authors describe Behçet's disease-like disorder due to high penetrance heterozygous mutations in *TNFAIP3* (tumor necrosis factor–induced protein 3; also known as A20). This protein has an N terminal ovarian tumour (OTU) domain, which is responsible for the deubiquinating activity of this protein, and seven zinc-finger (ZnF) domains, which have an E3 ubiquitin ligase function. Consequently, A20 regulates the ubiquitination status and, therefore, also regulates the levels and function of a number of intracellular proteins that are targeted for

proteasome-mediated degradation. The majority of disease-causing mutations (5/6) were found in the OTU domain, leading to a truncated protein which was not expressed in patients' PBMC. This appears to result in a haploinsufficient state of wild type A20, that is associated with increased activation of NF-κB and the mitogen-activated protein (MAP) kinases, p38 and JNK, due to increased degradation of IκBα, an NF-κB inhibitor. IκBα is normally deubiqunitated by A20, preventing its destruction by the proteasome. IκBα in turn prevents phosphorylation of IKKα/IKKβ, which is necessary for activation of NF-κB and nuclear translocation of the p65 component of the NF-κB complex. Altogether this leads to a strong pro-inflammatory state, which was illustrated by significantly increased levels of pro-inflammatory cytokines (IL-1β, TNF, IL-6, IL-18, IL-17) in patients' sera and also in supernatant from patient-derived PBMCs stimulated with LPS. Another potential effect of A20 haploinsufficiency was suggested by a murine study [18 19] that demonstrated a negative effect of this protein on NLRP3 inflammasome activation and this was independent of A20's effect on NF-κB regulation. Interestingly, constitutive activation of the NLRP3 inflammasome was also demonstrated in patient-derived PBMCs and the fact that anti-IL1 inhibition was used successfully in one patient, albeit at a very high dose, supports the notion that NLRP3 inflammasome dysregulation is one of the consequences of A20 haploinsufficiency.

Interferon-mediated autoinflammatory diseases

Although dysregulation of IL-1 release is still one of the main biological outcomes resulting from the genetic mutations associated with SAID, there is now a wide range of conditions, with an autoinflammatory clinical phenotype, but due to chronic excessive type I interferon (IFN) production. The term, type I interferonopathy, was proposed in 2011 [20], to describe this growing number of monogenic disorders, and, more recently, an umbrella term interferon-mediated autoinflammatory diseases (IMAD) was introduced. It is beyond the scope of this review to provide a detailed account of these conditions, and this topic was recently comprehensively covered [21], but it is worthwhile briefly mentioning some of the main biological and clinical aspects of these conditions that separate them from IL-1 mediated disorders. In Aicardi-Goutières syndromes (AGS) a range of genetic mutations lead to excessive IFN-producing responses, due to several different mechanisms. These include accumulation of endogenous nucleic acids, enhanced sensitivity or spontaneous activation of nucleic acids sensors, such as RIG-I or MDA5, dysregulated negative feedback regulation of nucleic acid-sensing pathways and, lastly, defects in pathways that modulate type I IFN responses, which are not due to nucleic acid sensing [21]. Another group of disorders that come under this category includes systemic inflammation, panniculitis, and myositis due to proteasome-associated autoinflammatory syndrome (PRAAS) or chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE). Initially, all affected individuals with PRAAS/CANDLE were found to have biallellic mutations in *PSMB8*, resulting in deficiency of proteasome subunit β 8 (PSMB8), leading to abnormal function of the immunoproteosome [22-24]. However, more recently a similar phenotype was also described in patients with loss of other proteasome subunits, respectively due to the mutations in *PSMA3*, *PSMB4* and *PSMB9* genes [25]. Patients with PRAAS present with a constellation of signs and symptoms that include recurrent fevers, joint contractions with muscle atrophy, partial lipodystrophy, hepatomegaly and basal ganglia calcification. Although IFNs are known to induce formation of immunoproteosome, it is still not entirely clear how immunoproteosome dysregulation leads to elevated IFNs, increased IFN signature, high serum IL-6 levels and constitutive STAT 1 phosphorylation, which have all been described in these patients. Many patients also produce non-specific autoantibodies, which are not linked with an obvious autoimmune process. Patients with AGS tend to present with a less obvious inflammatory phenotype. Often this disorder mimics in utero acquired viral infection, and patients classically present with leukoencephalopathy, typified by basal ganglia calcifications and progressive

cerebral atrophy, as well as lymphocytosis and elevated IFN-α in cerebrospinal fluid. Some clinical features of AGS tend to resemble SLE, including arthritis, thormobocytopenia, lymphopenia and antinuclear antibodies. SLE is considered to be a prototypic autoimmune disorder, in which the pathogenesis and breakdown of adaptive-immune control is linked with high IFN drive, but there are also studies which show direct effects on the innate immune cells in SLE [26]. It seems that high IFN drive can lead to either a clinical phenotype that is either predominantly autoinflammatory in character, or results in the breakdown of self-tolerance, which is a cardinal feature of classical autoimmune diseases. The immunological disease continuum (IDC) was proposed in 2006 [27], as a way of classifying the majority of inflammatory disorders according to their autoimmune or autoinflammatory characteristics, with monogenic autoimmune and monogenic autoinflammatory diseases located at opposite ends of the IDC spectrum.

It would certainly seem that IMADs could be classified in this fashion. In many chronic inflammatory disorders there is a significant overlap of autoinflammatory and autoimmune features, with rheumatoid arthritis [28]being one such example . It is probably an oversimplification, to an extent, to consider abnormalities of either the innate of adaptive immune systems in isolation, since, as demonstrated, by the IMADs, over time abnormalities within innate immune system will eventually trigger pathogenic responses in the adaptive immune system. This phenomenon has recently also been recognised to occur in IL-1 mediated autoinflammatory disorders. It has been known for some time that patients with CAPS have higher levels of TH-17 cells, which are typically linked with autoimmunity, but in these patients the presence of TH-17 cells has not resulted in clinically obvious autoimmune disease [29]. Nevertheless, it is thought that these cells might be responsible for the more chronic inflammatory response. A recent paper by Arbore *et al*, has demonstrated that NLRP3 inflammasome activity within CD4 T cells is necessary to drive TH-1 differentiation and for production of optimal IFN-γ responses during a viral infections [30]. They also showed that CAPS patients had hyperactive Th1 responses and that this was associated with increased IL-1β and IFN-γ production by Th1 CD4 T cells. Therefore it seems that overlap between the innate and adaptive immune responses is probably even greater than previously thought. With this in mind, a classification model such as IDC can still provide a useful template to classify chronic inflammatory diseases.

Immunodeficiency and immune dysregulation overlap

It has been recognised, for some time now, that immune-dysregulation is a frequent feature of many primary immunodeficiencies (PIDs). In the case of the newly described CTLA4 haploinsuficiency, in addition to the common variable immunodeficiency (CVID)-like phenotype, these patients also develop classical autoimmune complications, such as autoimmune cytopenias, and end organ damage, due to extensive autoreactive CD4+ T cell infiltrates [31]. On the other hand, another group of patients with immunodeficiency of CVID-like characteristics, due to gain-of–function mutations in phospholipase Cγ2 (*PLCγ2),* were found to suffer from cold-induced urticaria [32], which is also a features of FCAS, a prototypic autoinflammatory disorder. In this condition, known as cold-induced urticaria, atopy, and immune dysregulation due to alterations in PLCγ2 or PLAID, the mutated PLCγ2 leads to increased calcium flux, particularly at low temperatures. This appears to have a different effect on the activity of various immune cells, leading to reduced B cell function, but increased degranulation of mast and other cells of the innate immune system at cold temperatures. This overlap between immunodeficiency and immune dysregulation is also found in some fairly well-established disorders. Deficiency of recombinase-activating genes 1 and 2 (*RAG1/2*) is typically associated with various degrees of immunodeficiency, including severe combined immunodeficiency (SCID), and, more recently, some hypomorphic mutations have been associated with CVID-like phenotype [33]. However, RAG1/2 function is also necessary for elimination of autoreactive T and B

cells as well as development of both central and peripheral tolerance [34]. Therefore, it is not entirely surprising that patients with RAG deficiency are found to have a broad spectrum of antibodies against self-antigens, including cytokines [35]. In the case of chronic granulomatous disease (CGD), mutations in in any one of the five components of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex in phagocytes lead to a defective oxidative burst and susceptibility to invasive bacterial and fungal infections. However, many of these patients also develop severe inflammatory complications, including colitis. It has been demonstrated that macrophages from these patients also have impaired autophagy and, as a result of this, show increased IL-1β release [36]. Treatment of these patients with anakinra has resulted in rapid and sustained improvements in colitis.

We therefore propose to extend the IDC to include various PIDs that have either autoinflammatory or autoimmune manifestations (Figure 1). Although the list of conditions we propose to include is not exhaustive it provides a starting point towards more comprehensive classification of immune-mediated disorders.

Widening spectrum of pyrin-associated autoinflammatory diseases

Although FMF was the first SAID to be genetically defined it is only recently that the biological function of pyrin is being fully elucidated. It was the perceived autosomal recessive mode of inheritance for FMF that initially led to the impression that the mutations seen within *MEFV* were likely to result in loss of function, thus making pyrin a negative modulator of inflammation. However almost all 125 variants within *MEFV*, known to be causative, are of a missense nature, whereas null mutations are extremely rare [37]. Furthermore, about 30% of patients with clinical phenotype of FMF have only single mutated allele in *MEFV,* and there are also reports of seemingly autosomal dominant mode of inheritance in some families [38] [39]. Animal studies have shown that the FMF phenotype can only be achieved in knock-in-mice harbouring FMF-associated mutations of the gene encoding human pyrin, whilst MEFV knock-out, or pyrin deficient mice do not have any obvious clinical phenotype. All these observations suggest that the mutated pyrin has a pro-inflammatory or gain-of –function effect, but that the emergence of the full clinical phenotype is dependent on the gene dosage of the mutated proteins. These earlier murine studies have also shown that mutant pyrin causes increased release of IL-1β and that this was not dependent on NLRP3 but on ASC, suggesting the existence of separate pyrin inflammasome complex.

More recently Xu et al. have demonstrated that bacterial modifications, induced by toxins, such as Clostridium difficile toxin B (TcdB), in the switch I region of Rho GTPases (e.g. deamidation, glycosylation) were in some way 'sensed' by the pyrin inflammasome, resulting in IL-1β and IL-18 release [40]. Although they did not map out the entire pathway the authors did show that pyrin activation was depended on interaction with 14-3-3 protein; it had been shown previously that this interaction was dependent on the phosphorylation of S242 and S208 pyrin residues[41]. Desphosphorylation of these two residues, which occurs upon exposure to TcdB, leads to release of the inhibitory 14-3-3 protein and activation of pyrin inflammsome, which further is dependent on ASC and caspase-1.

The mapping of this pathway was carried out further by Park et al. who showed that inactivation of RhoA by bacterial toxin-mediated modifications leads to inactivation of RhoA effector kinases PKN1 and PKN2, which, in turn, maintain the phosphorylation status of S242 and S208 pyrin residues [9]. This process was recapitulated by demonstrating the range of effects certain *MEFV* mutations have on this pathway. For example, we have recently helped to describe a new autosomal dominant autoinflammatory disorder where patients present with a varied clinical phenotype, not suggestive

of classical FMF but presenting with pyoderma gangrenosum, neutrophilic dermatosis, myalgia, fevers, arthralgia and persistently raised acute-phase reactants [42]. All affected individuals were found to have heterozygous C-to-G substitution at c.726 in exon 2 of MEFV, which results in a serine-to-arginine substitution at position 242 (S242R) in the pyrin protein. Effectively, this change resulted in constitutive activation of the pyrin inflammasome, since the loss of serine at position 242 prevented phosphorylation of the protein and interaction with the inhibitory protein, 14-3-3. Park and colleagues also showed that mutations within the C-terminal B30.2 domain, where the majority of pathogenic FMF mutations are found, partially block the phosphorylation sites from access by kinases, such as PKN1. This could be due to conformational changes in the mutated proteins, but, nevertheless, the end result is to lower the threshold for activation of the pyrin inflammasome. They also showed that the defective geranylgeranylation, which occurs in HIDS, also leads to inactivation of the RhoA effector kinases, PKN1 and PKN2, producing similar effects on the pyrin inflammasome, as seen in FMF.

Vasculopathy as manifestation of autoinflammatory process

Work by two independent groups in 2014 led to the identification of autosomal recessive mutations in the CECR1 gene [43] [44]; these mutations resulting in adenosine deaminase 2 (ADA2) protein deficiency, hence the abbreviation DADA2. Since these initial reports, over 40 cases have been documented. ADA is an enzyme integral to maintenance of adenosine concentrations in the cell, the latter of which has a regulatory role in the activation or silencing of several intracellular pathways [45]. Although the pathology is well understood with regards to ADA1 deficiency resulting in severe combined immunodeficiency, ADA2 associated pathogenesis remains unclear. Increasing evidence demonstrates the additional role of ADA2 as a growth factor to ascertain the monocytic lineage [45], with zebrafish models also portraying the role of ADA2 in normal endothelial development. Taken together with abnormal endothelial development and given the results of the work by Zhou et al. [43] showing that normal ADA2 promotes the differentiation of monocytes into anti-inflammatory macrophages, mutant ADA2 therefore promotes the onset of vasculitis. The findings of another 2014 report indicated that reduced ADA2 activity leading to substantial endothelial cell damage, was due to an underlying process driven by neutrophils – the latter hypothesised by the increased expression of neutrophilic derived genes [46], although this finding has yet to be seen in other cases.

These recessive, loss-of-function mutations in CECR1 are associated with vasculitis, fevers, stroke and dysregulation of the immune system. However, the phenotypic variance observed between patients with DADA2, such as localised to severe vasculitis, indicates both epigenetic and environmental factors may play a role in development of severe disease manifestations [47]. This inherited type of early onset vasculitis bears resemblances to polyarteritis nodosa (PAN); a necrotising vasculopathy with its pathology also poorly understood.

Haemopoietic stem cell therapy (HSCT) presents as an efficacious choice of treatment, due to ADA2 expression primarily in bone marrow derived lineages. As demonstrated in the reports of Van Montfrans et al. [47] and Van Eyck et al. [48] and successful treatment of patients harbouring the p.Arg169Gln CECR1 mutation further reiterates the origination of ADA2 largely from monocytes and macrophages. Other therapeutic options include anti-TNF therapy, anti-IL-6 therapy and fresh frozen plasma containing ADA2, though the latter's practicality needs further assessment [49].

Broadening the spectrum of DADA2 associated conditions could perhaps present as an opportunity to unite an assortment of previously thought distinct syndromes. In addition to this, the possibility of identifying CECR1 mutations resulting in enzyme deficiency in other forms of vasculitis or indeed, different conditions associated with stroke and inflammation, could provide a deeper insight into the pathogenesis of such disorders.

Systemic autoinflammatory disorders of unknown etiology and somatic mosaicism/mutations

Despite the ongoing discovery of novel genetic causes for autoinflammatory disorders there is still a relatively large population of patients, estimated to be between 50-60%, who do not have a germline pathogenic mutation in any of genes associated with monogenic SAID [50]. This is particularly relevant in adult clinical practice where sporadic cases of patients with features of SAID increasingly are recognised. Some of these cases were later discovered to carry low frequency somatic mutations in known SAID related genes, arising in the haematopoetic stem cells within the bone marrow[51]. The first inkling of this phenomenon as a cause of sporadic SAID arose from the observation that somatic mosaicism of *NLRP3* was the cause of disease in previously genetically 'negative' cases of NOMID/CINCA. Somatic mosaicism, which is defined as the 'occurrence of two genetically distinct populations of cells' within an individual, is a mutation that occurs postzygotically and can either affect a proportion or subtype of cells [52]. Somatic mosaicism in exon 3 of the *NLRP3* gene was originally reported back in 2005, in a 15-year-old patient with CINCA, along with a negative family history for CAPS [53]. Of the two variants discovered in this patient, the frequency of Y570C was found to be 16.7% in whole blood, indicating that a mutated *NLRP3* gene in approximately a third of the cells were sufficient to trigger the disease; this particular Y570C variant was documented as being 'potent in inducing ASC dependent NF-kB activation', unlike the other variant (S196N) or the wild type (WT) gene alone. A case control study by Tanaka et al, indicated mosaicism as a major cause of CINCA (*NLRP3* mosaicism present in 18 out of 26 patients; completely absent in healthy controls). Notably, no mutations were detected in the controls using conventional Sanger sequencing previously [54]. More recently in 2015, Zhou et al. studied whole blood DNA from a previously identified *NLRP3* mutation-negative patient (via Sanger sequencing) who had presented with fever, chills and myalgia for several hours at a time; when blood was subjected to 'targeted deep sequencing' a potential mutation within the *NLRP3* gene was detected. Additional subcloning of the NLRP3 amplicons also validated the presence of a previously reported CAPS-related mutation 'p.Tyr570Cys', with a frequency of the mutant allele (G) at around 15% in monocytes and 17% in granulocytes, but being virtually absent in lymphocytes [51].

This disease mechanism is not limited to *NLRP3* but has also been reported with other genes associated with SAID. A 2015 study by Shinar *et al* described the first reported case of 'acquired' FMF, as opposed to the conventionally defined autosomal recessive hereditary disorder, in a patient with post polycythaemia myelofibrosis due to the rare, pathogenic variant c.1955G>A, p.Arg652His in exon 10, affecting the PRYSPRY domain of pyrin, which is known to interact with IL-1β and caspase-1 [55]. However, the presence of this variant was restricted to myeloid cells, initially at negligible levels before the onset of fever, but rising to 46% of the total *MEFV* alleles after the disease onset. It was presumed that this variant became co-segregated with JAK2 mutations, leading to a proliferating clone. On the other hand this case study demonstrated the presence of the suggested heterozygous, low penetrance variant c.2113C>A, p.Gln705Lys [56], which is likely to have served as a modifying gene in myeloid cells, thereby upregulating the proinflammatory effects of mutated *MEFV*. It should be noted that this patient did not experience an inflammatory phenotype prior to FMF development, and, therefore, this clinically silent variant is unlikely to be always pathogenic, despite reports to the contrary [57]. In line with these findings, clinical characterisation of 47 FMF mutation negative patients (i.e. no detectable mutation using PCR for common mutations in *MEFV*), compared with those heterozygous for the disease, saw that the mutation-negative patients were less likely to experience joint attacks [58]. As the reported phenotypes were only marginally distinctive, a number of conclusions could be derived; for example, there could be epigenetic

modifications of the *MEFV* gene product, which could suggest the involvement of other, unknown genes. Alternatively, the occurrence of somatic mosaicism may explain these findings in that there may be (are) mutations in *MEFV*, but only in a very small subset cells that have not been identified and sequenced.

The first case of somatic mosaicism in TRAPS was reported only recently in 2016 [59]. A 41-year-old patient exhibiting a wide spectrum of symptoms as seen with SAIDs underwent analysis of 4 genes *NLRP3*, *MEFV*, *MVK* and *TNFRSF1A*, where a 24 nucleotide deletion of the *TNFRSF1A* gene (c.255_278del) was detected in exon 3. Such deletions are associated with protein misfolding, which is likely to affect TNF binding. Although PCR and Sanger sequencing validated the presence of the mutated nucleotide peaks, the chromatogram peaks were evidently smaller than the wild-type. Targeted sequencing confirmed the presence of mosaicism, with a 7.6% mutant allele frequency in whole blood, and parental DNA corroborated with the wildtype sequence of *TNFRSF1A,* confirming the presence of somatic deletion in the patient.

The initial account of somatic mosaicism in Blau Syndrome (BS) was first reported by de Inocencio et al. in 2015 [60]. Targeted deep sequencing of both haematological and non-haematological derived DNA ascertained the presence of the p.Arg334Gln NOD2 mutation, first suspected during Sanger chromatogram analysis of a 'subtle' adenine peak at C.1001 as compared to a decrease in the wild type. The mutated allelic frequency (MAF) lay between 4.9%-11%, depending on the tissue of origin (monocytes showing the highest MAF), with parental screening showing an absence of this variant, thereby confirming its de-novo status. However more recently, the report of 'gonosomal mosaicism' – somatic mutations affecting both body and gonadal tissue [61] – where 3 members of the family across 2 consecutive generations are affected with BS, has brought new information to light [62]. Analysis of the same *NOD2* variant (p.Arg334Gln) showed presence in the embryonic layers and somatic cells; the former suggestive of the 'intrafamilial recurrence' as the patient's daughter is also affected, and also that somatic mutation transmission is very rare and unlikely to occur. The determination of the degree of gonadal mosaicism would not only indicate the level of variant distribution in the body, but could also establish transmission risk to offspring, unlike germline mutations where there is a 50% chance of transmitting the mutated allele; although this study was unable to derive this figure. The conclusion that there are milder phenotypes on the BS spectrum associated with de novo mutations than those phenotypes seen in BS patients with germline mutations is a concept that has also been seen in other SAID, such as CAPS [54 63] and TRAPS [59], as well as one BS case itself [60]. With this particular case, the presence of a somatic NOD2 mutation in the patient's haematopoietic cells is likely to be the cause of the main symptoms of an inflammatory nature. However, the later disease onset and a 'relatively benign course', with decreased joint involvement, in the 2015 study [60], as compared to other germline cases is indicative of a wider range of clinical phenotypes, albeit very dependent on the level of mosaicism.

Recognition and diagnosis of patients with somatic mutations in everyday clinical practice remains a challenge. Patients will present with variable expression of signs and symptoms and identification of somatic mutation is a technically highly demanding and expensive process. One option to help with selection of patients, in whom further investigation for somatic mutations is warranted, is to use response to therapy as part selection process. For example IL-1 blockade is a very effective treatment for a number of monogenic SAID with underlying dysregulation of IL-1 release, such as CAPS, FMF and TRAPS [10]. In these disorders administration of anti-IL1 therapies leads to rapid resolution of symptoms and disease control. We have recently used a trial of anakinra, which is a short acting IL-1 receptor antagonist, to identify patients presenting with SAID of unknown etiology [42]. These were adult patients presenting with features suggestive of SAID, but who did not

have any known germline mutations in a number of SAID-associated genes, and who did not meet the diagnostic criteria for polygenic SAID, such as Still's disease. In 9 out of 11 patients, administration of anakinra resulted in full symptom control within 4-6 weeks of starting treatment, and this was associated with a clinically significant reduction in C-reactive protein (CRP) levels. Such rapid and profound responses to a very targeted therapy implies that dysregulation of the IL-1 pathway is important in the pathogenesis of the disease in question. Therefore, it highly probable that some of these cases may turn out to have a somatic mutation in one of the relevant SAID genes.

Conclusions

It was not possible within the scope of this review to mention all interesting disorders of an autoinflammatory nature discovered in the last 12-18 months. However we have tried to provide an overview of those selected conditions and concepts which we believe are important to define this rapidly evolving field. We have briefly mentioned some of the more established therapies, such as biologicals directed against IL-1; however small molecular inhibitors targeting JAK kinases, or the recently discovered MCC950 which inhibits the NLRP3 inflammasome [64] are being introduced into clinical practice and may change the way we treat autoinflammatory disorders in the future.

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Figure 1. The Expanded Immunological Disease Continuum-including primary immunodeficiencies with autoinflammatory and/or autoimmune features

FMF – Familial Mediterranean Fever; TRAPS - TNF receptor associated periodic syndrome; CAPS-cryopyrin-associated periodic fever syndromes; HIDS- hyperimmunoglobulinaemia D with periodic fever syndrome; PAPA Pyogenic Arthritis, Pyoderma gangrenosum and Acne; DIRA-Deficiency of IL-1 Receptor antagonist; PAAND pyrin-associated autoinflammation with neutrophilic dermatosis; PRAAS-proteasome-associated autoinflammatory syndrome; AOSD-Adult Onset Still's disease; BD-Behçet's disease; sJIA- systemic juvenile idiopathic arthritis; ALPS-Autoimmune lymphoproliferative syndrome; APS-1 Autoimmune polyendocrine syndrome type 1 of APECED- autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy; IPEX Immunodysregulation polyendocrinopathy enteropathy X- linked syndrome