MANAGEMENT OF AUTOINFLAMMATORY DISEASES IN CHILDHOOD

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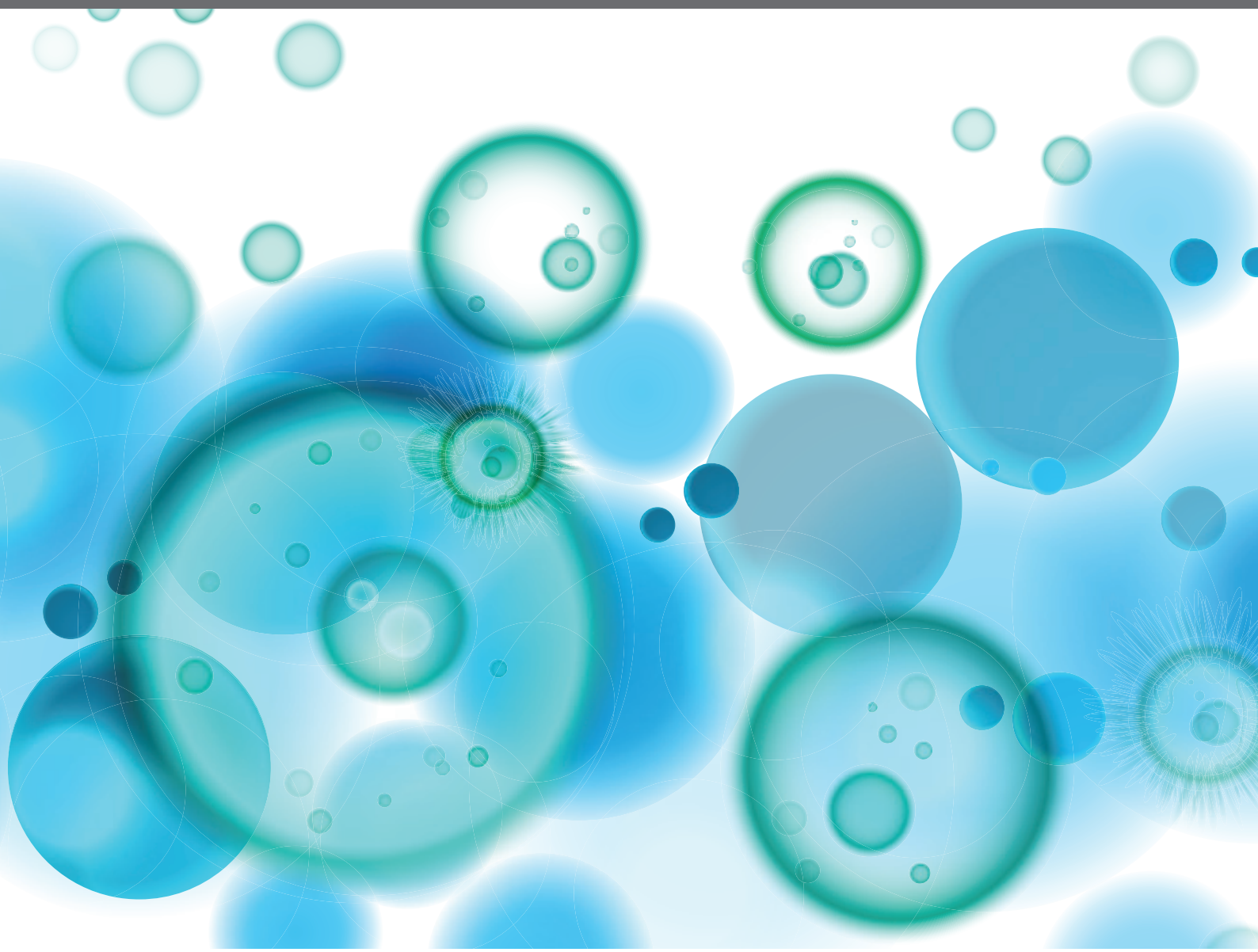

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MANAGEMENT OF AUTOINFLAMMATORY DISEASES IN CHILDHOOD

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REVIEW
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The Pyrin Inflammasome in Health and Disease

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The pyrin inflammasome has evolved as an innate immune sensor to detect bacterial toxin-induced Rho guanosine triphosphatase (Rho GTPase)-inactivation, a process that is similar to the "guard" mechanism in plants. Rho GTPases act as molecular switches to regulate a variety of signal transduction pathways including cytoskeletal organization. Pathogens can modulate Rho GTPase activity to suppress host immune responses such as phagocytosis. Pyrin is encoded by *MEFV*, the gene that is mutated in patients with familial Mediterranean fever (FMF). FMF is the prototypic autoinflammatory disease characterized by recurring short episodes of systemic inflammation and is a common disorder in many populations in the Mediterranean basin. Pyrin specifically senses modifications in the activity of the small GTPase RhoA, which binds to many effector proteins including the serine/threonine-protein kinases PKN1 and PKN2 and actin-binding proteins. RhoA activation leads to PKN-mediated phosphorylation-dependent pyrin inhibition. Conversely, pathogen virulence factors downregulate RhoA activity in a variety of ways, and these changes are detected by the pyrin inflammasome irrespective of the type of modifications. *MEFV* pathogenic variants favor the active state of pyrin and elicit proinflammatory cytokine release and pyroptosis. They can be inherited either as a dominant or recessive trait depending on the variant's location and effect on the protein function. Mutations in the C-terminal B30.2 domain are usually considered recessive, although heterozygotes may manifest a biochemical or even a clinical phenotype. These variants are hypomorphic in regard to their effect on intramolecular interactions, but ultimately accentuate pyrin activity. Heterozygous mutations in other domains of pyrin affect residues critical for inhibition or protein oligomerization, and lead to constitutively active inflammasome. In healthy carriers of FMF mutations who have the subclinical inflammatory phenotype, the increased activity of pyrin might have been protective against endemic infections over human history. This finding is supported by the observation of high carrier frequencies of FMF-mutations in multiple populations. The pyrin inflammasome also plays a role in mediating inflammation in other autoinflammatory diseases linked to dysregulation in the actin polymerization pathway. Therefore, the assembly of the pyrin inflammasome is initiated in response to fluctuations in cytoplasmic homeostasis and perturbations in cytoskeletal dynamics.

Keywords: autoinflammatory diseases, familial Mediterranean fever, pyrin inflammasome, RhoA GTPases, serine-threonine kinase, *Yersinia* toxins

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INTRODUCTION

The innate immune system forms molecular platforms to recognize components of pathogenic bacteria and to differentiate these danger signals from host motifs. The cells that form this first line of defense against pathogenic bacteria, namely macrophages, monocytes, dendritic cells, and neutrophils, express a variety of pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs). The membrane-bound family of Toll-like receptors (TLRs) is the most extensively studied group of PRRs and recognizes PAMPs in the extracellular milieu and in different types of intracellular endosomes (1). Signaling through these receptors leads to the expression of proinflammatory cytokine-inducing transcription factors, such as NF-κB. Additionally, TLR signaling triggers the activation of interferon regulatory factors that mediate the type I interferon-dependent antiviral response. A second set of pathogen recognition sensors is present in the cytosol and includes the family of nucleotide-binding domain leucine-rich repeat (NLR) proteins (NLRP1, NLRP3, NLRP7, and NLRC4), the protein absent in melanoma 2 (AIM2), and pyrin. These sensors are essential for detection of pathogens and endogenous danger-associated molecular patterns (DAMPs) inside the cell and their activation triggers the formation of multiprotein complexes, called inflammasomes (**Figure 1**) (2, 3).

NLRP1 was the first cytosolic sensor identified to form a caspase-1 activating inflammasome in response to the virulence factor lethal toxin produced by *Bacillus anthracis* (4). The antigen component of this toxin forms a membrane-inserted pore through which the anthrax lethal factor is delivered to the host cytosol. Upon cell entry, the anthrax lethal factor induces assembly and activation of the NLRP1 inflammasome. As NLRP1 plays a major role in host innate immune response, unsurprisingly, malfunctions in this inflammasome were shown to cause disease. Several common and low-penetrance polymorphisms in the *NLRP1* gene were associated with a number of autoimmune disorders, including vitiligo, systemic lupus erythematosus, inflammatory bowel disease, and celiac disease (5–7). The important role of NLRP1 was further highlighted in two publications from 2016 that for the first-time linked novel high-penetrance variants in *NLRP1* to human Mendelian monogenic disease (**Figure 1**) (8, 9).

The identification of the NLRP3 inflammasome was a major breakthrough in the field of innate immunity and autoinflammation (10). In 2004, Agostini et al. (10) showed that increased activity of the NLRP3 inflammasome is the molecular basis of the symptoms in patients with cryopyrin-associated periodic syndromes (CAPS). This study demonstrated that dominantly inherited gain-of-function (GOF) mutations in *NLRP3* cause activation of caspase-1, and an excessive release of IL-1β, which subsequently led to the recognition of IL-1 receptor antagonists and other IL-1 inhibitors as successful therapies for these disorders. NLRP3 is the best studied inflammasome and many distinct signals have been found to cause its activation including adenosine triphosphate (ATP), pore-forming bacterial toxins, crystalline and particulate structures, as well as cathepsin B released from lysosomes. The variety of activating stimuli indicates that the assembly of the NLRP3 inflammasome is likely stimulated through a common downstream signal. To date, several distinct unifying signals such as potassium efflux, mitochondrial reactive oxygen species (ROS), increased intracellular Ca^{2+}, and decreased cellular cyclic AMP (cAMP) have been proposed (8–10). However, the detailed mechanism of NLRP3 inflammasome activation has yet to be determined.

In contrast to NLRP3, the NLRC4/IPAF inflammasome responds to a limited set of stimuli and is triggered by virulence factors produced by Gram-negative pathogens known as type III (T3SS) and IV (T4SS) secretion systems (11, 12). The mechanism of NLRC4 activation is unique amongst inflammasomes in that it requires binding of another NLR-family member, the neuronal apoptosis inhibitor proteins (NAIPs). NAIPs act as sensors for PAMPs and upon direct binding to the ligands, NAIPs associate with NLRC4 to form the NAIP/NLRC4 inflammasome (**Figure 1**). This in turn induces recruitment and activation of caspase-1 and subsequently release of mature IL-1β and IL-18. Phosphorylation of NLRC4 by protein kinase Cδ is a critical event for inflammasome formation and was shown to induce a conformational change of NLRC4 (13). In 2014, two independent groups reported that novel and/or *de novo* GOF mutations in *NLRC4* cause autoinflammatory syndromes with distinct features of infantile enteropathy and macrophage activation syndrome (MAS) (14, 15).

The AIM2 protein is composed of a N-terminal pyrin domain (PYD) and a C-terminal HIN-200 domain and in contrast to NLRPs lacks the NLR/NACHT domain. Direct binding of cytosolic double stranded DNA (dsDNA) to the HIN-200 domain of AIM2 induces its release of the autoinhibitory conformation and allows inflammasome assembly and activation (16). To date, no GOF mutations in *AIM2* that cause human inherited autoinflammatory disease have been described. One possible explanation is that in contrast to NLR-containing inflammasomes that can form independently from ligands, AIM2-oligomerization is dependent of direct binding to DNA molecules.

The NLRP7 inflammasome has not been well-characterized, particularly in comparison to the other inflammasomes. One of the few described activation signals are microbial acylated lipopeptides that were shown to induce an ASC-dependent caspase-1 activation of NLRP7 (17). Interestingly, in addition to its proinflammatory role, NLRP7 also possesses properties that inhibit inflammation. Suggested mechanisms for its anti-inflammatory role include interaction with proteins that repress NF-κB signaling as well as direct sequestration of pro-caspase-1 and pro-IL-1β (18). Biallelic rare loss-of-function (LOF) mutations in *NLRP7* have been associated with recurrent hydatiform mole (19, 20).

Even though the presented inflammasomes differ in components and pattern recognition, they are all unified in their capability to mediate activation of caspase-1, which promotes the maturation of the proinflammatory cytokines IL-1β and IL-18 and the induction of inflammatory cell death (pyroptosis). Pyroptosis is morphologically different from apoptosis in that

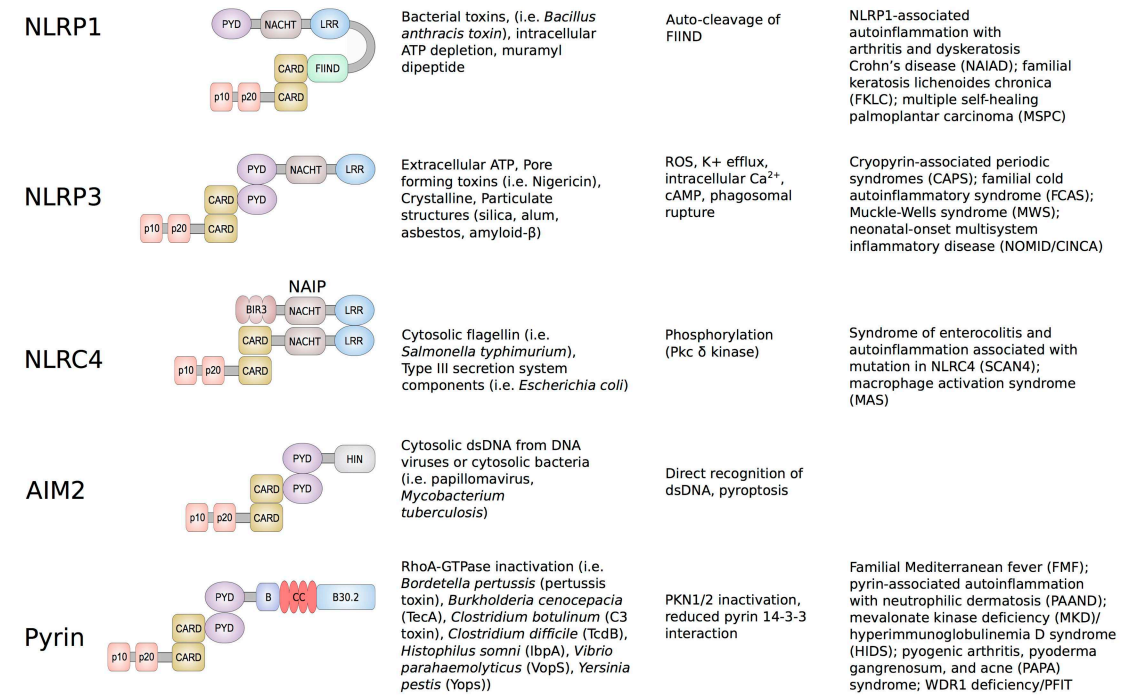

FIGURE 1 | Schematic representation of the structure and function of NLRP1, NLRP3, NLRC4/IPAF, AIM2, and Pyrin Inflammasomes. Shown are the minimal NLRP1, NLRP3, NLRC4/IPAF, AIM2, and pyrin inflammasomes, their corresponding stimulation signals, described resulting downstream effects and associated disorders. Formation of the inflammasome initiates autocatalytic activation of caspase-1, resulting in cleavage of the pro-enzyme into the active p20 and p10 subunits. The active enzyme is assembled in the form of heterodimers and cause release of mature IL-1β/IL-18 and gasdermin D cleavage.

it involves cell swelling and lysis. Pyroptosis involves caspase-1 mediated cleavage of gasdermin D (GSDMD), subsequent translocation of the N-terminal pore-forming domain to the cellular membrane and release of pro-inflammatory cytokines (21, 22). Pyroptosis can also be triggered by direct binding of LPS to caspase-11 in mouse cells and caspase-4 and 5 in human cells, which results in caspase oligomerization and cleavage of GSDMD. Cleavage of gasdermin E by caspase-3 was also shown to induce pyroptosis (23, 24). Thus, pyroptosis plays a major role in amplifying the protective immune responses during an infection (25).

Dysregulation or erroneous activation of the described inflammasomes can lead to autoinflammatory diseases, a group of genetically diverse but symptomatically similar disorders. Variability in clinical manifestations can be explained by cell-specific functions of these proteins. For example, NLRC4 is highly expressed in epithelial cells while NLRP1 is more abundant in keratinocytes, thus GOF mutations lead to severe inflammation in gastrointestinal and skin, respectively. In contrast to conventional autoimmune disorders, autoinflammatory diseases are not primarily mediated by the cells of adaptive immunity such as antigen-specific T-cells or antibodies producing B-cells. They are therefore considered the Mendelian disorders of the innate immunity (26).

IDENTIFICATION AND STRUCTURE OF PYRIN

The pyrin protein (also known as marenostrin; TRIM20), named after the Greek word for fever, is a 781-amino acid, ∼95 kDa protein that is encoded by *MEFV* on chromosome 16 (**Figure 2**). Pyrin expression is mainly confined to the cells of the innate immune system, namely granulocytes, eosinophils, monocytes, and dendritic cells. Homology analyses identified five different domains within the pyrin protein (**Figure 2**). The eponymous PYD domain (1–92) at the N-terminal end of the protein is found in more than 20 human proteins that are mainly involved in inflammatory processes. Via its PYD domain, the protein binds to the inflammasome adaptor protein, apoptosis-associated speck-like protein with a caspase recruitment domain (ASC), which subsequently causes caspase-1 mediated production of IL-1β (27).

Due to the presence of a bZIP transcription factor domain (266–280) and of two overlapping nuclear localization signals, early structural analyses suggested a nuclear function for pyrin (28). This hypothesis was further supported by a study that demonstrated that a variant protein, lacking a domain encoded by exon 2, indeed translocated to the

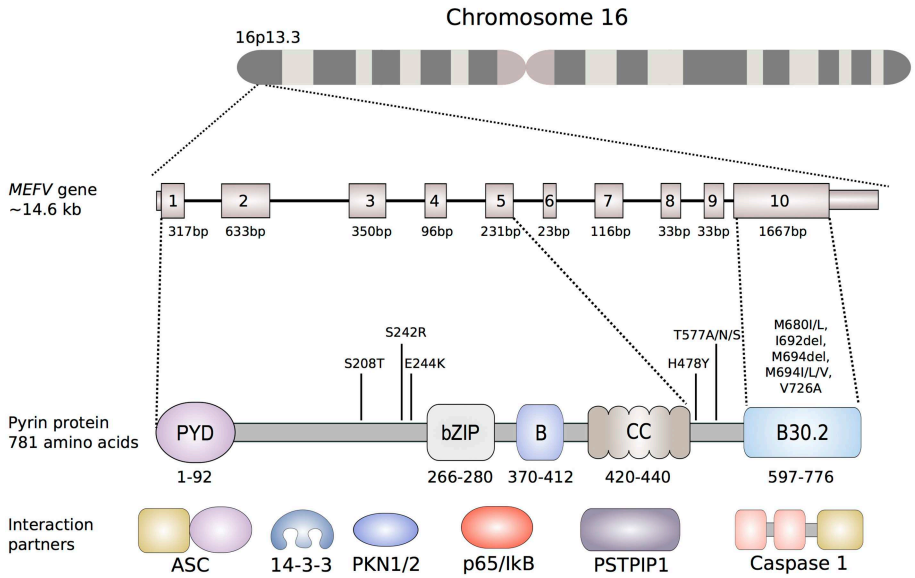

FIGURE 2 | Schematic representation of the *MEFV* gene and the encoded pyrin protein. Of the more than 300 nucleotide variants described in the *MEFV* gene, only shown are the ones that are clearly associated to disease phenotypes. The most common FMF-associated mutations reside in exon 10, that encodes the B30.2 domain (B30.2). Mutations in the B30.2 domain tend to be transmitted in an autosomal-recessive fashion, while the mutations in exons 2, 3 and 5 more often exhibit an autosomal-dominant pattern of inheritance. Known pyrin interaction partners are depicted next to their corresponding or putative interaction domain of the pyrin protein: ASC (apoptosis-associated speck-like protein containing a CARD), 14-3-3 (14-3-3 protein), PKN1/2 (serine-threonine kinases PKN1 and PKN2), p65 (transcription factor p65), IκB (NF-κB inhibitor), PSTPIP1 (proline serine threonine phosphatase-interacting protein).

nucleus and that the N-terminal fragment of pyrin interacts with the p65 subunit of NF-κB (29, 30). However, later studies investigating localization and function of pyrin found that full-length pyrin is mainly located in the cytosol and that the N-terminal half of pyrin colocalizes with both microtubules and the actin cytoskeleton (31). Furthermore, the localization of pyrin was also shown to be dependent on the expressing cell type and further research is needed to decipher the possible cell-type specific functions of pyrin (32).

The B-box (370–412) and the α-helical, coiled-coil (420–440) domain may play a role in the oligomerization of pyrin (33). These two domains were also shown to interact with the proline serine threonine phosphatase-interacting protein (PSTPIP1/CD2BP1), a protein that is important for the organization of the cytoskeleton (34). Missense mutations in *PSTPIP1/CD2BP1* cause a dominantly inherited autoinflammatory syndrome called pyogenic arthritis, pyoderma gangrenosum, and acne (PAPA) (35).

The C-terminal B30.2 domain of pyrin is of particular importance since most of the FMF-associated mutations are clustered in this domain and it is therefore essential for the molecular mechanisms leading to FMF. *In vitro* overexpression studies showed that the B30.2 domain of pyrin directly interacts with caspase-1. However, studies investigating the effect of FMF-associated mutations on the binding affinity of B30.2 to caspase-1 led to conflicting results (36, 37).

FAMILIAL MEDITERRANEAN FEVER

FMF, the prototypic autoinflammatory disease, is characterized by recurrent episodes of fever with serosal inflammation manifesting with severe abdominal or chest pain, arthralgia, monoarticular arthritis and limited erythematous skin rash. The onset of symptoms is typically in childhood and the episodes of fever with abdominal/chest pain usually resolve within 48–72 h. Laboratory findings of FMF resemble an attack of acute inflammation with elevated erythrocyte sedimentation rate and C-reactive protein, leukocytosis, thrombocytosis, as well as fibrinogen and immunoglobulins in the blood (38). Inflammation in FMF patients is well-controlled by treatment with colchicine or IL-1 inhibitors. The most severe complication of FMF is a secondary serum amyloid A (SAA) protein amyloidosis that can affect various tissues, commonly kidneys. These patients usually have severe and chronic inflammation lasting for many years and are often resistant or non-compliant to treatment with colchicine. The mortality rate was very high in the era before colchicine and IL-1 inhibitors. The incidence of SAA amyloidosis is far lower nowadays with improved health care for FMF patients in most affected populations.

MODE OF INHERITANCE OF FAMILIAL MEDITERRANEAN FEVER

In 1958, Heller et al. (39) described FMF as a genetic disease that exhibits autosomal dominant inheritance with incomplete penetrance, due to the high prevalence of disorder in the

non-Ashkenazi Jewish population. Later studies with larger cohorts postulated that FMF is an autosomal recessively inherited disorder and pseudodominance was suspected as the reason for divergent results in earlier studies (40, 41). Based on these segregation analyses FMF has long been considered a recessive illness and the *MEFV* positional cloning studies were therefore based on an autosomal-recessive model of inheritance (28). *MEFV*, as the causative gene for FMF, was identified by two independent groups in 1997 (28, 42).

The advent of genetic testing and the increase in diagnosed patients led to the recognition that approximately 30% of all cases clinically diagnosed with FMF carry only one demonstrable mutation despite extensive search for a second disease-causing variant (43–45). This observation suggested that a single pathogenic mutation in *MEFV* in the presence of other genetic or environmentally permissive factors might be sufficient to trigger excessive activation of the pyrin inflammasome. In addition, it was shown that asymptomatic carriers for monoallelic FMF mutations, for instance unaffected parents of FMF patients, exhibit a biochemical phenotype such as elevated inflammatory biomarkers (46, 47). The described findings, together with the fact that a recessive model of inheritance would favor disease-associated variants that are null mutations, prompted a re-evaluation of the LOF recessive model of FMF inheritance.

FMF-associated missense mutations reside in exon 10, which encodes the B30.2/SPRY domain (**Figure 2**). Within this domain, an FMF mutation hot-spot is identified between amino acid residues 680 and 726, with Met680Ile, Met694Val, and Val726Ala as the most frequent disease-causing variants. The carrier frequency is as high as 10% in multiple populations in the Middle East and Mediterranean basin, raising the possibility of balancing selection.

THE PROINFLAMMATORY ROLE OF THE PYRIN INFLAMMASOME

Early studies with mice expressing a truncated form of pyrin showed an increase in caspase-1 activation and therefore suggested an anti-inflammatory function of pyrin (48). However, later studies postulated the existence of a pyrin inflammasome and a potential proinflammatory role for pyrin, but the mechanisms that lead to its activation remained elusive (49). The first study showing that pyrin regulates IL-1 processing and release in human myeloid cells was published in 2007 (50). Subsequently in 2009, Gavrilin et al. (51, 52) postulated based on siRNA knockdown experiments that pyrin forms an inflammasome in human monocytes and transfected THP1 cells upon infection with *Francisella tularensis* and *Burkoholderia cenocepacia*. Another important series of experiments that helped to distinguish between an anti- or proinflammatory function of pyrin were performed in 2011 in animal model studies (53). Chae et al. (48) initially generated pyrin knock-out mice that lack both copies of a murine ortholog of pyrin and these mice developed normally and had no signs of inflammation, which strongly argues against a LOF model. Subsequently, pyrin knockin (KI) mice were generated with murine pyrin fused to the human B30.2 domain carrying the most common FMF mutations, Met680Ile, Met694Val, and Val726Ala (53). Because murine pyrin lacks the B30.2 domain, the generation of the fusion protein was necessary to investigate pyrin's role in the pathogenesis of FMF. Only homozygous KI mice, carrying two copies of the mutated fusion protein, developed a severe inflammatory phenotype. These findings indicate a GOF mechanism for pyrin inflammasome activation.

ACTIVATION AND FUNCTION OF THE PYRIN INFLAMMASOME

The engagement of pyrin through the appropriate stimulus leads to the assembly of an inflammasome, and the subsequent activation of caspase-1 and release of IL-1β and IL-18. An important step in this process is the recruitment of ASC to pyrin. Via its N-terminal PYD domain, ASC enters a PYD-PYD homotypic interaction with pyrin, which induces its oligomerization of micrometer-sized assemblies, the ASC specks (49, 54–56). Subsequently, pro-caspase-1 is recruited to the specks via interaction of the caspase recruitment domain (CARD) of ASC with the CARD domain of the pro-caspase-1. The resulting clustering of pro-caspase-1 molecules promotes a proximity-induced autoproteolytic induction of caspase activity (3, 57). The autocleavage of pro-caspase-1 leads to the formation of active caspase-1 p10/p20 tetramer, which processes pro-IL-1β and pro-IL-18 to their mature forms. Another mechanism contributing to inflammation in FMF is GSDMD-mediated pyroptosis, which results in the release of cytoplasmic content, including mature IL-1β and IL-18 (21, 58). These cytokines then work as potent initiators and amplifiers of innate immune responses and induce a variety of defense processes including fever, hematopoiesis, lymphocyte activation, leukocyte attraction, and antibody synthesis (59). The described pyroptotic mechanisms also lead to the release of ASC specks into the extracellular space where these specks exhibit "prionoid" features to further promote the inflammatory response (55, 60).

The type of ligands or signals that trigger pyrin activation remained unknown until 2014, when Xu et al. (61) demonstrated that pyrin can sense pathogen-induced modifications of host Rho guanosine triphosphatases (Rho GTPases). This study showed that TcdB, a virulence factor of *Clostridium difficile*, known to glycosylate and thereby downregulate the activity of a small Rho GTPase, RhoA, can activate the pyrin inflammasome (**Figure 3**) (62). Bone marrow-derived macrophages (BMDMs) treated with wildtype TcdB exhibit a strong pyrin-mediated inflammasome reaction and increased caspase-1 activity, resulting in pyroptosis. This effect was abolished when a glucosyltransferase-defective mutant form of TcdB was used. The described modification of RhoA is not restricted to TcdB. Other bacterial proteins, such as C3 toxin (*Clostridium botulinum*), pertussis toxin (*Bordetella pertussis*), VopS (*Vibrio parahaemolyticus*), IbpA (*Histophilus somni*), as well as the TecA toxin of *Burkholderia cenocepacia*, were also shown to add distinct modifications to the switch I

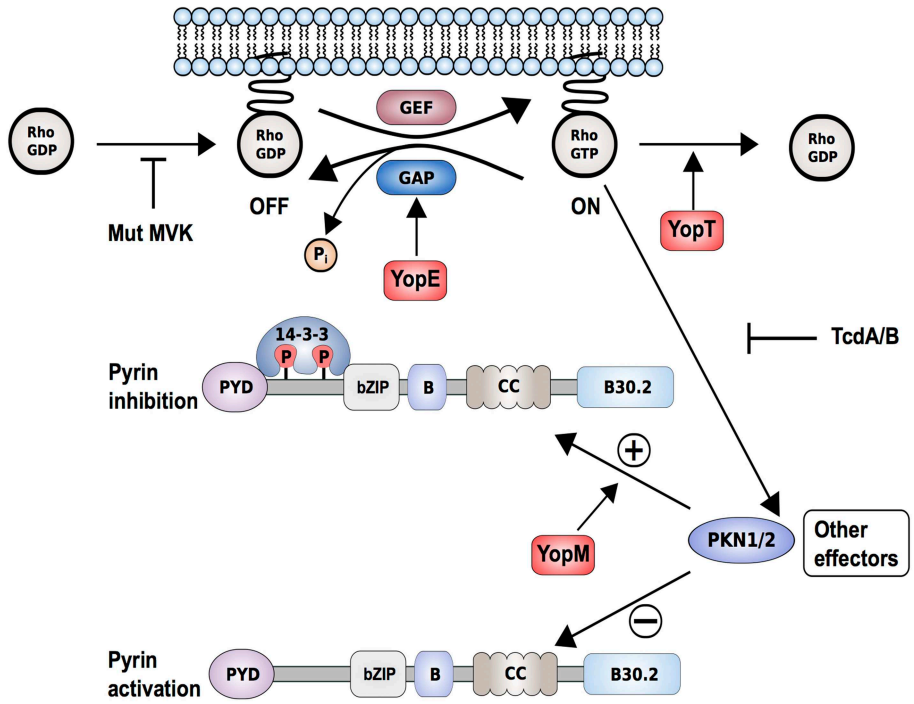

FIGURE 3 | Proposed interaction model of *Yersinia pestis* effectors, Yops, with the pyrin inflammasome. *Yersinia pestis* effectors YopE (by promoting GTP hydrolysis) and YopT (by catalyzing cleavage and detachment of RhoA from the plasma membrane) inactivate RhoA. This results in reduced PKN1/2 activity, which in turn promotes the assembly of a pyrin inflammasome. Other bacterial toxins such as Clostridium difficile toxin B/TcdB (by glycosylation) or deleterious mutations in key enzymes of the mevalonate kinase pathway (Mut-MVK) also cause RhoA-inactivation. By delivering the additional effector YopM to the host cell, *Yersinia pestis* maintains virulence. YopM stimulates the PKN-1/2-mediated phosphorylation of pyrin and thereby the inhibition of pyrin inflammasome by hijacking host kinases PKN-1/2.

region domain of RhoA (63–66). Due to the variety of post-translational modifications and the lack of direct interaction between pyrin and RhoA, it was proposed that pyrin does not directly recognize specific modifications but rather is triggered by an indirect signal downstream of RhoA. The fact that Rho GTPases control many aspects of the actin cytoskeleton dynamics led to a hypothesis that pyrin may sense changes in the cytoskeleton organization. Further support for this postulation came from studies on WDR1, a regulator of actin-cytoskeleton dynamics. Mice and humans deficient for the *WDR1* gene present with a distinct IL-1 independent, but IL-18 dependent autoinflammatory phenotype and thrombocytopenia (67). WDR1 deficiency leads to an increase in actin polymerization, and these alterations are in part detected by the pyrin inflammasome (68).

MOLECULAR MECHANISMS OF PYRIN INFLAMMASOME ACTIVATION

Four recent publications elucidated the molecular mechanisms of pyrin regulation downstream of RhoA and demonstrated how changes in host Rho GTPase activity trigger pyrin inflammasome activation (69–72). Park et al. (70) showed that the RhoA-dependent serine/threonine-protein kinases PKN1 and PKN2 directly phosphorylate pyrin at positions Ser208 and Ser242. This results in an interaction of pyrin with the chaperone proteins 14-3-3ε and 14-3-3τ. This interaction keeps pyrin in an inactivate state and prevents the formation of an active inflammasome. The inactivation of RhoA through bacterial toxins causes a decrease in PKN1 and PKN2 activity and results in reduced levels of phosphorylated pyrin. This in turn releases pyrin from the inhibitory 14-3-3 proteins and facilitates the formation of an active pyrin inflammasome.

These findings were confirmed by Gao et al. (71) through experiments in murine BMDMs and dendritic cells. They showed that 14-3-3 protein binding to murine pyrin is dependent on phosphorylation of the corresponding residues (Ser205 and Ser241) in the murine pyrin ortholog. The stimulation with RhoA inactivating toxins causes a reduction in phosphorylated pyrin, the dissociation of 14-3-3 protein and subsequently the formation of a pyrin inflammasome complex.

Further evidence for the important role of phosphorylation in the regulation of pyrin came through the recent description of a new dominantly inherited disorder called pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND) (69, 73). PAAND is caused by the amino acid substitution in exon 2 of *MEFV* at position 242 (Ser242Arg) or 244 (Glu244Lys) that are critical for PKN-mediated phosphorylation of pyrin (**Figure 2**). Overexpression of Ser242Arg or Glu244Lys mutated proteins in

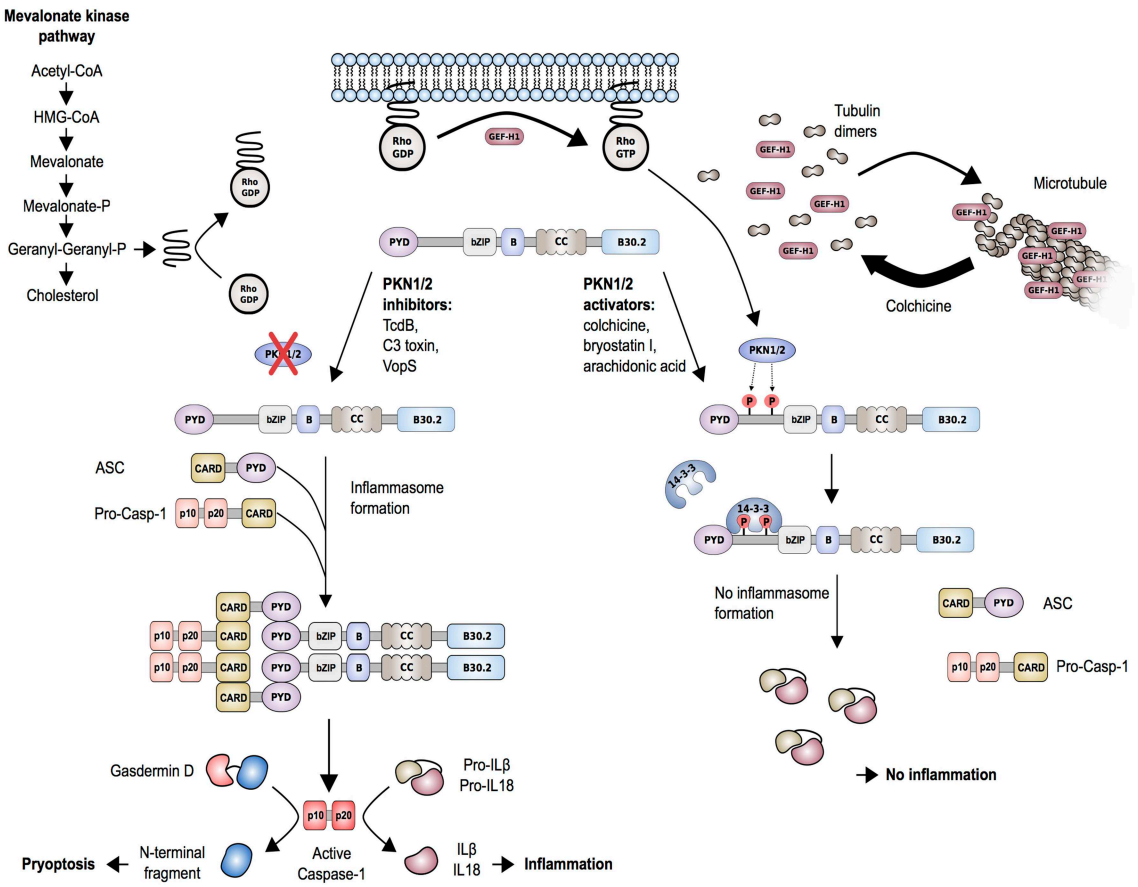

FIGURE 4 | Proposed molecular mechanism of regulation of the pyrin inflammasome. Geranylgeranylation (mevalonate kinase pathway) and release of GEF-H1 (i.e., through the microtubule polymerization inhibitor colchicine) induce RhoA activity. RhoA effector kinases, PKN1 and PKN2, cause subsequent phosphorylation of pyrin and binding to inhibitory proteins 14-3-3. This mechanism is enhanced by PKN1/2 activating substances and release of GEF-H1, while low GEF-H1 or defective MVK-pathway function leads to inactivation of PKN1/2. Decreased phosphorylation of pyrin is associated with pyrin inflammasome activation and release of mature IL-1β and IL-18. The release of IL-1β and IL-18 is further facilitated by the production of the plasma membrane pore-forming N-terminal fragment of gasdermin D.

HEK293T or THP1 cells demonstrated spontaneous ASC-speck formation, higher caspase-1 activity, and increased inflammatory cell death suggesting constitutive activation of pyrin. Additional *in vitro* experiments showed that these substitutions cause reduced binding to 14-3-3 proteins and impair the self-regulatory mechanism of pyrin.

Studies of another autoinflammatory disorder caused by mevalonate kinase (MVK) deficiency provided additional support for the described mechanism of pyrin inflammasome regulation. MVK is a key enzyme of the mevalonate/cholesterol pathway and biallelic hypomorphic mutations in *MVK* cause mevalonate kinase deficiency (MKD)/hyperimmunoglobulinemia D syndrome (HIDS) (74). Besides cholesterol, the mevalonate pathway also synthesizes other intermediates, including geranylgeranyl pyrophosphate, which serves as a substrate for a specific type of post-translational lipid modification, called protein geranylgeranylation. Akula et al. (72) showed that geranylgeranylation of the small GTPase Kras is essential for the TLR-induced activation of PI3K-Akt signaling that maintains pyrin in the inhibitory state. Loss of Kras-geranylgeranylation causes an unchecked TLR-induced inflammatory response and leads to a constitutive activation of the pyrin inflammasome. RhoA is also a subject to geranylgeranylation and the translocation of RhoA from the cytosol to the cellular membrane, an essential step for its activation, is dependent on this modification (**Figure 4**). Park et al. (70) showed that the inhibition of the MVK pathway in BMDMs induces the release of membrane-bound RhoA and pyrin inflammasome-dependent secretion of IL-1β. The IL-1β production was blocked through the addition of geranylgeranyl pyrophosphate or through chemical activation of PKN1 and PKN2. Thus, the inflammation in patients with MKD/HIDS is mediated by the pyrin inflammasome.

The described studies demonstrated that the pyrin inflammasome does not directly interact with PAMPs, but rather indirectly senses pathogen-induced changes in RhoA activity. This indirect mechanism represents a paradigm shift for the sensing of pathogens in the mammalian immune system and resembles the "guard hypothesis" by which innate immunity is triggered by resistance proteins in plants (75). Resistance

proteins recognize the downstream effects of pathogen virulence factors rather than the factors themselves and therefore fulfill a surveillance function in cellular homeostasis. The indirect pyrin inflammasome activation can therefore be considered as an example of the "guard" mechanism in humans.

MOLECULAR PATHOGENESIS OF FAMILIAL MEDITERRANEAN FEVER

The described studies on the molecular mechanism regulating the pyrin inflammasome also were essential to better understand the pathogenesis of FMF and how mutations in *MEFV* can lead to excessive inflammasome activation. The fact that FMF-associated mutations cluster in the B30.2 domain implies that mutations might interfere with an important regulatory role of this domain. Park et al. (70) demonstrated that binding of PKN1 to the mutant pyrin knock-in mice with common FMF-associated mutations was substantially decreased relative to binding of PKN1 to wild-type mouse pyrin, which lacks a B30.2 orthologous domain. This finding was confirmed by studies in HEK293T cells, ectopically expressing wildtype or mutated pyrin, as well as in macrophages, differentiated from PBMCs of FMF patients. In both model systems, binding of inhibitory protein 14-3-3ε to human mutant pyrin was substantially reduced relative to binding to wildtype pyrin.

These experiments indicated that binding of 14-3-3 proteins to phosphorylated pyrin acts as a molecular block to keep the pyrin inflammasome in an inactive state. Disturbances that influence 14-3-3 binding to pyrin, either through bacterial pathogens that change the phosphorylation status of pyrin or through mutations in the B30.2 domain, lead to an activation of pyrin. To date it is not clear how the B30.2 domain keeps pyrin in an inactivated state, but different hypotheses have been proposed. The B30.2 domain might function as a platform that allows binding of PKN1/2 and/or 14-3-3ε/τ and mutations in this domain interfere with the efficient docking of these proteins. A second hypothesis proposes that B30.2 domain is essential for the formation of a secondary structure that keeps pyrin in an autoinhibitory state. Phosphorylation of Ser208 and Ser242 and subsequent 14-3-3 protein binding may induce an intramolecular interaction of the B30.2 domain with the B-box/coiled-coil or other regions of pyrin. FMF-associated mutations in the B30.2 domain might result in a steric hindrance of the autoinhibition and thereby favor the active confirmation of pyrin. Interestingly, a mechanistically similar, self-inhibitory regulation was described recently for the NLRP1 inflammasome in the context of two inflammatory skin-disorders (9).

The described findings also help to better understand the mode of action of drugs used for the treatment of FMF, such as colchicine. Colchicine is an alkaloid with microtubule toxic properties and has been proven very effective as a prophylactic treatment of FMF and gout (76). Colchicine is a known RhoA activator and was shown to function through the release and thereby activation of guanine-nucleotide-exchange factor (GEF)-H1 from depolymerized microtubules (**Figure 4**) (77). In line with that, Park et al. (70) showed that treatment of LPS-primed BMDMs with clinically therapeutic doses of colchicine not only activated RhoA but also reversed the C3 toxin-induced inhibition of RhoA activity. Furthermore, colchicine treatment increased the interaction of 14-3-3ε to pyrin in lysates of BMDMs from mice carrying FMF-associated mutations. These findings indicate that colchicine works through the GEF-H1-dependent activation of RhoA that leads to pyrin phosphorylation and inhibition.

Interestingly, other groups proposed a microtubule-dependent and phosphorylation-independent mechanism of pyrin inflammasome activation as an alternative mechanism of action for colchicine (71, 78, 79). They confirmed the previous finding that colchicine attenuates bacterial toxin-induced caspase-1 activation, IL-1β release and pyroptosis in mouse BMDMs. However, they did not find changes in pyrin phosphorylation or 14-3-3 binding upon colchicine treatment. Gao et al. (71) showed that colchicine works downstream of pyrin phosphorylation and suggested a mechanism of action for colchicine through its inhibitory effect on ASC-speck formation. Van Gorp et al. (78) demonstrated that FMF-associated mutations allow microtubule-independent oligomerization of ASC and therefore prime pyrin for ASC-binding and inflammasome formation without microtubule-related signals.

One explanation for the discrepancies seen in colchicine downstream effects could be due to the differences in colchicine concentrations used in the respective studies. Further studies are necessary to delineate precise mechanisms of colchicine effect on the pyrin inflammasome, specifically in human cells.

Pyrin-induced pyroptosis was shown to be critical for neutrophilia and production of IL-1β in a murine model of FMF (80). Deletion of GSDMD in FMF KI mice abolished bacterial toxin-induced *ex vivo* IL-1 production from BMDMs and development of spontaneous inflammatory disease in these mice.

OTHER PYRIN INFLAMMASOME-MEDIATED DISORDERS

Dominantly Inherited Pyrin Mediated Disorders

Pyrin-Associated Autoinflammation With Neutrophilic Dermatosis (PAAND)

In 2016, Masters et al. (69) identified a dominantly inherited autoinflammatory disorder, PAAND, in a three-generation Belgian family caused by a serine-to-arginine substitution at position 242 of pyrin (Ser242Arg). In contrast to FMF, patients with PAAND present with longer fever episodes and severe neutrophil-mediated dermatosis and cystic acne. Subsequently, another family with PAAND was identified and the disease-causing variant was found to be the heterozygous Glu244Lys mutation (73). Monocytes from patients with PAAND have a significantly higher spontaneous production of IL-1β and IL-18 than cells from healthy controls or FMF patients. Thus, patients with PAAND should respond to treatment with IL-1 or IL-18 inhibitors, although the latter therapy has not been evaluated yet.

Interestingly, the homozygous mutation Ser208Thr, affecting the other residue that is phosphorylated by PKN1/2, has been associated with another phenotype manifesting with failure to

thrive, lymphadenopathy, transient purpuric rashes, arthralgia, oral ulcerations and mixed lymphocytic/eosinophilic infiltrates in bone marrow. Elevated levels of IL-1β and IL-18 cytokines were found in serum samples and supernatants of LPS stimulated monocytes. In addition, stimulated patients' PBMCs released increased levels of C-C-motif chemokine ligand 5 (CCL5), a potent chemotactic agent for eosinophils, which likely explains the observed eosinophilia (81). The fact that biallelic mutations that cause an amino acid change at position 208 are necessary to activate pyrin suggests that the Ser208 residue is less critical for pyrin inhibition than the Ser242 residue. Collectively, these studies have shown how identification of patients with a rare mutation can be instrumental in understanding the physiological function of a protein.

Pyrin-Associated Dominant Disease (PADD)

A severe autosomal-dominant periodic inflammatory disease without neutrophilic dermatosis has been reported in a three-generation family from Spain. The main findings in the five affected individuals were long fever episodes, renal amyloidosis, and colchicine resistance. All individuals were found to be heterozygous carriers for the novel pathogenic variant His478Tyr in *MEFV* (82). The His478Tyr amino acid substitution is located between the coiled-coil and the B30.2 domain of pyrin, but the exact molecular effect of this variant is still unknown (**Figure 2**).

Another residue in pyrin, associated with an autosomal dominant autoinflammatory syndrome, is Thr577 (83). Stoffels et al. (83) found four different heterozygous substitutions at the amino acid position 577 of pyrin in two families and two single individuals (Thr577Asn, Thr577Ala, and Thr577Ser). All patients presented with an autoinflammatory phenotype, including fever and systemic inflammation, which was similar to FMF but also showed some differences. Recently, another three-generation family of Japanese ancestry has been described carrying the Thr577Asn mutation and presenting with low-grade fevers, serositis and amyloidosis (84). PBMCs of a patient with the Thr577Asn mutation exhibit increased IL-1β secretion after LPS stimulation indicating that Thr577Asn acts through a GOF mechanism. Because of the vicinity of 577 residue to CC domains, which are known to play a role in oligomerization, it is possible that these heterozygous mutations lead to a constitutive pyrin activation, which would explain a more severe phenotype (**Figure 2**). The crystal structure of this region of the protein is still unresolved, therefore it is still unknown how exactly these variants cause a hyperactivation of the pyrin inflammasome.

Most FMF-associated mutations described to date are missense variants except for a couple in frame single amino-acid deletions, including Ile692del and Met694del. Interestingly, one of these variants, namely Met694del, was reported in families with dominantly inherited FMF despite intensive search for a second causal variant (85, 86).

Pyogenic Arthritis, Pyoderma Gangrenosum, and Acne (PAPA) Syndrome

PAPA syndrome is an autosomal dominantly inherited autoinflammatory disorder and was first described in an extended family in 1997 (87). Manifestations of PAPA syndrome encompass early-onset flares of sterile arthritis characterized by neutrophilic infiltrates. Cutaneous manifestations are variable and may include ulcerations, pyoderma gangrenosum, or cystic acne. Increased acute-phase reactants and increased production of IL-1β and TNFα in peripheral blood leukocytes are common laboratory findings in this disorder (88, 89).

Wise et al. (35) found that heterozygous mutations in CD2-binding protein 1 (*CD2BP1*), also known as proline serine threonine phosphatase-interacting protein 1 (*PSTPIP1*), are the genetic cause of PAPA syndrome. The PAPA syndrome causing mutations in *PSTPIP1* impair its association with PEST (rich in proline, glutamic acid, serine, and threonine)-type protein tyrosine phosphatase (PTP-PEST). Pyrin interacts with PSTPIP1 via its B-box/coiled-coil domain (34). The authors further demonstrated that the two most common PAPA-associated mutations (Ala230Thr and Glu250Gln) induce phosphorylation of PSTPIP1, likely due to its reduced affinity for PTP-PEST, and that the hyperphosphorylation increases the affinity of PSTPIP1 to pyrin. This may in turn induce the activation of the pyrin inflammasome and increase IL-1β production. The mechanism by which this occurs has yet to be fully understood. Pyrin and PSTPIP1 co-localize with the tubulin cytoskeleton and mutant PSTPIP1 proteins are recruited by pyrin to form ASC specs (90). Another study suggested that binding of PSTPIP1 activates pyrin by unmasking its pyrin domain, which leads to increased ASC-mediated oligomerization and inflammasome formation (33). With the identification of PKN1/2 and their role in the regulation of the pyrin inflammasome, it remains to be investigated whether binding of PKN1/2 or 14-3-3 proteins to pyrin might be affected by the differential affinity of mutant PSTPIP1. In addition, PSTPIP1 localizes to actin-rich regions in the cell and functions as an important factor for the organization of the cytoskeleton (91). This finding is particularly relevant in the view of the hypothesis that changes in cytoskeletal organization caused by invading pathogens might be one of the pyrin inflammasome triggering signals.

Recessively Inherited Pyrin Mediated Disorders

Mevalonate Kinase Deficiency (MKD)/ Hyperimmunoglobulinemia D Syndrome (HIDS)

Mevalonate Kinase Deficiency (MKD)/Hyperimmuno-globulinemia D syndrome (HIDS) is a rare recessively inherited autoinflammatory disease with onset in infancy or early childhood and the first disease episode is often provoked by immunization. MKD/HIDS is characterized by recurrent fever lasting 3–7 days, cervical lymphadenopathy, abdominal pain, hepatosplenomegaly, diarrhea, arthralgia/arthritis, and erythematous maculopapular rash. Most but not all MKD/HIDS patients present with high levels of IgD, while during flares laboratory findings also include elevated urinary mevalonic acid levels and increased acute-phase reactants. The disease-associated gene *MVK* was reported in 1999 by two independent groups (92, 93). Subsequent work demonstrated that patients with nearly absent MVK enzymatic activity manifest a more

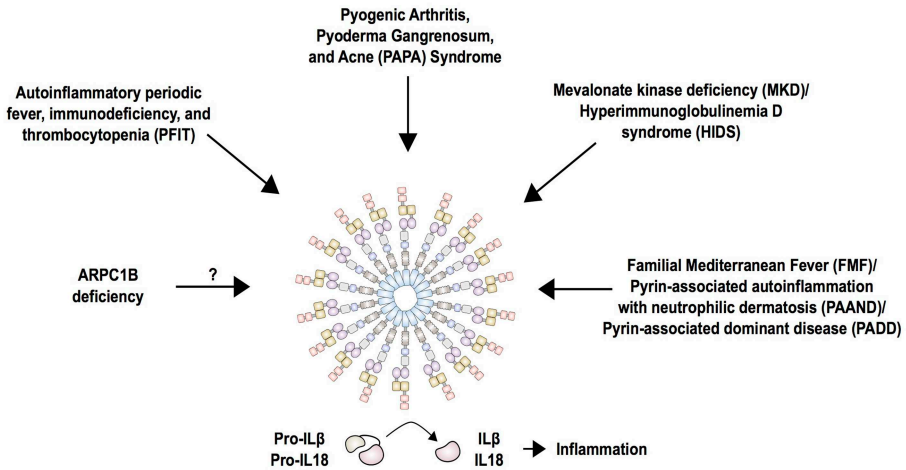

FIGURE 5 | Current summary of autoinflammatory diseases mediated by the pyrin inflammasome. Pyrin interacts with ASC and caspase-1 to form the canonical inflammasome that process pro-IL-1β and pro-IL-18 into its mature forms, IL-1β and IL-18. Mutations in pyrin increase activity of the inflammasome and cause familial Mediterranean fever (FMF) and pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND). While FMF mutations only enhance the activity of pyrin, PAAND-associated mutations lead to constitutive activation of pyrin, which is reflected by a higher production of IL-1 and IL-18 in PAAND than FMF. The molecular consequence of other dominantly inherited pyrin mutations (PADD) is still unknown. Mutations in MVK associated with MKD/HIDS act by downregulating RhoA activity that is important for pyrin inhibition. Mutations in PSTPIP1 (PAPA syndrome), WDR1 (PFIT), and ARPC1B affect actin polymerization, thus it is likely that these perturbations in the cytoskeleton are detected by the pyrin inflammasome.

severe phenotype and present with developmental disabilities and inflammation (mevalonic aciduria), while a partial deficiency causes milder, autoinflammatory MKD/HIDS (92, 94). The deficiency in MVK function seen in MKD/HIDS results in the reduction of geranylgeranyl pyrophosphate (95–97). As described earlier, reduced geranylgeranylation of Rho GTPases, RhoA, and Kras, leads to increased pyrin activity (**Figure 4**). As expected, the inflammation in MKD/HIDS patients is controlled with IL-1 inhibitors (98).

Autoinflammatory Periodic Fever, Immunodeficiency, and Thrombocytopenia (PFIT)

In 2007, a new autoinflammatory and thrombocytopenia phenotype was described in mice that is caused by a LOF mutation in the actin-depolymerizing cofactor *Wdr1* (67). Wdr1 is a WD40 repeat protein that is required for the cofilin-dependent disassembly of actin filaments and the disease-causing mutation, a T-to-A transversion in the second nucleotide of intron 9, was shown to affect mRNA-splicing. Further analyses showed that the phenotype of Wdr1 deficiency is IL-18 dependent, but IL-1β independent, and that the pyrin inflammasome is the main inflammatory mediator of this disease (68). Neutrophils and macrophages of *Wdr1*-deficient mice have elevated levels of polymerized actin compared with wild-type mice and the high levels of polymerized actin induce ASC oligomerization, caspase-1 activation, and IL-18 secretion in these cells. The treatment of these cells with latrunculin-b, a marine toxin that disrupts actin polymerization, or with colchicine, a known microtubule depolymerizing agent, caused a reduction in LPS-induced caspase-1-mediated IL-18 secretion.

Subsequently, homozygous missense mutations in *WDR1* were identified in two siblings who presented with autoinflammatory recurrent fevers, thrombocytopenia, and immunodeficiency. Standing et al. (99) show that LPS-stimulated patient monocyte-derived dendritic cells, CD14-lymphocytes, and Epstein-Barr virus-transformed lymphoblasts exhibit elevated levels of polymerized actin and produced higher levels of IL-18. Furthermore, HEK293T cells transfected with mutated WDR1 exhibited abnormal aggregate formation that co-localized with pyrin in fluorescent microscope analyses. These findings indicate that the association of mutated WDR1 with pyrin might cause spontaneous ASC oligomerization and pyrin inflammasome activation (**Figure 5**). The effect of WDR1 deficiency on neutrophil morphology, motility, and function was demonstrated in several additional affected individuals, presenting with recurrent infections, neutropenia, impaired wound healing and severe stomatitis (100). The detailed molecular mechanisms of how WDR1 deficiency leads to pyrin inflammasome activation remains to be determined.

ARPC1B Deficiency

Loss of function mutations in the ARPC1B subunit of actin related protein complex 2/3 (ARP2/3) have been identified in patients with early-onset immunodeficiency, low platelet count, eosinophilia, elevated IgE and IgA levels, small vessel vasculitis and predisposition to inflammatory bowel disease (101–103). The ARP2/3 complex is ubiquitous in eukaryotic cells and is essential for mitotic integrity, cell survival, and a variety of cellular functions (104). This multi-system phenotype is similar to the phenotype of patients with Wiskott-Aldrich syndrome that is caused by mutations in the WASP protein. WASP promotes

actin polymerization and branching of F-actin via the ARP2/3 complex. ARPC1B expression is restricted to hematopoietic cells and consequently patients with absent or low protein expression manifest variable degrees of thrombocytopenia and immune dysregulation. The inflammatory phenotype has not yet been studied, but it is tempting to speculate that perturbations in actin polymerization might trigger an activation of the pyrin inflammasome (**Figure 5**). One study showed that murine *Wdr1* hypomorphic monocytes secrete significantly less IL-18 cytokine in the presence of the Arp2/3 inhibitor CK-666. The production of IL-18 was dependent on ASC, caspase-1 and pyrin. Contribution of other inflammasomes, including NLRP1, NLRP3, and AIM2, was excluded by generating double knockout/mutant mouse strains in this disease model (68).

EVOLUTIONARY ASPECTS

Population studies in multiple Mediterranean populations recognized high carrier frequencies of FMF-associated mutations and suggested a selective advantage of these genotypes, probably to an endemic pathogen (105, 106). Although haplotype data showed that FMF carrier chromosomes from different ethnic groups share a common progenitor, implicating a founder effect, the unexpectedly high frequency of several distinct mutations in different populations indicates evolutionary selection. Further support for the selective advantage model comes from genetic studies of the B30.2 domain during primate evolution (107). Schaner et al. (107) showed that pyrin is not evolving at a constant rate as it would be expected for neutral evolution, but rather evolves at different rates across species. The authors hypothesized that these episodes of positive selection in different species might have been provoked by novel environmental pathogens. Moreover, wild-type pyrin of non-human primates often exhibits amino acid residues that are associated with human FMF suggesting that primates likely tolerate viral or bacterial pathogens against which mutated human pyrin confers resistance.

The recent recognition that pyrin senses changes in a variety of cellular processes might contribute to a better understanding of the proposition for selective advantage of FMF heterozygous mutations. A broad spectrum of bacterial pathogens use Rho GTPase-inactivating toxins to compromise cytoskeleton-dependent host cell defense mechanisms such as immune cell migration and phagocytosis. Other pathogenic toxins, such as the cholera toxin or pertussis toxin, alter the cytosolic concentration of cAMP to impair or to deactivate a variety of basic cellular processes and functions. These perturbations of cytoplasmic homeostasis were recently termed "homeostasis-altering molecular processes" (HAMPs) and the fact that the pyrin inflammasome senses changes of cellular homeostasis rather than directly recognizing pathogens might provide the indispensable flexibility to detect evolutionarily novel infections (108). The balancing selection of FMF-associated mutations might therefore be due to an increased "alertness" of the pyrin inflammasome for cellular changes in individuals carrying these mutations.

Along these lines, two recent publications demonstrated how pathogens develop mechanisms to evade the inflammasome-triggered immune response and showed, through the example of *Yersinia* infections, how host-pathogen co-evolution might occur (109, 110). Previous studies had shown how *Yersinia* species, including *Yersinia pestis*, the causative agent of the plague, use pathogenicity factors, so-called *Yersinia* outer proteins (Yops), to counteract multiple defense responses in the infected host cell (111). Recent work demonstrated that the pyrin inflammasome successfully recognizes *Yersinia* infections through changes in Rho GTPase activity (**Figure 3**). Both YopE and YopT downregulate RhoA activity and thus prevent activation of PKN1/2, which result in pyrin inflammasome activation (112). YopE and YopT-dependent pyrin activation is efficiently neutralized or hijacked by YopM, an additional Yop that is delivered into the host cell. The leucine-rich repeat (LRR)-containing protein YopM was shown to inhibit caspase-1 activation but the exact mechanism remained elusive (113). Recent findings suggested that YopM works as a scaffold protein to interact with different host proteins, including PKN1 and PKN2. By recruiting and activating these kinases, YopM facilitates the phosphorylation and inhibition of pyrin. Chung et al. (109) further speculated that the high carrier frequencies for FMF-associated variants in Mediterranean and Middle Eastern populations might have emerged because the heterozygous carriers are more protected against pathogenic *Yersinia* species.

PERSPECTIVES AND OPEN QUESTIONS

Following the identification of pyrin and its physiologic role many steps have been made toward a better understanding of the complexity of the pyrin inflammasome. Unequivocally, the discovery of the mechanisms of how bacterial toxins and other pathogens can indirectly trigger the pyrin inflammasome and how these signals are transmitted to downstream effectors were important achievements. However, other questions remain unanswered and new ones have arisen with these recent findings.

An important question is how the B30.2 domain regulates pyrin function and whether it acts through an autoinhibitory or proinflammatory mechanism. Murine studies implicated that the B30.2 domain has a role in autoinhibition and that disease-causing mutations confer poor affinity to regulatory 14-3-3 proteins. However, this is less clear in humans. Current consensus is that FMF-associated mutations lower a threshold for activation of the pyrin inflammasome but whether increased pyrin activity results from a loss of autoinhibition or from a facilitated activation remains unclear (114).

The major impediment in understanding the function of human pyrin and the effect of FMF mutations is in that X-ray crystallography has failed to solve the complete pyrin structure, due to the protein insolubility. Admittedly, crystal structure of the B30.2/SPRY domain has been solved and identified a conserved peptide-binding site in the vicinity of FMF-associated mutations (115–117). Putative binding partners have not been found, however it is unlikely that the binding pocket directly recognizes PAMPs. Solving the crystal structure of pyrin, ideally

at different states of activation and in association with binding partners, such as 14-3-3 protein, PKN1/2 or ASC, could help to answer this question. Recent advances in cryo-electron microscopy may facilitate these studies (118, 119).

As discussed earlier, the binding of PKN1 to murine pyrin fused to the wildtype human B30.2 domain is strongly reduced relative to PKN1 binding to wildtype murine pyrin lacking the B30.2 domain. This finding indicates that the B30.2 domain regulates pyrin phosphorylation/inhibition and suggests that murine pyrin, which lacks B30.2 domain, mostly exists in an inhibited state in the cell. This raises the question, how the pyrin inflammasome can be activated in mice. Park et al. (70) propose the presence of phosphatases that dephosphorylate pyrin to release it from its inactive state. It is also possible that mouse pyrin interacts with a yet to be identified protein that fulfills similar functions as the B30.2 in human and therefore compensates for the absence of this protein domain.

Still little is known about the triggers that cause the outbreak of autoinflammatory attacks. Different factors have been associated with disease flares including infection, trauma, physical and emotional stress, menstruation, and exposure to cold (120–122). The observation that elevated levels of cAMP trigger pyrin inflammasome activation through repression of RhoA might provide a first hint toward understanding the underlying mechanism (70, 123). The cAMP/PKA signaling pathway is an important cellular integrator for a variety of different signals, including hormones and neurotransmitters. These signals bind and stimulate G protein-coupled receptors that subsequently trigger cAMP production (124). In 2015, a study demonstrated the direct association between cAMP/PKA signaling pathway activity and stress-induced behavioral responses (125). Loss of cyclin-dependent protein kinase 5 in the forebrain of mice induced elevated cAMP concentrations and PKA activation in striatal neurons and also affected the behavioral responses to acute or chronic stress. Conversely, it is possible that physical and/or emotional stress affects cAMP/PKA signaling and consequently induces inflammation. The question of what explains the fluctuation in inflammation and what is natural progression toward termination of these episodes of inflammation in FMF and other autoinflammatory disorders remains quite relevant.

A better understanding of the molecular mechanisms that regulate pyrin and other intracellular inflammasomes will ultimately guide development of new therapies for patients with immune dysregulation and other diseases that may benefit from modulations in inflammatory and immune responses.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Natural Killer Cells in Systemic Autoinflammatory Diseases: A Focus on Systemic Juvenile Idiopathic Arthritis and Macrophage Activation Syndrome

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Natural killer (NK) cells are innate immune lymphocytes with potent cytolytic and immune-regulatory activities. NK cells are well-known for their ability to kill infected and malignant cells in a fast and non-specific way without prior sensitization. For this purpose, NK cells are equipped with a set of cytotoxic molecules such as perforin and apoptosis-inducing proteins. NK cells also have the capacity to produce large amounts of cytokines and chemokines that synergize with their cytotoxic function and that ensure interaction with other immune cells. A less known feature of NK cells is their capacity to kill non-infected autologous cells, such as immature dendritic cells and activated T cells and monocytes. Via the release of large amounts of TNF-α and IFN-γ, NK cells may contribute to disease pathology. Conversely they may exert a regulatory role through secretion of immuno-regulatory cytokines such as GM-CSF, IL-13, and IL-10. Thus, NK cells may be important target and effector cells in the pathogenesis of autoinflammatory diseases, in particular in those disorders associated with a cytokine storm or in conditions where immune cells are highly activated. Key examples of such diseases are systemic juvenile idiopathic arthritis (sJIA) and its well-associated complication, macrophage activation syndrome (MAS). sJIA is a chronic childhood immune disorder of unknown etiology, characterized by arthritis and systemic inflammation, including a daily spiking fever and evanescent rash. MAS is a potentially fatal complication of autoimmune and autoinflammatory diseases, and most prevalently associated with sJIA. MAS is considered as a subtype of hemophagocytic lymphohistiocytosis (HLH), a systemic hyperinflammatory disorder characterized by defective cytotoxic pathways of cytotoxic T and NK cells. In this review, we describe the established features of NK cells and provide the results of a literature survey on the reported NK cell abnormalities in monogenic and multifactorial autoinflammatory disorders. Finally, we discuss the role of NK cells in the pathogenesis of sJIA and MAS.

Keywords: natural killer cell, sJIA, MAS, autoinflammation, immune-regulation

INTRODUCTION

Natural killer (NK) cells are granular innate lymphocytes best known for their ability to kill infected and malignant cells in a fast and non-antigen specific manner. In humans, there is a general consensus for the existence of 2 NK cell subtypes based on the relative expression of CD56, a cell adhesion molecule, and CD16, also known as FcγRIII. In healthy donors, $CD56^{dim}CD16^{+}$ NK cells comprise around 90% of NK cells in peripheral blood and are mainly cytotoxic. The other 10% are $CD56^{bright}CD16^{dim/-}$ NK cells and produce greater amounts of cytokines than $CD56^{dim}CD16^{+}$ NK cells (1). NK cells originate from hematopoietic stem cells that are differentiated to common lymphoid precursor cells and in a traditional view the $CD56^{bright}$ NK cells are considered as precursor cells of the $CD56^{dim}$ subset. However, this concept has been challenged as there is evidence that the two subsets can be seen as separate populations with different origin and characteristics (2).

More recently, NK cells have been classified as a group 1 innate lymphoid cell (ILC), comprising conventional (c)NK cells and ILC1s (3). ILC1s are tissue-resident, also known as tissue-resident (tr)NK cells, and are virtually found in all organs, including liver, lung, and uterus, whereas cNK cells are circulating via the blood stream (3). Although ILC1s and NK cells have multiple common features, they are functionally and phenotypically different. cNK cells have a stronger cytotoxic potential with higher expression of perforin compared to ILC1s. In contrast, ILC1s have only weak cytotoxic function with low perforin levels, but produce high levels of cytokines like interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (4, 5). In addition, trNK cells have been shown to confer adaptive features, i.e., hapten- and virus-induced memory (6–8). Besides these differences in function, ILC1s and NK cells have a distinct phenotype. Both cell types express characteristic NK cell markers, i.e., CD56, NKp46, CD122 (IL-2 receptor β), and activating receptor NKG2D, whereas other markers can be used to discriminate subsets of NK cells (9, 10). The subtypes of NK cells and their characteristics have been reviewed extensively elsewhere (2, 9), of which we provide a short overview here. In the peripheral blood, $CD56^{bright}$ NK cells can be distinguished from $CD56^{dim}$ NK cells via the higher expression of CD56 and the absence of CD16 (**Figure 1A**). In addition, $CD56^{bright}$ NK cells are characterized by the expression of NKG2A, CXCR3, and CCR7. In contrast, $CD56^{dim}$ NK cells express CXCR1, CXCR2, S1P5, and have a higher expression of killer immunoglobulin-like receptors (KIR), and maturation markers KLRG1 and CD57 (9, 11). Furthermore, peripheral blood NK cells can be distinguished from tissue-specific NK cells via the expression of CD49e (9, 12). Specialized tissue-specific NK cells have been described in liver, lung, and spleen (**Figure 1B**) (4, 12–17). In general, trNK cells largely resemble $CD56^{bright}$ NK cells with a tissue-specific expression of adhesion and tissue-retention markers, such as CD69 and chemokine-receptors CXCR6 and CCR5 (4, 9, 16). In addition, trNK cells lack the expression of CD49e, KIR, CD16, and maturation marker CD57 (4, 9, 12). Unlike for murine trNK cells, the expression of CD49a is not specific for human trNK cells (4, 16, 18). A more distinct subtype of NK cells can be found in the uterus (**Figure 1B**). Uterine NK cells have a CD56 "superbright" phenotype, express CD9 and KIR, and show more characteristic tissue-resident features by the expression of CD69 and CD49a (9, 19). Uterine NK cells produce growth-promoting factors, have an important placental vascular remodeling function during pregnancy and are thought to provide memory for this vascular remodeling in subsequent pregnancies (19, 20).

NK cells can exert their cytotoxic function either via perforin-release or via the engagement of death receptors TNF-related apoptosis-inducing ligand (TRAIL) or Fas ligand (21). NK cells also have an important immune-modulatory function by the release of cytokines and chemokines (22). The most prominent cytokines produced by NK cells are IFN-γ and TNF-α. NK cells also secrete immuno-regulatory cytokines such as GM-CSF, IL-13, and IL-10 (23–25). In addition, NK cells also produce a variety of chemokines, including CXCL8 (IL-8), CCL2 [monocyte chemoattractant protein (MCP)-1], CCL3 [macrophage inflammatory protein (MIP)-1α], CCL4 (MIP-1β), CCL5 (RANTES), and CXCL10 [IFN-inducible protein (IP)-10] (25, 26). As part of the innate immune system, NK cells do not need prior sensitization to exert these functions, though activation with cytokines, i.e., type I IFN, IL-2, IL-12, IL-15, and IL-18, greatly enhances their activity (1). IL-18 is an important cytokine for stimulation of NK cell cytotoxicity and IFN-γ production by NK cells (27). Also IFN-γ has been shown to drive NK cell function. The importance of the IL-18/IFN-γ axis in NK cell function is further highlighted by the impaired NK cell function in IL-18- and IFN-γ-deficient mice (28, 29). Next to the innate function of NK cells, recent reports have demonstrated adaptive features of NK cells, and more specifically trNK cells, with a hapten-, virus-, and cytokine-induced memory function, thereby enabling NK cells to respond with higher efficacy and enhanced cytotoxic and cytokine-producing activity upon restimulation (6–8, 30).

NK cell activity is tightly regulated via a large repertoire of inhibitory and activating NK cell receptors to avoid off-target effects (31). NK cell receptors can be classified in different subsets based on their receptor function or by the nature of their ligands (**Figure 1C**). Signaling by most inhibitory and activating receptors is mediated via conserved sequences in the cytoplasmic region of the NK cell receptor. Inhibitory receptors share an immunoreceptor tyrosine-based inhibitory signaling motif (ITIM), whereas activating receptors have an immunoreceptor tyrosine-based activation motif (ITAM) (31). The CD94/NKG2-family, including NKG2A, and the murine Ly49 or human KIR-family recognize MHC class I ligands. Both inhibitory and activating receptors are found in these families. The activating receptor NKG2D recognizes MHC class I-related proteins, including retinoic acid early inducible (Rae)-1 in mice and UL16 binding protein (ULBP) and MHC class I polypeptide-related sequence (MIC)A/B in humans. CD48 is the ligand for 2B4 (CD244) and is expressed on all hematopoietic cells. 2B4 can act as an inhibitory or activating receptor depending on the expressed isoform. For many NK cell receptors (e.g., KLRG1 and NKp46), their function or ligand-specificity remains unknown. The family of natural cytotoxicity receptors, i.e.,

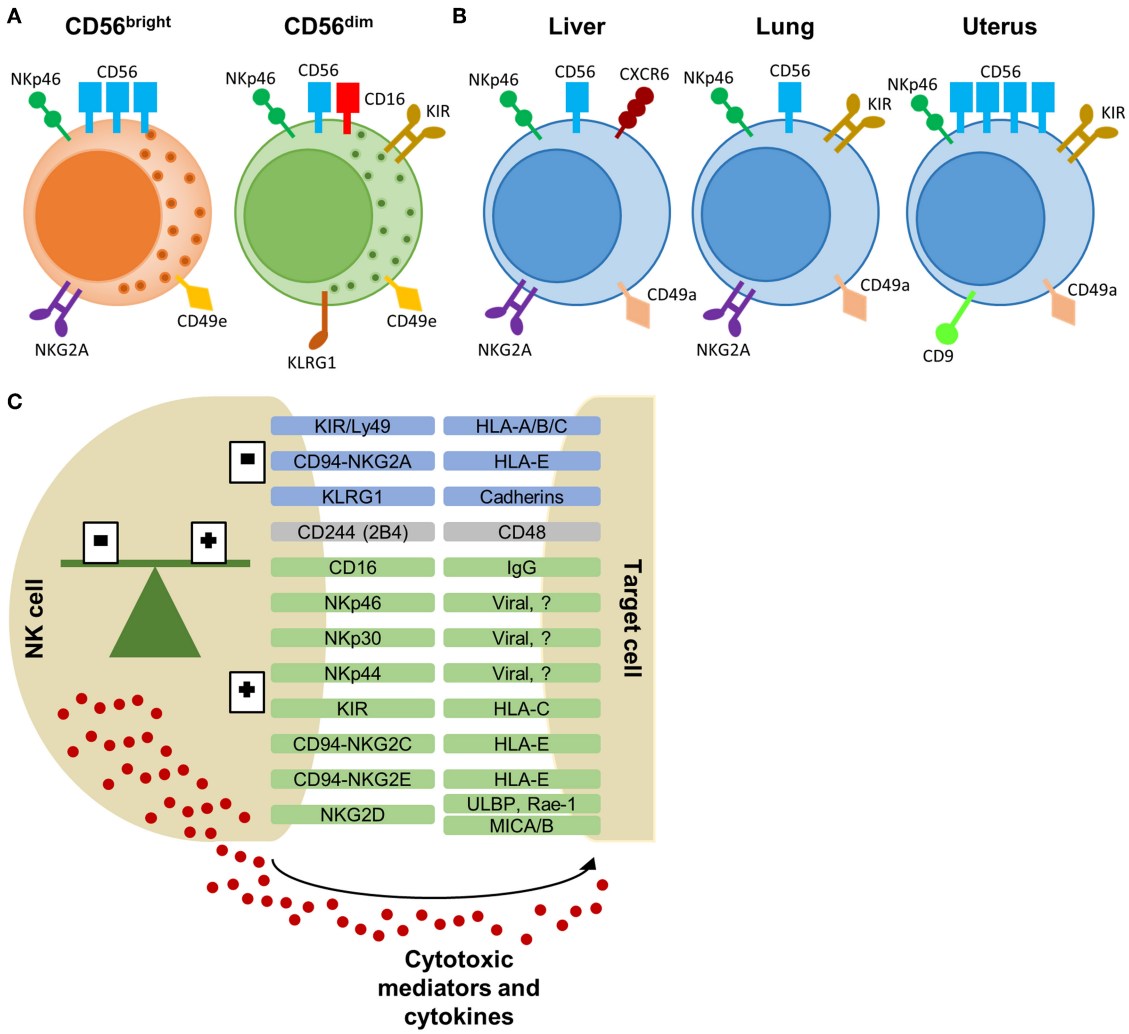

FIGURE 1 | NK cell subtypes and receptor-ligand interaction between NK cell and target cell. **(A)** CD56^{bright} and CD56^{dim} NK cells, primarily found in the peripheral blood, with a selection of characteristic markers. **(B)** Tissue-resident NK cells from the liver, lung, and uterus express overlapping markers with peripheral NK cells. Each tissue-specific NK cell subtype displays a characteristic phenotype which allows differentiation from peripheral NK cells. **(C)** Selection of NK cell receptors and their corresponding ligands on target cells. Inhibitory receptors are in blue, activating receptors are in green and receptors with dual function are in gray. The balance of inhibitory and activating signals define the activity or tolerance of NK cells. Activated NK cells will release cytotoxic proteins and cytokines to eliminate the target cells. KIR, killer-immunoglobulin receptor; HLA, human leukocyte antigen; KLRG1, killer cell lectin-like receptor G1; ULBP, UL16 binding protein; Rae, Retinoic acid early inducible; MIC, MHC class I polypeptide-related sequence.

NKp46, NKp44, and NKp30, are specifically expressed by NK cells. These receptors can activate the NK cell via recognition of pathogen-derived proteins and self-ligands (1, 31–33).

During maturation, NK cells undergo an educational process that allows them to discriminate between healthy cells and target cells via two mechanisms. Firstly, downregulation of MHC class I molecules by transformed and infected cells results in a lack of inhibitory signals (missing-self recognition). On the other hand, stressed cells can upregulate expression of stimulatory ligands, overruling the signals of inhibitory NK cell receptors (induced-self recognition). Both mechanisms result in a shifted balance toward activation of the NK cells and target cell lysis (30, 34). The latter mechanisms allows NK cells to regulate activated cells. It has been shown that NK cell cytotoxicity has an important role in homeostasis and induction or down-regulation of the cellular immune response (35, 36). On the one hand, NK cells can stimulate the adaptive immune response via IFN-γ and chemokine production. On the other hand, NK cells can kill autologous activated T cells, dendritic cells, and monocytes (1, 37–40), which could be important in terminating the immune response when necessary. Together with the secretion of anti-inflammatory cytokine IL-10, NK cells are now considered as complex immune-regulatory cells in striking contrast to their pathogenic effector role via the release of IFN-γ and the induction of tissue damage via cytolysis (1, 41, 42).

Autoinflammatory syndromes comprise a group of rare, genetically diverse, but clinically distinct pathologies characterized by recurrent fever, rash, and lymphadenopathy, accompanied by cutaneous, mucosal, serosal, and osteoarticular inflammation. Autoinflammatory diseases are associated with constitutive inflammasome activation and a dysregulation of the innate immunity. The classification, etiology, and pathogenesis of the diverse monogenic and multifactorial autoinflammatory diseases has been extensively reviewed elsewhere (43–48). The role of NK cells in autoinflammatory diseases remains largely unknown. In this review, we will give an overview of the pathogenic and regulatory features of NK cells in the context of autoinflammation.

THE INFLAMMASOME AND NATURAL KILLER CELL ACTIVATION

Activation of innate immune pathways is a hallmark of autoinflammation. The innate immune system protects the body from pathogens in a fast and non-specific manner, in contrast to the antigen-specific adaptive immune system. Innate immune cells, such as macrophages, dendritic cells and neutrophils, detect pathogens or cell damage via pattern recognition receptors (PRRs) which recognize pathogen- or damage- associated molecular patterns (PAMPs or DAMPs). Activation of PRRs initiates the expression of chemokines, cytokines, enzymes, and adhesion molecules and the recruitment of leukocytes. The PRR family includes multiple receptors, amongst which are TLRs, NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). The TLR family exists of 11 receptors recognizing various extracellular or endolysosomal bacterial and viral PAMPs. These receptors initiate a signaling cascade via the Toll/IL1 receptor (TIR) domain and the MyD88 adaptor, except for TLR3, which signals via the TIR-domain-containing adapter-inducing interferon-β-dependent pathway. The RLR family mediates the expression of pro-inflammatory cytokines via the recognition of pathogenic nucleic acids in the cytoplasm. Finally, the NLR family consist of intracellular PRR proteins, which upon triggering activate signaling through the NF-κB pathway (NOD1/2) or through inflammasome complexes (NLRP1, NLRP3, and NLRC4) (49, 50). Multiple autoinflammatory disorders are associated with mutations in inflammasome-related genes (49, 51), resulting in constitutive activation of the inflammasome and cleavage of pro-IL-1β and pro-IL-18 via caspase-1 into active IL-1β and IL-18. The latter have been identified as main drivers of disease in autoinflammatory disorders (49, 52, 53).

More recently, inflammasome activation and the release of IL-18 has been shown to be a critical checkpoint in the activation of NK cells and the induction of memory NK cells in the liver (54, 55). Activation of the NLRC4 inflammasome (via intracellular bacteria) primed NK cells to efficiently kill infected hepatocytes in a perforin-dependent manner and thereby enhancing control of the infection (56). Alternatively, carcinoma-derived proteins were found to activate the NLRP3 inflammasome, which resulted in FasL-mediated NK cell cytotoxicity against metastatic tumor cells and effective tumor suppression after IL-18 activation of NK cells (57). Also, the NLRP3 inflammasome in tissue-resident macrophages was involved in the induction of hapten-dependent memory function of NK cells and was necessary to establish contact hypersensitivity against monobenzone (58). Inflammasome-derived IL-18 efficiently primed NK cells resulting in higher cytotoxic potential (56–58).

These reports can be of great value for understanding the role of NK cells in autoinflammatory syndromes with constitutive activation of the inflammasome.

NATURAL KILLER CELLS IN MONOGENIC AUTOINFLAMMATORY DISEASES

Monogenic autoinflammatory syndromes are associated with genetic defects in a single gene. Reports on the functionality or numbers of NK cells in this group are scarce or even non-existing for the majority of monogenic autoinflammatory diseases (**Table 1**), most likely due to the low prevalence of most of the monogenic autoinflammatory diseases.

Conflicting reports exist on the number of NK cells in patients with familial mediterranean fever (FMF, mutation in *MEFV,* resulting in abnormal regulation of IL-1β activation). One study reported higher NK cell numbers in patients with FMF (59), whereas a recent study demonstrated decreased $CD16^+$ NK cells as compared to the control group (60). The presence of activating KIR2DS2 was found to be associated with FMF (61).

In a patient with TNF receptor-associated periodic syndrome (TRAPS, mutation in *TNFRSF1A*, resulting in abnormal TNF-receptor function) a *de novo* missense variant in the *TNFRSF1A* gene was found. Interestingly, the mosaic variant allele was detected specifically in B cells, NK cells, and neutrophils, but not monocytes and T cells, potentially indicating an important role for NK cells along neutrophils in the TRAPS pathogenesis (62). Another study reported decreased numbers of NK cells in patients with TRAPS as compared to healthy controls (63).

Ombrello et al. described a defective NK cell function and signaling in patients with autoinflammation and phospholipase Cγ2-associated antibody deficiency and immune dysregulation (APLAID, mutation in *PLCγ2*, resulting in abnormal B cell function) syndrome. In these patients, NK cell numbers and the CD107a degranulation were decreased and a reduced signaling activity was observed downstream of the receptors NKG2D and 2B4 (64).

We found no reports on NK cells in patients with cryopyrin-associated periodic syndrome (CAPS), including familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome, and neonatal-onset multisystemic inflammatory disorder (NOMID), mevalonate kinase deficiency/hyperimmunoglobulinemia D syndrome (MKD/HIDS), deficit of IL-1 receptor antagonist (DIRA), pyogenic arthritis pyoderma gangrenosum and acne syndrome (PAPA), familial cold autoinflammatory syndrome 2 (FCAS2) Majeed syndrome, Blau syndrome, deficiency of IL-36 receptor antagonist

TABLE 1 | NK cells in monogenic autoinflammatory disorders.

Monogenic autoinflammatory disorder (44, 45, 47)		Gene	Reports on NK cells
FMF	Familial mediterranean fever	*MEFV*	↑ NK cell numbers (59) ↓ CD16$^+$ NK cells (60) KIR2DS2 association (61)
TRAPS	TNF receptor-associated periodic syndrome	*TNFRSF1A*	*De novo* missense variant (62) ↓ NK cell numbers (63)
CAPS	Cryopyrin-associated periodic syndrome		/
-FCAS	Familial cold autoinflammatory syndrome	*NLRP3*	/
-MWS	Muckle-Wells syndrome	*NLRP3*	/
-NOMID	Neonatal-onset multisystemic inflammatory disorder	*NLRP3*	/
MKD/HIDS	Mevalonate kinase deficiency/hyperimmuno-globulinemia D syndrome	*MVK*	/
DIRA	Deficit of IL-1 receptor antagonist	*IL1RN*	/
PAPA	Pyogenic arthritis pyoderma gangrenosum and acne syndrome	*PSTPIP1*	/
FCAS2	Familial cold autoinflammatory syndrome 2	*NLRP12*	/
Majeed syndrome		*LPIN2*	/
Blau syndrome		*NOD2/CARD15*	/
DITRA	Deficiency of IL-36 receptor antagonist	*IL36RN*	/
JMP	Joint contractures, muscle atrophy, and panniculitis-induced lipodystrophy syndrome	*PSMB8*	/
CANDLE	Chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature syndrome	*PSMB8*	/
NNS	Nakajo-Nishimura syndrome	*PSMB8*	/
CAMPS	CARD-14-mediated pustular psoriasis	*CARD14*	/
NALP12-associated periodic fever		*NALP12*	/
APLAID	Autoinflammation and phospholipase Cγ2-associated antibody deficiency and immune dysregulation	*PLCγ2*	↓ NK cell numbers ↓ NK cell degranulation ↓ NKG2D and 2B4 signaling (64)

↑, increased; ↓, decreased compared to control group; /, no reports available. References are indicated in the table.

TABLE 2 | NK cells in multifactorial autoinflammatory disorders.

Multifactorial autoinflammatory disorder (43, 44)		Reports on NK cells
PFAPA	Periodic fever, aphthous stomatitis, pharyngitis, and adenopathy syndrome	= numbers of CD57$^+$ NK cells (65)
Schnitzler's syndrome		↑/= percentage NK cells (66)
SAPHO	Synovitis acne pustulosis hyperostosis osteitis syndrome	↓ NK cell numbers (67)
CRMO	Chronic recurrent multifocal osteomyelitis	/
Sweet's disease		/
Behçet's disease		↓(68, 69) / = (70) / ↑ (71) NK cell numbers ↓/= NK cell cytotoxicity (69, 71–74) ↑/= CD107a degranulation (68, 70, 72) ↓ perforin and granzyme B expression (69) KIR association (75–77) ↑ IFN-γ production (68, 72, 78, 79)
Crohn's disease and ulcerative colitis (UC)		↓ (80) / = (81) NK cell numbers ↓ (81, 82) / = (83, 84) NK cell activity ↓ NKG2D$^+$ NK cells in lamina propria (85) ↑ NKp46$^+$ (Crohn) /NKp44$^+$ (UC) NK cell in mucosa (86) ↑ risk: KIR2DL2, KIR2DS2, KIR2DL5 and KIR2DS1 (UC) (87) ↓ risk: KIR2DS3 (Crohn's) (88)

↑, increased; ↓, decreased; =, equal compared to control group; /, no reports available. References are indicated in the table.

(DITRA), joint contractures, muscle atrophy and panniculitis-induced lipodystrophy (JMP) syndrome, chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE) syndrome, Nakajo-Nishimura syndrome (NNS), CARD-14-mediated pustular psoriasis (CAMPS), and NALP12-associated periodic fever.

NATURAL KILLER CELLS IN MULTIFACTORIAL AUTOINFLAMMATORY DISEASES

Next to monogenic autoinflammatory disease, another group of autoinflammatory diseases present with an uncertain genetic etiology and are considered to have a polygenic or multifactorial cause (43, 44). Also for multifactorial autoinflammatory disorders, NK cell studies remain scarce (**Table 2**). More reports are available on the more prevalent multifactorial autoinflammatory disorders, including Behçet's disease, Crohn's disease and Still's disease. The latter will be discussed in the next section.

In patients with periodic fever, aphthous stomatitis, pharyngitis and adenopathy (PFAPA) syndrome, staining of tonsillar tissue revealed normal numbers of CD57$^+$ NK cells (65). In a small study of patients with Schnitzler syndrome, one out of the two included patients presented with a highly

increased percentage of NK cells (66). A relatively large study of 19 patients with synovitis acne pustulosis hyperostosis osteitis (SAPHO) syndrome reported reduced numbers of NK cells and increased numbers of Th17 cells in SAPHO patients compared to controls, suggesting an imbalance of NK cells and inflammatory Th17 cells may underlie the immune inflammation in patients with SAPHO (67). We found no reports on NK cells in patients with chronic recurrent multifocal osteomyelitis (CRMO) and Sweet's disease.

Reports on peripheral NK cells function in Crohn's disease and ulcerative colitis (UC) demonstrated normal to lower NK cell numbers and NK cell cytotoxic activity, during active disease and remission (80–83). Although NK cell activity was elevated upon intravenous IFN-γ infusion in a clinical trial with a limited number of patients with Crohn's disease, no clinical improvement was reported, suggesting a limited role for IFN-γ-induced NK cell activity in the pathogenesis of Crohn's disease (84). Nevertheless, the association of KIR genes in the susceptibility for Crohn's disease and UC suggests involvement of NK cells in disease development. KIR2DL2, KIR2DS2, KIR2DL5, and KIR2DS1 were found to confer a higher UC susceptibility, whereas a negative association was found between KIR2DS3 and Crohn's disease risk (87, 88). More recent reports have focused on NK cells in tissues in contrast to the peripheral circulating NK cells, resulting in the identification of organ-specific and tissue-resident NK cells. In the gut, a novel subset of mucosal NK cells has been identified. These mucosal NK cells are characterized by the expression of the transcription factor retinoic acid-related orphan receptor C (RORC), CD127 (IL-7Rα), and the production of IL-22 (89). In patients with Crohn's disease, $NKp46^+$ mucosal NK cells were increased which produced IFN-γ upon IL-23 stimulation. In contrast, in patients with UC, $NKp44^+$ IL-22-producing mucosal NK cells were elevated (86, 90). The potential pathogenic or protective role of these NK cells in chronic gut inflammation is still unknown (90). A potential pathogenic role for NK cells in Crohn's disease was established via the successful treatment of patients with Crohn's disease with anti-NKG2D antibody (91). Expression of NKG2D, predominantly on T cells, and its ligands, MHC class I polypeptide-related sequence (MIC)A/B and UL16 binding protein (ULBP), were highly elevated on lesions of patients with active Crohn's disease and UC (92). Upregulation of these ligands, can activate NK cells to kill these activated or stressed autologous cells, indicating a potential pathogenic pathway (92, 93). Nevertheless, in a dextran sulfate sodium (DSS)-induced mouse model of UC, $NKG2D^+$ NK cells were found to be decreased in the lamina propria during active disease, proposing a regulatory role for NK cells in this mouse model of UC (85). In general, NK cells are thought to have a dual role in gut inflammation with a pathogenic role in Crohn's disease and UC via cytotoxic activity and cytokine secretion, and a protective role against the development of cancer (90, 93).

Also in patients with Behçet's disease, there are several conflicting reports on NK cell numbers, phenotype and activity. NK cell numbers were reported to be lower (68), normal (70), or even increased (71) in patients with Behçet's disease compared to controls. One study described the decreased presence of NK cells in bronchoalveolar lavage (BAL) fluid in patients with Behçet's disease with pulmonary manifestations (69). Phenotypically, NK cells of patients with Behçet's disease showed a slightly skewed NK cell receptor repertoire with increased NKG2D, decreased perforin and granzyme B expression, and abnormal KIR expression (69, 70, 75, 76). Association of KIR2DL3 gene expression suggests that NK cell activity is involved in the pathogenesis of Behçet's disease (77). Nevertheless, other reports state a normal NK cell phenotype with normal NKG2D (72), NKG2A, NKp30 or NKp46 expression (70) or normal perforin and granzyme B expression (68). Polymorphisms in *CD94/NKG2A*, *CD94/NKG2C*, *ERAP1*, *KLRC4*, *CCR1,* and *STAT4* were associated with Behçet's disease (94–96). The exact effect of these genetical variations on Behçet's disease remains unknown. Since the genes were either directly or indirectly linked to NK cell activity, it was hypothesized that potential defects in NK cells would result in diminished NK cell function and persistent inflammation following a pathogenic trigger (96, 97). Indeed, a normal to decreased cytotoxic activity was observed in multiple studies in patients with Behçet's disease (69, 71–74). In contrast, a normal to high degranulatory capacity was observed in patients with Behçet's disease after tumor cell stimulation (68, 70, 72). Interestingly, patients with active Behçet's disease showed high IFN-γ production by NK cells, which was thought to contribute to disease relapse (68, 72, 78, 79). In accordance, patients with inactive Behçet's disease had an impaired IL-12-induced STAT4 phosphorylation, associated with lower IFN-γ production. NK cells from inactive Behçet's patients were also able to suppress IFN-γ production by $CD4^+$ T cells, suggesting a regulatory role for NK cells in disease remission (74).

NATURAL KILLER CELLS IN SJIA AND MAS

Systemic juvenile idiopathic arthritis (sJIA), or Still's disease, is a severe immune-inflammatory childhood disorder, classified as one of the subtypes of juvenile idiopathic arthritis (JIA). According to ILAR classification, sJIA is diagnosed in the presence of arthritis in one or more joints with or preceded by quotidian fever of at least 2 weeks duration, and accompanied by evanescent erythematous rash, enlargement of lymph nodes, liver, and/or spleen or serositis (98). In adults, a comparable disorder to sJIA can occur and is referred to as Adult-onset Still's disease (AOSD) (99). sJIA is associated with the potentially life-threatening complication macrophage activation syndrome (MAS). Around 10% of sJIA patients develop MAS, with subclinical MAS reported in up to 50% of the patients (100, 101).

MAS is a potentially life-threatening hyperinflammatory syndrome associated with excessive activation and proliferation of macrophages and $CD8^+$ T cells leading to an overwhelming cytokine storm and hemophagocytosis (102). MAS closely resembles hemophagocytic lymphohistiocytosis (HLH) and is therefore classified as a form of secondary HLH (sHLH). Primary or familial HLH and secondary forms of HLH, including MAS, share most clinical and biological manifestations. Both conditions are characterized by severe inflammation with high morbidity and increased mortality risk. Patients

present with a persistent high fever, lymphadenopathy, and hepatosplenomegaly. In addition, liver dysfunction and central nervous system involvement are frequently observed. Similar to familial or genetic HLH, MAS is characterized by a decrease of several blood cell lines, leading to anemia, thrombocytopenia, and leukopenia. CRP levels are increased. Further, patients present with increased liver enzymes, including AST, ALT, and LDH, increased bilirubin levels, hypoalbuminemia, hyponatremia, and hypertriglyceridemia. Patients also present with increased D-dimers and decreased fibrinogen levels, resulting in a decreased ESR. A severe coagulopathy may ensue and cause multi-organ failure and death in 8–22% of patients (103, 104). An important marker of MAS is the highly increased levels of ferritin. At last, hemophagocytic macrophages are found in bone marrow and tissue biopsies (102, 105–107).

Genetic or fHLH is due to mutations in genes associated with the cytotoxic pathway of cytotoxic T cells (CTL) and natural killer (NK) cells. Conversely, sHLH is not associated with monogenic defects in the cytotoxic pathway and can occur as a complication of infections, malignancies, immunosuppressive therapy, and autoimmune and autoinflammatory diseases (108). In the context of autoimmune and autoinflammatory diseases, sHLH is referred to as MAS. MAS can occur in the context of rheumatic diseases, including systemic lupus erythematosus, Kawasaki disease, AOSD and rheumatoid arthritis, and in monogenic autoinflammatory diseases, amongst which CAPS and FMF (105, 109). Nevertheless, MAS is most frequently reported in patients with sJIA. It has been suggested that NK cell dysfunction represents a common pathway in patients with sJIA, MAS complicating sJIA and HLH (35). In the following sections, we will review NK cell-linked genetical abnormalities and NK cell function in patients with sJIA and MAS complicating sJIA (**Table 3**).

TABLE 3 | NK cells in sJIA, MAS, and fHLH.

	sJIA/AOSD	MAS/sHLH*	fHLH
GENETIC ABNORMALITIES IN CYTOTOXICITY-RELATED GENES			
PRF1		(110–113)	fHLH-type2
UNC13D	(114)	(112, 113, 115–117)	fHLH-type3
STX11		(113)	fHLH-type4
STXBP2		(112, 117)	fHLH-type5
LYST		(111, 117)	Chédiak-Higashi syndrome
Rab27A		(118)	Griscelli syndrome
AP3B1			Hermansky-Pudlak syndrome type 2
SH2D1A		(113)	XLP-1
BIRC4			XLP-2
other		(111, 117)	
NUMBERS OF NK CELLS			
Total	↓ (119–124) = (125–129)	↓ / = (35, 130)	= (35)
$CD56^{dim}$	↓ (119, 122) / = (125)		
$CD56^{bright}$	↓ (121, 128) / = (124, 125)		
NK CELL CYTOTOXICITY AND RELATED PROTEINS			
Cytotoxicity	↓ (110, 120, 121, 123, 128, 129, 131) = (125)	↓ (130)	↓ (132)
CD107a	↓ (121) / = (125)	= (133)	↓(133)
Perforin	↓ (110, 120, 121, 123, 131) = (125)	↓ (110, 130) = (130)	↓ (134) = (35)
Granzyme A	= (125)		
Granzyme B	↓ (120, 123) / ↑ (125)		
Granzyme K	↓ (125)		
CYTOKINE PRODUCTION BY NK CELLS			
IFN-γ	↓° (121, 125) ↑# (120, 124)		↑ (135)
TNF-α	↓# (120)		

XLP, X-linked lymphoproliferative syndrome;, MAS comprises sJIA-associated MAS and sHLH comprises virus-associated HLH and late-onset onset HLH; ↑, increased compared to control group; ↓, decreased compared to control group; =, equal with control group; °, after stimulation with IL-18; #, after stimulation with PMA and ionomycin. References are indicated in the table.*

Genetical Abnormalities Linked to NK Cells in sJIA and MAS

The pathogenesis of MAS has been deducted from HLH due to its clinical and biological similarity. In fHLH, mutations in genes regulating granule-dependent cytotoxicity cause defective cytolysis by NK cells and CTLs underlying the excessive inflammation (136). The exact mechanism that links defective cytolysis with excessive and ongoing inflammation remains elusive. It is hypothesized that NK cells and CTLs in patients with fHLH/MAS fail to eliminate infected cells, which leads to persistent antigenic stimulation. This ongoing stimulation enhances immune activation, excessive proliferation of T cells and production of cytokines, resulting in a self-amplifying inflammatory activity (35, 137). Another hypothesis suggests that defective cytotoxic NK cells and CTLs fail to induce apoptosis to remove activated antigen-presenting cells (APC) and T cells. This failure to terminate the immune response leads to a persistent inflammatory response (35, 42, 137).

fHLH has been linked to mutations in 9 genes which are inherited in an autosomal recessive or X-linked manner. Each of the underlying mutations affects a different protein involved in the granule-mediated cytolytic pathway and is therefore associated with a different subtype of HLH or an HLH-related immunodeficiency syndrome (108, 137). Although the subtypes all share a similar clinical phenotype, disease severity and onset of disease is different according to the affected cytotoxicity-related protein (138, 139). Mutations in *PRF1*, encoding cytotoxic effector protein perforin, is associated with subtype fHLH-2 and early onset of disease (108, 138). Defective perforin expression results in failure to induce apoptosis in the targeted cell, contributing to a prolonged synapse time and an impaired cytotoxic activity (140). Mutations in *UNC13D* are associated with fHLH-type 3. The gene encodes Munc13-4, a protein with a non-redundant role in the priming of the cytolytic granules and consequently fusion of the granule with the

plasma membrane. Mutations in *STX11*, encoding Syntaxin-11, and *STXBP2*, encoding Munc18-2, are associated with fHLH subtype 4 and 5, respectively. Syntaxin-11 and Munc18-2 interact and regulate fusion of granules with the plasma membrane (108).

HLH-related primary immunodeficiency syndromes are associated with mutations in genes involved in the cytotoxic pathway, including *LYST* (encoding for Lyst, involved in Chédiak-Higashi syndrome), *RAB27A* (Rab27a, Griscelli syndrome 2), *AP3B1* (AP3, Hermansky-Pudlak syndrome type 2), *SH2D1A* (SAP, X-linked lymphoproliferative syndrome (XLP)-1), and *BIRC4* (XIAP, XLP-2). These mutations are linked to more general defects in trafficking and exocytosis of lysosomes, resulting in impaired functions in multiple cell types, including neurons, melanocytes, platelets, granulocytes, and lymphocytes. Of note, mutations in *UNC13D*, *STX11,* and *STXBP2* also influence exocytosis processes in platelets and neutrophils, in addition to cytolytic degranulation (108, 136).

With regard to MAS, no loss-of-function mutations in fHLH-associated genes have been described. Nevertheless, numerous studies have documented polymorphisms in genes associated with granule-mediated cytotoxicity in MAS patients. Vastert et al. reported heterozygous missense mutations in *PRF1* in 20% of sJIA patients with a history of MAS, compared to only 9.8% of sJIA patients without MAS (110). Zhang et al. reported polymorphisms in the *UNC13D* gene in 11 out of 18 patients with MAS complicating sJIA. Two patients presented with bi-allelic sequence variants, 9 of the 16 other patients had a common pattern of sequence variants comprising 12 single nucleotide polymorphisms (SNPs). The genetic variations were highly associated with MAS-complicated sJIA (57%) compared to uncomplicated sJIA (8.2%) and healthy controls (12%) (115). SNPs in *UNC13D* were also found in 2 sJIA-associated MAS patients in another cohort (116). Remarkably, a single-patient study described heterozygous mutations in the *UNC13D* gene associated with reduced NK cell cytotoxic function in sJIA (114). More recently, Schulert et al. described a novel heterozygous intronic variant of *UNC13D* associated with impaired NK cell degranulation in a patient with sJIA and recurrent MAS episodes (141). In contrast, another study in 133 sJIA patients found no association between SNPs in *PRF1*, *UNC13D*, *GZMB,* and *Rab27a* and sJIA (142). Whole exome sequencing in 14 MAS-complicated sJIA patients revealed heterozygous protein-altering variants in fHLH-associated genes (*LYST*, *UNC13D,* and *STXBP2*) in 35.7% of patients compared to only 13.7% in uncomplicated sJIA patients. The functional significance of these variants was not investigated. Next to these known genes, heterozygous protein-altering variants and SNPs were found in a number of genes indirectly associated with cytotoxicity, including *SLAC2B*, *XIRP2*, *MICAL2*, *CADPS2*, *ARHGAP21*, *CCDC141*, *FAM160A2,* and *LRGUK*, through an effect on microtubule reorganization and vesicle transport (117).

Also in virus-associated HLH, heterozygous mutations in *LYST* and *PRF1* have been described in patients with fatal sHLH following H1N1 influenza infection. No protein-altering variants were found in *UNC13D*, *STX11*, *STXBP2,* or *Rab27a*. The study also identified other protein-altering variants in genes associated with cytoskeleton stabilization (*XIRP2*) and microtubule structure (*LRGUK*), which were described as MAS-associated genes by Kaufman et al. (111, 117). Also NK cell receptor-related genetic variations, especially KIR polymorphisms were found in EBV-associated HLH, with higher susceptibility in carriers of KIR2DS5 or KIR3DS1 (143).

The identification of heterozygous variants in genes directly and indirectly linked to cytotoxicity pathways in MAS and infection-associated HLH blurs its distinction from late-onset fHLH. A heterozygous *Rab27a* mutation was identified in 2 adolescents with HLH (118). A large study on adult HLH patients found hypomorphic mutations in *PRF1*, *UNC13D,* and *STXBP2* in 14% of patients correlating with a later-onset of disease (112). Another study identified mutations in HLH-related genes in 18 out of a total of 252 adolescent and adult patients. The majority of these patients (50%) presented with mutations in *PRF1*, followed by 38.8% of patients with *STX11* missense mutations. *SH2D1A* mutations were found in 2 patients of whom 1 patient also had a *PRF1* mutation, and 1 patient had a *UNC13D* missense mutation. No variations were found in *STXBP2* or *BIRC4*. The biallelic and monoallelic mutations corresponded with a very low to low, respectively, NK cell activity compared to controls. The authors suggest that these "milder" genetic mutations, in a functionally unimportant region of the protein, may be a predisposing factor to late-onset HLH (113). In contrast, high-throughput sequencing of genetically undiagnosed late-onset HLH patients found no enriched mono-allelic variations compared to the healthy population. The authors suggest caution with the interpretation of causality and identification of genetic variants in disease (144). Indeed, other factors than genetics can trigger the development of HLH. The presentation of HLH as a genetic or secondary form has recently shifted toward considering it as a threshold-disease. A combination of predisposing factors (i.e., genetics, underlying diseases, immunosuppression, infection,…) are accumulated until a certain threshold is reached, leading to uncontrolled inflammation. This model comprises a wide spectrum of HLH, from fHLH, to MAS, and sHLH (108, 145, 146).

Although no clear monogenic defects in cytotoxicity-related genes can be associated with MAS complicating sJIA, the genetic variations observed in some studies can reflect subpopulations of patients and further highlight the role of NK cell dysfunction in the development of MAS. In addition, these observations suggest the involvement of inflammation-driven factors underlying cytotoxic defects of NK cells in sJIA and MAS.

NK Cell Dysfunction in sJIA

The role of NK cells in the pathogenesis of sJIA remains incompletely understood. Contradictory results concerning numbers or activity of NK cells in sJIA patients have been reported, which at least partially can be explained by the small numbers of patients included in these studies together with a high heterogeneity of the disease course of sJIA patients (147).

Most studies report low to mildly decreased numbers of NK cells in the PBMC fraction of sJIA and AOSD patients (119–124). Nevertheless, other research groups described normal numbers of NK cells (125–129). Correspondingly, the proportion of NK cell subsets was also altered in sJIA patients. Some studies found decreased $CD56^{dim}$ NK cells (119, 122), whereas others reported decreased $CD56^{bright}$ NK cells (121, 128). In contrast, equal numbers of $CD56^{dim}$ and $CD56^{bright}$ NK cells have been reported when comparing sJIA or AOSD patients and healthy controls (124, 125). Phenotypically, only subtle alterations in the expression of NK cell receptors are observed in sJIA patients (120, 121, 125).

NK cell dysfunction in sJIA is thought to be one of the contributory features for its strong association with MAS. Nevertheless, whether an intrinsic cytotoxic defect can be found in patients with sJIA and AOSD remains undecided. Although, multiple studies reported decreased NK cell cytotoxicity in PBMCs from sJIA patients (110, 120, 121, 123, 128, 129), normal NK cell cytotoxicity was detected when the cytotoxicity was calculated relative to the (decreased) numbers of NK cells (125). Lower expression of cytotoxic proteins (i.e., perforin and granzyme B) was associated with this decrease in NK cell function (110, 120, 121, 123, 148). Interestingly, autologous stem cell transplantation in sJIA patients restored this decreased expression of perforin (148). Contradictory, RNA sequencing of NK cells of sJIA patients revealed normal transcriptional expression of perforin and granzyme A and B. In the same study, protein levels of these cytotoxic proteins in NK cells were found to be normal-to-increased, further supporting the reported intact NK cell cytotoxicity. Furthermore, Put et al. described decreased expression of granzyme K in $CD56^{bright}$ NK cells at protein and transcriptional level (125). The expression of granzyme K by $CD56^{bright}$ NK cells has been linked to the killing of autologous activated T cells in patients with multiple sclerosis (149). This regulatory aspect of NK cells has not been investigated so far in patients with sJIA. However, by using a novel mouse model for sJIA (150), we recently found a cytotoxic defect in NK cells of the diseased animals and further provided evidence that NK cells play a regulatory role in the development of the disease via a NKG2D-dependent control of inflammatory monocytes (151).

Since the functional, phenotypical and transcriptional data have not allowed to identify an intrinsic cytotoxic defect in NK cells of patients with sJIA, a transient NK cell dysfunction induced by the continuous inflammatory environment has been proposed (125, 152). This effect is mainly thought to be driven by the excessive levels of IL-18 found in patients with sJIA. Although IL-18 is a stimulatory cytokine for cytotoxicity and cytokine production by NK cells, it failed to induce a cytotoxic response, an increased perforin expression and degranulation by NK cells (121, 153). More surprisingly, stimulation with IL-18 did not elicit production of IFN-γ by sJIA NK cells, which was caused by a reduced phosphorylation downstream of the IL-18 receptor β (121, 125). Of note, NK cells of sJIA and AOSD patients were still capable of IFN-γ and TNF-α production after triggering with other stimulatory factors (120, 124, 125). This discordance between the high plasma levels of IL-18 and relatively low IFN-γ expression are most likely reflecting a state of hyporesponsiveness toward IL-18 in patients with active sJIA. At the time of MAS, patients seem to recover IL-18 responsiveness resulting in high plasma levels of IFN-γ in contrast to sJIA (125, 152, 154, 155). Next to IL-18, the high levels of IL-6 during sJIA are also associated with decreased cytotoxicity with decreased perforin and granzyme B expression, which could be recovered by tocilizumab (131). The NK cell dysfunction seen in patients with sJIA could be a consequence of the systemic inflammation, but whether this is a predictive factor of MAS development in all of these patients remains unclear.

NK Cell Deficits in MAS

Functional deficits of NK cells are generally accepted to be part of the pathogenesis of MAS (as discussed above). Indeed, Grom et al. reported decreased cytotoxicity of NK cells in MAS-complicated sJIA patients. Interestingly, two patterns were observed, mimicking secondary HLH and genetic HLH. Firstly, some patients presented with low NK cell activity associated with drastically decreased NK cell numbers but mildly increased perforin expression. On the other hand, very low NK cell cytotoxicity was associated with mildly decreased NK cell numbers but highly decreased expression of perforin (130). Contradictory, NK cell degranulation assays demonstrated abnormal NK cell activity in only 22% of patients with secondary HLH, including patients with MAS (133). Phenotypical studies of NK cells of MAS patients are scarce. Increased NKG2A and decreased NKG2D expression on NK cells were reported in a study on sHLH patients, including MAS patients (156).

CONCLUSION

The role of NK cells in autoinflammatory disorders remains elusive. In general, a defective NK cell function and diminished NK cell numbers are observed. Nevertheless, for many monogenic and multifactorial autoinflammatory diseases (i.e., Crohn's disease, UC and Behçet's disease) no decisive conclusion can be made, due to the low number of studies or the contradictory results.

The role of NK cells in sJIA and MAS has been studied extensively. Nevertheless, there are no robust genetical or transcriptional defects observed in the cytotoxic pathways of NK cells in patients with sJIA and MAS complicating sJIA. Although, functional defects in cytotoxicity and/or cytokine production have frequently been observed in these patients, a number of conflicting data have been reported for patients with sJIA, which probably reflects the disease heterogeneity in sJIA (122, 157, 158). In MAS, the data are conclusive for functional deficits of NK cells. Since inflammasome activation and the release of IL-18 efficiently activates NK cell function, one would assume highly active NK cells in patients with sJIA and MAS complicating sJIA, both characterized by extremely high IL-18 levels. Nevertheless, an inflammation-induced NK cell exhaustion, mediated via the constitutively high levels of cytokines (IL-18 and IL-6), has been

hypothesized to be part of the pathogenesis of sJIA and MAS. The resulting cytokine-induced NK cell dysfunction, leads to failure to terminate the immune response and thus an ongoing inflammation (35, 42, 137).

AUTHOR CONTRIBUTIONS

JV wrote the first draft of the review. JV, CW, and PM wrote and revised the final version of the review. All authors contributed to manuscript revision, read and approved the submitted version.

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frontiers in Immunology

ORIGINAL RESEARCH
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Released Exosomes Contribute to the Immune Modulation of Cord Blood-Derived Stem Cells

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Background: Clinical studies demonstrated the immune modulation of cord blood-derived stem cells (CB-SC) for the treatment of type 1 diabetes and other autoimmune diseases, with long-lasting clinical efficacy. To determine the molecular mechanisms underlying the immune modulation of CB-SC, the actions of exosomes released from CB-SC were explored in this study.

Methods: Exosomes were isolated from CB-SC cultures using ultracentrifugation and confirmed with different markers. The activated T cells and purified monocytes from peripheral blood mononuclear cells (PBMC) were treated with CB-SC in the presence or absence of the purified exosomes, followed by functional and flow cytometry analysis of phenotypic changes with different immune cell markers.

Results: CB-SC-derived exosomes displayed the exosome-specific markers including CD9, CD63, and Alix, at the size of 85.95 ± 22.57 nm. In comparison with the treatment of CB-SC, functional analysis demonstrated that the CB-SC-derived exosomes inhibited the proliferation of activated PBMC, reduced the production of inflammatory cytokines, downregulated the percentage of activated $CD4^+$ T and $CD8^+$ T cells, and increased the percentage of naive $CD4^+$ T and $CD8^+$ T cells. Using the fluorescence dye DiO-labeled exosomes, flow cytometry revealed that exosomes preferably bound to the monocytes in the PBMC, leading to an improvement of mitochondrial membrane potential of treated monocytes. Further study indicated that the purified monocytes gave rise to spindle-like macrophages displaying type 2 macrophage (M2) surface markers and upregulating an expression of immune tolerance-related cytokines after the treatment with exosomes.

Conclusions: CB-SC-derived exosomes display multiple immune modulations and primarily on monocytes, contributing to the immune education of CB-SC in the clinical treatment of autoimmune diseases.

Keywords: exosome, immune modulation, CB-SC, T cell, monocyte, M2 macrophage, stem cell educator therapy

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INTRODUCTION

Autoimmunity, leading to the destruction of pancreatic islet β cells in type 1 diabetes (T1D), is mediated by abnormalities in multiple types of immune cells, including T cells, B cells, regulatory T cells (Treg), monocytes/macrophages (Mo/Mφ), and dendritic cells (DC) (1). While insulin therapy allows T1D patients to manage blood sugar, it does not address the underlying immune

dysfunction. Pancreatic and islet transplantations can overcome the shortage of insulin-producing islet β cells. However, donor scarcity and risk of immune rejection severely limit the use of these treatment modalities. Insulin-producing cells have been generated from embryonic stem (ES) cells and induced pluripotent stem cells (iPSC) through *ex vivo* inductions (2). However, transplanting these stem cells can also cause immune rejection (3), raising ethical and safety concerns. Alternative approaches are needed to correct the underlying autoimmunity of T1D.

We developed the Stem Cell Educator (SCE) therapy, which harnesses the unique therapeutic potential of cord blood-derived stem cells (CB-SC) to treat the multiple immune dysfunctions in diabetes (4–7). SCE therapy circulates patient's blood through a blood cell separator, cocultures the patient's lymphocytes with adherent CB-SC *in vitro*, and returns "educated" lymphocytes to the patient's circulation (4, 5, 7). In contrast to conventional immune therapies, SCE therapy modifies rather than destroys the cells responsible for autoimmunity. Our clinical phase 1/2 trials indicate that SCE therapy reverses autoimmunity, promotes regeneration of islet β cells, and improves metabolic control for the treatment of T1D (4–7) and T2D (6, 7). SCE therapy elicits immune tolerance by altering autoimmune T cells and pathogenic Mo/Mφs through the autoimmune regulator (AIRE) and other molecular pathways (8). Exosomes are the smallest extracellular vesicles (EVs) (30–150 nm) that are produced by a variety of cells, and existing in all biological fluids, with diverse biological functions in the maintenance of homeostasis (9–11) To understand the molecular mechanisms underlying the immune education of SCE therapy to improve its clinical efficacy for the treatment of T1D and other autoimmune diseases, we explored the action of CB-SC-derived exosomes in this study. The data demonstrated multiple immune modulations of CB-SC-derived exosomes on immune cells.

MATERIALS AND METHODS

Cell Culture for CB-SC

The culture of CB-SC was performed as previously described (4). In brief, human umbilical cord blood units (50–100 ml/U) were collected from healthy donors and purchased from Cryo-Cell International blood bank (Oldsmar, FL). Cryo-Cell has received all accreditations for cord blood collections and distributions, with hospital institutional review board (IRB) approval and signed consent forms from donors. Mononuclear cells were isolated with Ficoll-Hypaque ($\gamma = 1.077$, GE Health), and red blood cells were removed using ammonium–chloride–potassium (ACK) lysis buffer (Lonza). The remaining mononuclear cells were washed three times with phosphate-buffered saline (PBS) and seeded in 150 × 15 mm style non-tissue culture-treated Petri dishes (Becton Dickinson Labware) at 1×10^6 cells/ml. Cells were cultured in X-Vivo 15 chemical-defined serum-free culture medium (Lonza) and incubated at 37°C with 8% CO_2 for 10–14 days. To characterize the phenotype and purity of CB-SC, the detached CB-SC were performed by flow cytometry with associated markers, including leukocyte common antigen CD45, ES cell markers OCT3/4 and SOX2, hematopoietic stem cell marker CD34, and the immune tolerance-related markers CD270 and CD274. Isotype-matched immunoglobulin G (IgGs) served as control.

Isolation of Exosomes From CB-SC Culture

At 80–90% of confluence, CB-SC were washed with PBS three times to remove all cellular debris, supplied with 25 ml fresh serum-free medium, and continued culturing. After 4 days, the conditioned medium was harvested for purifying exosomes by ultracentrifugation in an Optima L-100XP ultracentrifuge (Beckman Coulter) as previously described (12, 13). Initially, the conditioned medium was centrifuged at 2,000 g for 20 min to remove cellular debris and other components, and followed by centrifugation at 10,000 g for 30 min to remove mitochondria. Consequently, the supernatant was collected and transferred to a new tube with a 10-kDa Amicon® Ultra-15 Centrifugal Filter Unit (Millipore Sigma) and centrifuged at 4,000 g for 30 min to concentrate the supernatant, followed by centrifugation at 100,000 g for 70 min. Finally, the pellets were rewashed once with PBS at 100,000 g for 70 min. The purified exosomes were resuspended in 100 μl PBS and transferred to a new centrifuge tube with a 0.22-μm filter and centrifuged at 2,000 g for 2 min. All centrifugation was performed at 4°C. The purified exosomes were kept at −80°C for later applications.

CHARACTERIZATION OF EXOSOME

Electron Microscopy

The concentrated exosomes were loaded onto the electron microscope grids coated with Formvar. After being contrasted with uranyl–acetate solution and embedded in methylcellulose, exosomes were observed and photographed with a FEI Titan Themis 200 kV scanning transmission electron microscope (Thermo Fisher Scientific).

Dynamic Light Scattering

The size of the exosomes was determined using a dynamic light scattering (DLS) method, performed with a Nano-ZS Zetasizer Analyzer (Malvern Instruments Ltd, Malvern, united Kingdom) with a refractive index (RI) at 1.39.

Western Blotting

Purified exosomes or cells were treated with radioimmunoprecipitation assay (RIPA) buffer; protein concentration was determined by a bicinchoninic acid (BCA) protein assay. Proteins were separated by 10% Tris–HCl gel (Bio-Rad) and transferred to the polyvinylidene fluoride (PVDF) membrane, blotted overnight with anti-human Calnexin (Biolegend) and anti-human Alix monoclonal antibodies (mAb) (Biolegend), followed by anti-rat or anti-mouse horseradish peroxidase (HRP)-conjugated secondary mAb (Thermo Fisher Scientific). Membranes were incubated with chemiluminescent substrate (Millipore Sigma), and chemiluminescent signal was detected upon exposure to autoradiographic films.

FACS Assessment of Exosomes Characterization

To further confirm the release of exosomes from CB-SC, we performed the flow cytometry analysis after being captured with Exosome-Human CD63 Isolation/Detection Beads (Thermo Fisher, Waltham, MA). Owing to the small size of CB-SC-derived exosomes and the limitation (>200 nm) of particle size detected by the Gallios Flow Cytometer (Beckman Coulter), the human CD63 isolation/detection beads (4.5-μm size) were utilized to isolate the exosomes from the ultracentrifuge-concentrated supernatant of CB-SC cultures for flow cytometry. Exosomes (10–20 μg protein) were incubated with 20 μl of 4.5-μm size anti-human CD63 beads in 100 μl volume and incubated overnight at 4°C under 500 rpm agitation. Consequently, exosomes bound with CD63-capturing beads were place in the DynaMag™-2 Magnet Stand (Thermo Fisher) and washed twice with PBS, and followed by the preparation for flow cytometry. Exosomes captured by anti-CD63 beads were aliquoted and incubated with phycoerythrin (PE)-conjugated anti-human CD63 (BD Bioscience), fluorescein isothiocyanate (FITC)-conjugated anti-human CD81 (BD Bioscience), and PE-conjugated anti-human CD9 mAb (BD Bioscience), respectively, for 45 min at room temperature and then washed twice with PBS in the DynaMag™ -2 Magnet Stand, followed by flow cytometry.

PBMC Isolation and Proliferation Assay

Human buffy coat blood units were purchased from the New York Blood Center (New York, NY). Human peripheral blood-derived mononuclear cells (PBMC) were harvested as previously described (5). PBMC were stained with carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies) according to the manufacturer protocol, then stimulated with Dynabeads coupled with anti-CD3 and anti-CD28 antibodies (Life Technologies) for 72 h in the presence of treatment with exosomes at 10 μg/ml, 20 μg/ml, and 40 μg/ml in duplicate, respectively, and were incubated at 37°C in 5% CO_2 in the tissue culture-treated 96-well plate. Exosome-untreated cells served as control.

Flow Cytometry

Flow cytometric analyses of surface and intracellular markers were performed as previously described (6). Samples were preincubated with human BD Fc Block (BD Pharmingen) for 15 min at room temperature and then directly aliquoted for different antibody staining. Cells were incubated with different mouse anti-human mAb from Beckman Coulter (Brea, CA), including PE-conjugated anti-CD56 and anti-CCR7; PE-Cy5-conjugated anti-CD19; PE-Cy7-conjugated anti-CD11c, and anti-CD45, anti-CD45RO; allophycocyanin (APC)-conjugated anti-CD80; APC-Alexa Fluor 750-conjugated anti-CD8 and anti-CD86; Krome Orange-conjugated anti-CD14; and PC 5.5-conjugated human leukocyte antigen DR isotype (HLA-DR). From BD Biosciences (San Jose, CA), mAb include the following: the APC-conjugated anti-human CD4 antibody, PE-conjugated anti-CD63 and anti-CD163, FITC-conjugated anti-CD81 and anti-CD206, Alexa Fluor 488-Sox2 and BV421-conjugated anti-CD209, and propidium iodide (PI). The eFluor 660-conjugated rat anti-human OCT3/4 and isotype-matched IgG Abs and FITC-conjugated CD9 were from Thermo Fisher. Pacific Blue (PB)-conjugated anti-human CD3 Ab was from BioLegend. For surface staining, cells were stained for 30 min at room temperature and then washed with PBS at 2,000 rpm for 5 min before flow analysis. Isotype-matched mouse anti-human IgG antibodies (Beckman Coulter) served as a negative control for all fluorescein-conjugated IgG mAb. For intracellular staining, cells were fixed and permeabilized according to the PerFix-nc kit (Beckman Coulter) manufacturer's recommended protocol. After staining, cells were collected and analyzed using a Gallios Flow Cytometer (Beckman Coulter) equipped with three lasers (488 nm blue, 638 nm red, and 405 nm violet lasers) for the concurrent reading of up to 10 colors. The final data were analyzed using the Kaluza Flow Cytometry Analysis Software version 2.1 (Beckman Coulter).

BCA Assay

Exosome concentration was measured by protein quantification using BCA assay (14), using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). For protein concentration quantification, 10 μl of exosome samples were incubated with 200 μl BCA reagent (Thermo Fisher Scientific) at 37°C for 30 min. Absorbance was read at 562 nm within 10 min. The protein concentration was determined by interpolating test sample concentrations relative to the standard concentration curve using bovine serum albumin.

Assessment of Exosome Uptake by Different Subpopulation PBMC

To explore the interaction of CB-SC-derived exosomes with different cell compartments of PBMC, PBMC were incubated in non-tissue-treated hydrophobic 24-well plates (avoiding the attachment of monocytes), with the green fluorescent lipophilic dye 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) (Millipore Sigma)-stained exosomes. Four hours later, different cell populations in PBMC were labeled with mAb specific for lineage markers including Pacific Blue-conjugated anti-human CD3 (Thermo Fisher Scientific), APC-conjugated anti-human CD4 (BD Bioscience), AF750-conjugated anti-human CD8 (Beckman Coulter), PE-Cy7-conjugated anti-human CD11c (Beckman Coulter), Krome Orange-conjugated anti-human CD14 (Beckman Coulter), PC5-conjugated anti-human CD19 (Beckman Coulter), and PE-conjugated anti-human CD56 mAb (Beckman Coulter). To determine T-cell population and remove $CD4^+$ monocytes, anti-CD3 Ab was employed for gating out $CD4^+$ monocytes, in addition to the consideration of cell-size difference.

Action of CB-SC Derived Exosomes on Monocytes

Monocytes were purified from PBMC using $CD14^+$ microbeads (Miltenyi Biotec) according to the manufacturer's instruction, with purity of $CD14^+$ cells >95%. The purified $CD14^+$ monocytes were initially seeded in the tissue culture-treated six-well plate at 5×10^5 cells/well and cultured in X-Vivo 15 serum-free medium, at 37°C, 5% CO_2 conditions. After adhering for 2 h, the attached monocytes were washed

twice with PBS to remove all floating cells and cell debris, followed by treatment with or without CB-SC-derived exosomes (40 μg/ml) in X-Vivo 15 serum-free medium, at 37°C, 5% CO_2 conditions. After treatment for 3 days, both the supernatant and detached cells were collected for ELISA and flow cytometry, respectively. The morphological change was photographed by phase-contrast microscope before cells were detached with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) (Corning, New York) or an 18-cm cell scraper (BD Falcon). The exosome-treated and untreated cells were immunostained with a combination of mAbs, including Krome Orange-conjugated anti-human CD14 (Beckman Coulter), APC-conjugated anti-human CD80 (Beckman Coulter), AF750-conjugated anti-human CD86 (Beckman Coulter), PE-conjugated anti-human CD163, FITC-conjugated anti-human CD206, and BV421-conjugated anti-human CD209 (BD Bioscience) for 30 min at room temperature and then washed with PBS at 2,000 rpm for 5 min before flow analysis. Isotype-matched mouse anti-human IgG antibodies (Beckman Coulter) served as a negative control.

To examine the effects of CB-SC-derived exosomes on mitochondrial function of monocytes, 3×10^5 fresh PBMC were plated on non-tissue-treated hydrophobic 24-well plates (avoiding the attachment of monocytes); PBMC were cultured with or without CB-SC-derived exosomes. After incubation for 3 h, the PBMC were harvested to be stained with anti-human CD14-Krome Orange-conjugated antibody and cytoplasmic Ca^{2+} dye (Fluo-4) (Thermo Fisher Scientific) and mitochondrial Ca^{2+} dye (Rhod-2) (Thermo Fisher Scientific) and tetramethylrhodamine, ethyl ester (TMRE) (Abcam) for the detection of mitochondrial membrane potential.

ELISA Assay

To detect the cytokine production by PBMC, 1×10^5 PBMC were stimulated with anti-CD3/anti-CD28 beads in the presence or absence of CB-SC-derived exosomes at 10, 20, and 40 μg/ml in triplicate in a 96-well plate with a total of 200 μl X-Vivo 15 serum-free culture medium (Lonza) per well. After the treatment for 72 h, the supernatants were collected to examine the inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) using human TNF-α and IFN-γ enzyme-linked immunosorbent assay (ELISA) kits (Biolegend) according to the manufacturer protocols, respectively.

To examine the cytokine production by monocytes, the purified $CD14^+$ monocytes were initially seeded in the tissue culture-treated six-well plate at 5×10^5 cells/well and cultured in X-Vivo 15 serum-free medium, at 37°C, 5% CO_2 conditions. After adhering for 2 h, the attached monocytes were washed twice with PBS to remove all floating cells and cell debris, followed by treatment with or without CB-SC-derived exosomes (40 μg/ml) in X-Vivo 15 serum-free medium, at 37°C, 5% CO_2 conditions. After treatment for 3 days, supernatants were collected to test the transforming growth factor (TGF)-β1 and interleukin (IL)-10 using ELISA kits (Biolegend) according to manufacturer's recommended protocol, respectively.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 8 (version 8.0.1) software. The normality test of samples was performed by the Shapiro–Wilk test. Statistical analyses of data were performed by the two-tailed Student's *t*-test to determine statistical significance for parametric data. Mann–Whitney *U*-test was utilized for non-parametric data. Values were given as mean ± standard deviation (SD). Statistical significance was defined as $P < 0.05$, with two sided.

RESULTS

Characterization of CB-SC-Derived Exosomes

Initially, the phenotype and purity of CB-SC were characterized by flow cytometry with CB-SC-associated markers (8, 15) including leukocyte common antigen CD45, ES cell markers OCT3/4 and SOX2, hematopoietic stem cell marker CD34, and the immune tolerance-related markers CD270 and CD274. CB-SC highly express CD45, OCT3/4, SOX2, and CD270, with medium level of CD274 and no expression of CD34 (**Figure 1A**). CD45 and OCT3/4 are regularly utilized for the purity test of CB-SC, at ≥95% of $CD45^+OCT3/4^+$ CB-SC.

Next, exosomes were purified from CB-SC cultures using serial centrifugations (**Figure 1B**). Phenotypic characterization confirmed the expression of exosome-specific markers such as CD9 and CD81 on the CB-SC-derived exosomes by flow cytometry, which were analyzed following the purification by conjugation with anti-CD63 beads (**Figure 1C**). The presence of exosomes was demonstrated by transmission electron microscopy (**Figure 1D**), with the size of 85.95 ± 22.57 nm (**Figure 1E**). Western blot further proved the expression of the exosome-associated universal marker Alix, but failed to exhibit the endoplasmic reticulum (ER)-associated marker calnexin (**Figure 1F**). The data indicated that CB-SC release exosomes.

Suppression of PBMC Proliferation by CB-SC-Derived Exosomes

To explore the immune modulation of CB-SC-derived exosomes, the anti-CD3/CD28 bead-activated PBMC were initially treated with different dosages of CB-SC-derived exosomes ranging from 10 to 40 μg/ml. The PBMC proliferation was evaluated by carboxyfluorescein succinimidyl ester (CFSE) staining and flow cytometry analysis. The data demonstrated that the proliferation of PBMC was markedly declined after the treatment with CB-SC, with a percentage reduction about of 61.55 ± 6.43% (**Figures 2A,B**). In comparison, treatment with different dosages of exosomes declined the percentage of PBMC proliferation at 5.54% for the dosage of 10 μg/ml exosomes, 10.99% for 20 μg/ml, and 15.37% for 40 μg/ml, respectively (**Figure 2A**). There were significant differences for the dose groups at 20 μg/ml ($P < 0.01$) and 40 μg/ml ($P < 0.005$) relative to the group of anti-CD3/CD28-activated PBMC (**Figure 2B**).

In addition, we tested the inhibitory effect of CB-SC-derived exosomes at high doses such as 80 and 160 μg/ml on the anti-CD3/CD28 bead-activated PBMC. The inhibitory effect of

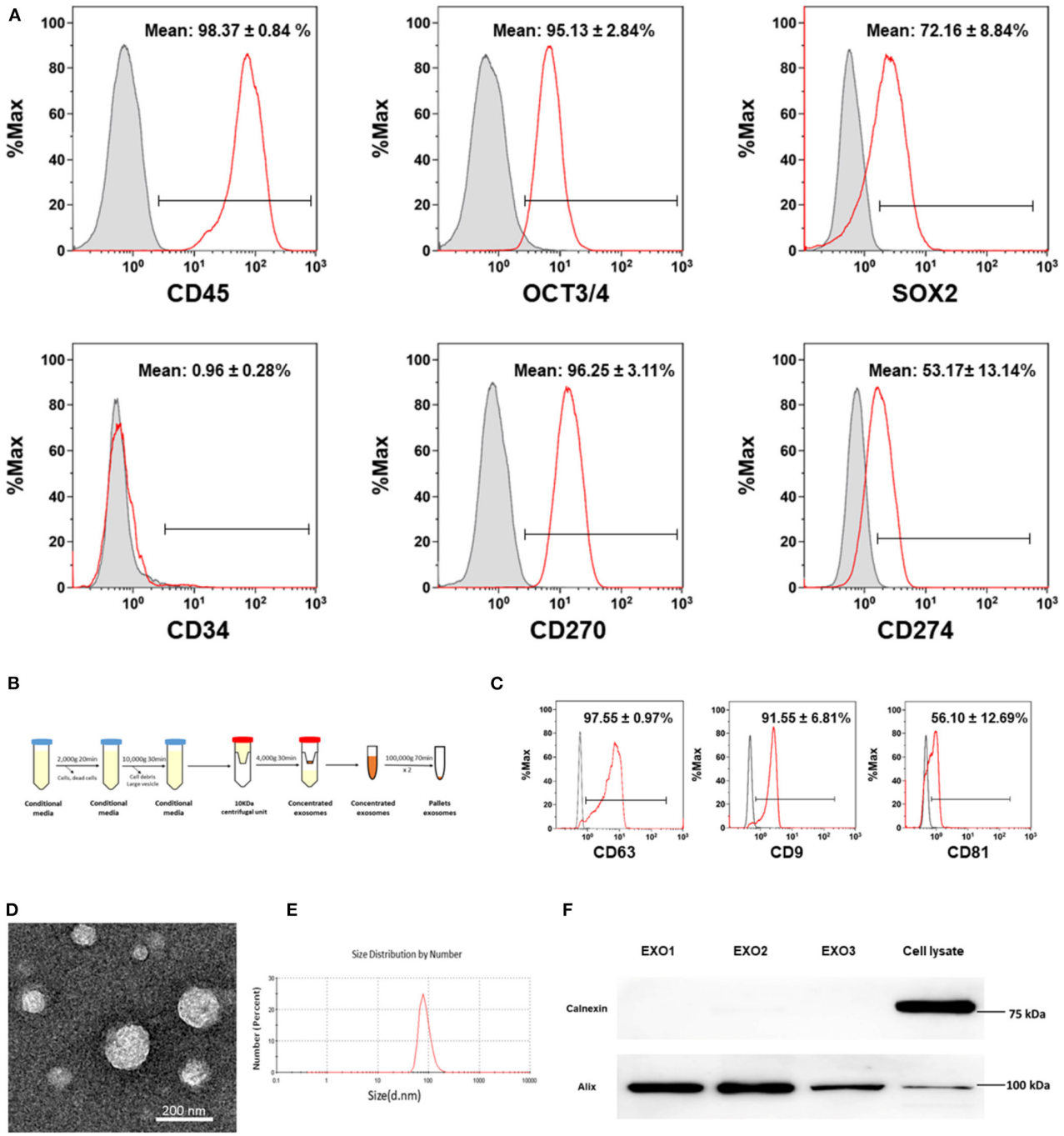

FIGURE 1 | Characterization of cord blood multipotent stem cell (CB-SC)-derived exosomes. **(A)** Phenotypic characterization of CB-SC with a high purity. The detached CB-SC were performed by flow cytometry with associated markers including leukocyte common antigen CD45, embryonic stem (ES) cell markers OCT3/4 and SOX2, hematopoietic stem cell marker CD34, and the immune tolerance-related markers CD270 and CD274. Isotype-matched immunoglobulin G (IgGs) served as control. Data were represented from three experiments with similar results. **(B)** Outline the protocol for an isolation of CB-SC-derived exosomes. **(C)** Flow cytometry analysis of the expression of CB-SC-derived exosome-specific markers CD63, CD9, and CD81. The CB-SC-derived exosomes ($N = 4$) were initially concentrated by ultracentrifugation and followed by capturing with anti-CD63 Dynabeads. Isotype-matched IgGs served as control (gray histogram). **(D)** Image of CB-SC-derived exosomes by electron microscopy. **(E)** Size distribution of CB-SC-derived exosomes. **(F)** Western blotting of CB-SC-derived exosomes with exosome marker Alix and ER-associated marker calnexin.

exosomes on the proliferation of anti-CD3/CD28 bead-activated PBMC was markedly improved at these high dosages (**Figure S1**). In consideration of the clinical design (4, 7), the 9- or 12-layer Stem Cell Educators are utilized for the treatment of patients. Based on the current protocol, a maximum of 80 μg exosomes was normally purified from 240 ml supernatants of CB-SC

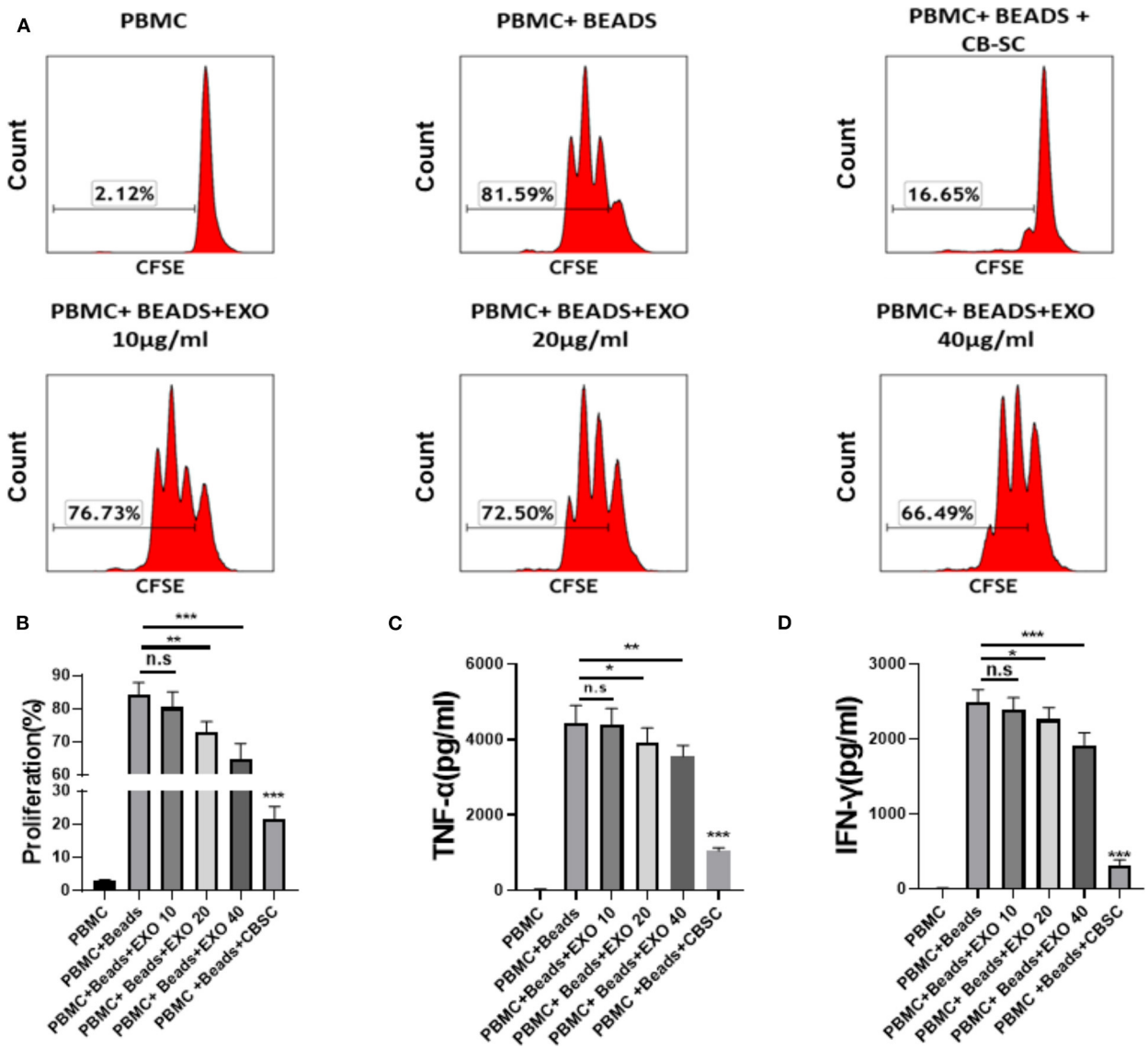

FIGURE 2 | Modulation of cord blood-derived stem cell (CB-SC)-derived exosomes on peripheral blood mononuclear cell (PBMC). **(A–D)** Health donor-derived PBMC ($N = 3$) were treated with CB-SC-derived exosomes ($N = 3$) for 3 days in the presence of T-cell activator CD3/CD28 Dynabeads. **(A)** Suppression of PBMC proliferation by CB-SC-derived exosomes. The carboxyfluorescein succinimidyl ester (CFSE)-labeled PBMC were stimulated to proliferate with T-cell activator CD3/CD28 Dynabeads in the presence of different dosages of CB-SC-derived exosomes. Treatment with CB-SC served as positive control. Untreated PBMC served as negative control. Histograms of flow cytometry were representative of nine experiments with similar results. **(B)** Quantitative analysis of PBMC proliferation showed a marked reduction in PBMC expansion after the treatment with CB-SC-derived exosomes at the dosage of 10 μg/ml ($p > 0.05$), 20 μg ($p < 0.01$)/ml, and 40 μg/ml ($p < 0.005$), respectively. **(C)** Suppression of inflammatory cytokine tumor necrosis factor alpha (TNF-α) production in presence of different dosages of CB-SC-derived exosomes at 10, 20, and 40 μg/ml. **(D)** Decrease in the level of cytokine IFN-γ in PBMC after the treatment with different dosages of CB-SC-derived exosomes at 10, 20, and 40 μg/ml. Data were given as mean ± SD (standard deviation). *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$.

cultures (20 ml per layer for 12 layers). Therefore, the optimal dose of exosomes at 40 μg/ml was utilized for the following experiments. Meanwhile, inflammatory cytokines were examined by ELISA assay. We found that the levels of TNF-α and IFN-γ were markedly decreased following treatment with CB-SC-derived exosomes at 20 μg/ml ($P < 0.05$) and 40 μg/ml ($P < 0.01$ and $p < 0.005$ respectively) (**Figures 2C,D**). In addition, the CD4/CD8 ratio was analyzed, and it failed to display a marked change after the treatment with exosomes, with CD4/CD8 ratios at 2.49 ± 0.97 for the PBMC group, 2.53 ± 0.94 for the PBMC + beads group, and 2.49 ± 0.96 for the PBMC + beads + exosomes group ($P > 0.05$, $N = 5$).

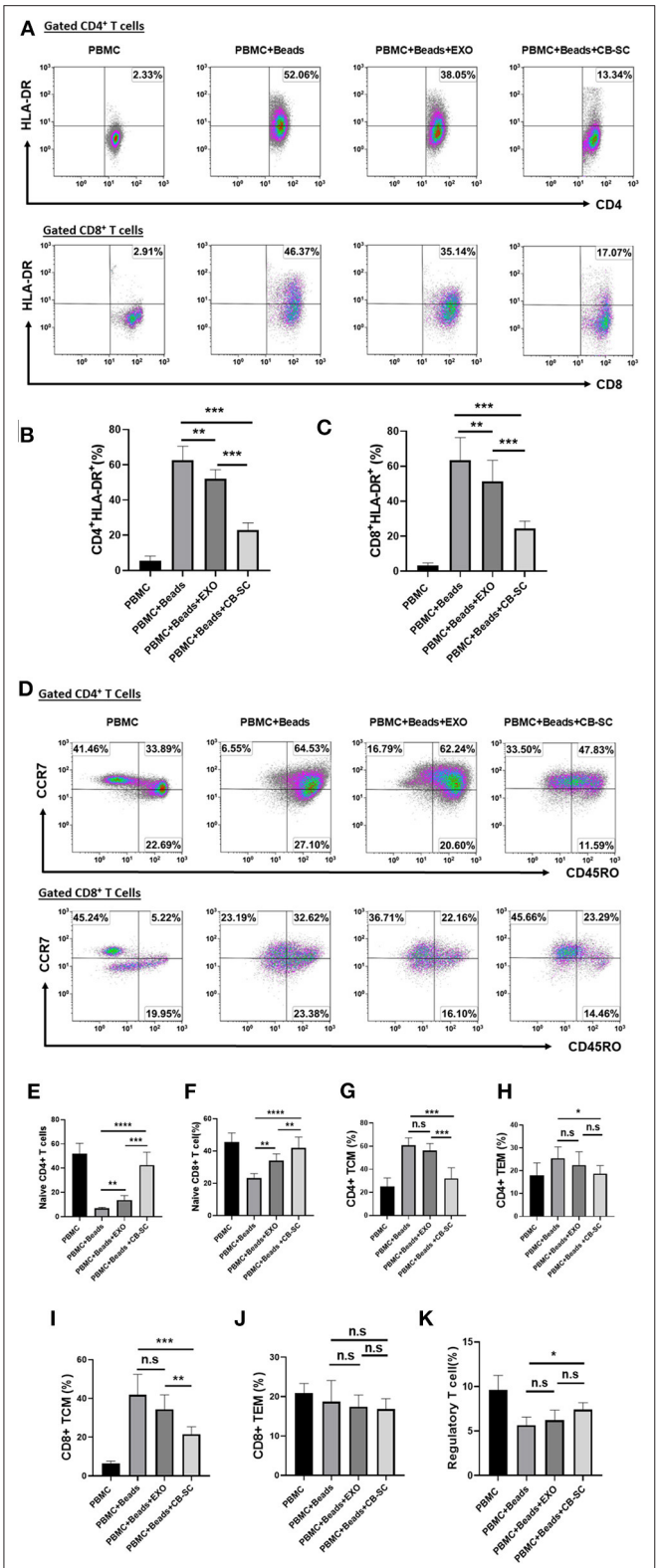

FIGURE 3 | Modulation of different T-cell subpopulations by cord blood-derived stem cell (CB-SC)-derived exosomes. **(A–J)** Peripheral blood mononuclear cells (PBMC) ($N = 3$) were activated with T-cell activator

(Continued)

FIGURE 3 | CD3/CD28 Dynabeads in the presence of 40 μg/ml exosomes derived from CB-SC cultures ($N = 8$). Treatment with CB-SC served as positive control. Untreated PBMC served as negative control. **(A)** Downregulation of activated $CD4^+$ and $CD8^+$ T cells by CB-SC-derived exosomes. Histograms of flow cytometry were representative of five experiments with similar results. **(B)** Decline the level of human leukocyte antigen DR isotype (HLA-DR) expression on the activated $CD4^+$ T cells by CB-SC-derived exosomes. **(C)** Decline the level of HLA-DR expression on the activated $CD8^+$ T cells by CB-SC-derived exosomes. **(D)** Upregulation of the percentage of naive $CD4^+$ and $CD8^+$ T cells by CB-SC-derived exosomes. Histograms of flow cytometry were representative of five experiments with similar results. **(E)** Increase in the percentage of naive $CD4^+$ T cells by CB-SC-derived exosomes. **(F)** Increase in the percentage of naive $CD8^+$ T cells by CB-SC-derived exosomes. **(G–K)** There were no significant effects on the percentages of $CD4^+$ T_{CM} cells **(G)**, $CD4^+$ T_{EM} cells **(H)**, $CD8^+$ T_{CM} cells **(I)**, $CD8^+$ T_{EM} cells **(J)**, and $CD4^+CD25^+CD127^{dim/-}$ Treg cells **(K)** after the treatment with CB-SC-derived exosomes. Results were given as mean ± SD. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$; $^{****}p < 0.001$.

Downregulation of Activated T Cells and Increasing Naive T Cells by CB-SC-Derived Exosomes

A previous work demonstrated CB-SC modulation of activated and memory T cells after the treatment with SCE therapy in type 1 diabetic subjects (5). To explore the action of CB-SC-derived exosomes, the anti-CD3/CD28-activated PBMC were examined after the treatment with CB-SC-derived exosomes. Flow cytometry revealed that both percentages of activated $CD4^+HLA\text{-}DR^+$ and $CD8^+HLA\text{-}DR^+$ T cells were reduced about 8–14% in the presence of 40 μg/ml CB-SC-derived exosomes (**Figures 3A–C**). Further analysis with memory T-cell markers indicated that both percentages of naive $CD4^+$ T cells ($CD4^+CD45RO^-CCR7^+$) and naive $CD8^+$ T cells ($CD8^+CD45RO^-CCR7^+$) were increased posttreatment with CB-SC-derived exosomes (**Figures 3D–F**), but failed to show significant changes on the percentages of $CD4^+$ central memory ($CD4^+$ T_{CM}, **Figure 3G**), $CD4^+$ effector memory T_{EM} ($CD4^+$ T_{EM}, **Figure 3H**) cells, $CD8^+$ T_{CM} (**Figure 3I**), $CD8^+$ T_{EM} (**Figure 3J**), and $CD4^+CD25^+CD127^{dim/-}$ Tregs (**Figure 3K**). In contrast, treatment with CB-SC displayed the significant downregulation of percentages of $CD4^+$ T_{CM}, $CD4^+$ T_{EM}, and $CD8^+$ T_{CM} (**Figures 3G–I**), and upregulation of the percentage of $CD4^+CD25^+CD127^{dim/-}$ Treg (**Figure 3K**).

CB-SC-Derived Exosomes Primarily Target Monocytes in PBMC

To further explore the action of CB-SC-derived exosomes on other immune cells, PBMC were treated with CB-SC-derived exosomes labeled with DiO dye. Different blood lineage cells were characterized and gated with cell-specific markers such as CD3 for T cells, CD4 for $CD3^+CD4^+$ T cells, CD8 for $CD3^+CD8^+$ T cells, CD11c for myeloid dendritic cells (DC), CD14 for monocytes, CD19 for B cells, and CD56 for NK cells (**Figure 4A**). After an incubation for 4 h, flow cytometry demonstrated that different blood cell compartments displayed at different levels of DiO-positive exosomes (**Figure 4B**). Notably, monocytes exhibited higher fluorescence intensity of DiO-positive exosomes

than those of other immune cells, about six times higher than that of DC (**Figure 4C**). It suggested that CB-SC-derived exosomes were mainly found in monocytes.

Differentiation of Monocytes Into Type 2 Macrophage (M2) After *ex vivo* Treatment With CB-SC-Derived Exosomes

To determine the direct effects of CB-SC-derived exosomes on monocytes, the purified $CD14^+$ monocytes from PBMC were treated with CB-SC-derived exosomes. Phase contrast microscopy showed that about 50.80 ± 1.70% of exosome-treated monocytes turned into spindle-like morphologies after the treatment for 3 days (**Figures 5A,B**), which was similar to previously characterized fibroblast-like macrophages (16). In contrast, most of the untreated monocytes were round and spread with a few elongated cells (**Figure 5A**). Next, we tested their phenotype through gating the viable (PI^-) monocytes. In comparison with untreated monocytes (**Figure 5C**, green histogram and **Figure 5D**), expressions of M2-associated markers including CD163, CD206, and CD209 were markedly increased after the treatment with CB-SC-derived exosomes, specifically for the level of CD206 expression (**Figure 5C**, red histogram and **Figure 5D**). There were no substantial changes in the expression levels of CD14, CD80, and CD86 (**Figures 5C,D**).

In addition, the ELISA assay further indicated the increase in the production of immune tolerance-associated cytokines such as TGF-β1 and IL-10 in the supernatant of exosome-treated monocytes (**Figure 5E**). Thus, these data imply that monocytes gave rise to macrophages with M2 phenotype after the treatment with CB-SC-derived exosomes.

Owing to the metabolic status of mitochondria contributing to the polarization of M1 and M2 macrophages (17, 18), we examined mitochondrial mass and associated markers following the treatment with CB-SC-derived exosomes. Labeling with MitoTracker deep red for the detection of mitochondrial mass failed to show marked difference between exosome-treated and untreated groups (**Figure 5F**). There were no significant differences in levels of cytoplasmic Ca^{2+} (**Figure 5G**) and mitochondrial Ca^{2+} (**Figure 5H**). However, the mitochondrial membrane potential ($\Delta\psi m$) was considerably upregulated after the treatment with CB-SC-derived exosomes (**Figure 5I**). This may contribute to polarizing the differentiation of M2 macrophages after the treatment with CB-SC-derived exosomes.

DISCUSSION

Exosomes are becoming one of the hot topics for pharmacology and translational medicine. The current studies demonstrated the releasing of exosomes from CB-SC cultures, with specific exosome markers. *Ex vivo* data confirmed the direct modulation of CB-SC-derived exosomes on activated PBMC and monocytes. In comparison with the substantial effects of CB-SC, the data demonstrated that CB-SC-derived exosomes inhibited the proliferation of activated PBMC, reduced the production of inflammatory cytokines, and could be effective on specific subsets of T cells such as in downregulating the percentage of activated $CD4^+$ and $CD8^+$ T cells and increasing the percentage of naive $CD4^+$ and $CD8^+$ T cells. Notably, the differentiation of purified monocytes into M2 macrophages following the treatment with CB-SC-derived exosomes provide additional molecular mechanisms underlying the immune education and induction of immune tolerance observed in the clinical use of SCE therapy for the treatment of T1D (4–6) and other autoimmune diseases like alopecia areata (19).

Previous studies have demonstrated the long-lasting clinical efficacy of SCE therapy for the treatment of T1D and T2D patients, with a complete recovery of islet β-cell functions in some subjects after a single treatment with SCE therapy sustained for 4 years (6), as well as in the treatment of alopecia areata (19). These observations suggest that SCE therapy has the potential to fundamentally correct the immune dysfunction of these subjects with autoimmune diseases. Several possible molecular mechanisms underlying the immune education of SCE therapy were reported elsewhere (8), such as an expression of autoimmune regulator (AIRE) transcription factor in CB-SC, displaying high levels of the programmed death-ligand 1 (PD-L1) and CD270 on the cell surface of CB-SC, and the release of TGF-β1 and nitric oxide (NO) (20). Current data confirmed that the released CB-SC-derived exosomes contributed to the immune down-modulation of activated CD4 and CD8 T cells, along with a corresponding increase in naive CD4 and CD8 T cells. In the process of clinical treatment, SCE therapy circulates a patient's blood through a blood cell separator, cocultures the patient's T cells and monocytes with adherent CB-SC *in vitro*, and returns "educated" autologous cells to the patient's circulation (4, 7). During the cocultures, CB-SC may release exosomes that target dysfunctional monocytes and/or T cells, leading to the tolerization of these cells and expanding the therapeutic potential of SCE therapy after infusing the cells back to the subjects. CB-SC-derived exosomes would also be transferred back to the subjects along with the CB-SC-treated patient cells, leading to the expansion of the therapeutic potential of SCE therapy in patients. Owing to their small size, the circulating CB-SC-derived exosomes may penetrate into the damaged tissues such as pancreatic islets and target the pathogenic T cells and/or macrophages, contributing to the induction of tolerance and the clearance of residential autoimmune memory T cells (21). In this respect, previous clinical study established the modulation of autoimmune T-cell memory (e.g., $CD4^+$ T_{CM} and $CD8^+$ T_{EM}) by SCE therapy in T1D subjects (5). Current *ex vivo* study demonstrated the marked downregulation of percentages of $CD4^+$ T_{CM}, $CD4^+$ T_{EM}, and $CD8^+$ T_{CM} after the treatment with CB-SC, but only slight reductions in the presence of CB-SC-derived exosomes. To overcome this limitation and improve the clinical efficacy of SCE therapy, it is necessary to further explore by which additional CB-SC-derived signals (8) contribute to the synergistic effect with exosomes in the modulation of autoimmune memory T cells. To mimic the clinical setting, it will be better to utilize T1D patient-derived PBMC, instead of using the anti-CD3/CD28-activated PBMC.

Monocytes/macrophages (Mo/Mφ) are frontline immune cells defending against viral and bacterial infections and maintaining homeostasis, with diverse functions and

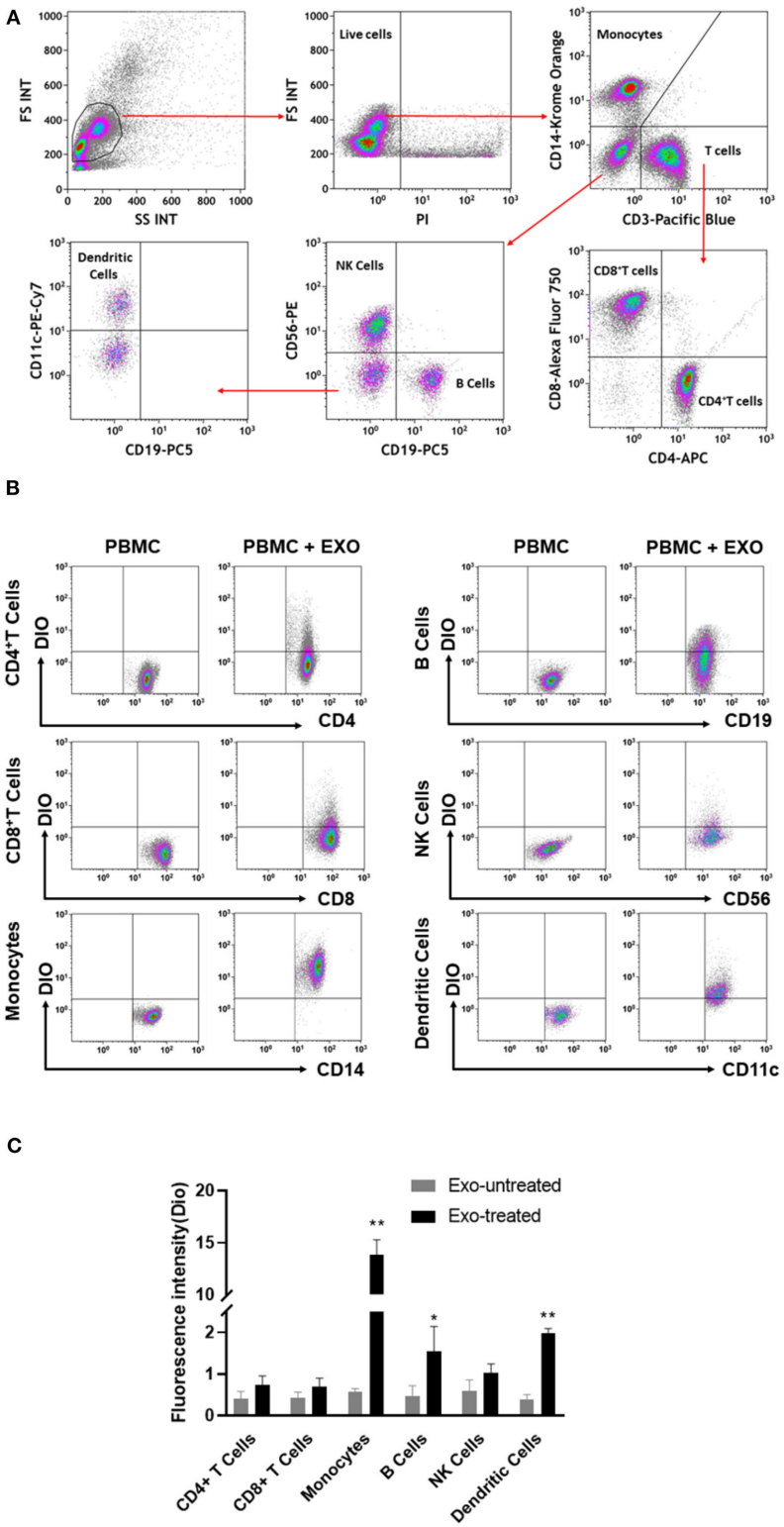

FIGURE 4 | Interaction of cord blood-derived stem cell (CB-SC)-derived exosomes with different compartments of immune cells. **(A)** Flow cytometry analysis and the gating strategy with the lineage-specific surface markers for different cell compartments in PBMC ($N = 3$), including CD3/CD4/CD8 for T cells, CD19 for B cells, CD14 for monocytes, CD11c for dendritic cells, and CD56 for NK cells. **(B)** Flow cytometry revealed the distributions of DiO-labeled CB-SC-derived exosomes ($N = 3$) among different cell populations at different levels. **(C)** CB-SC-derived exosomes ($N = 3$) primarily targeted on monocytes with high fluorescence intensity. Data were representative of three experiments with six preparations of CB-SC-derived exosomes.

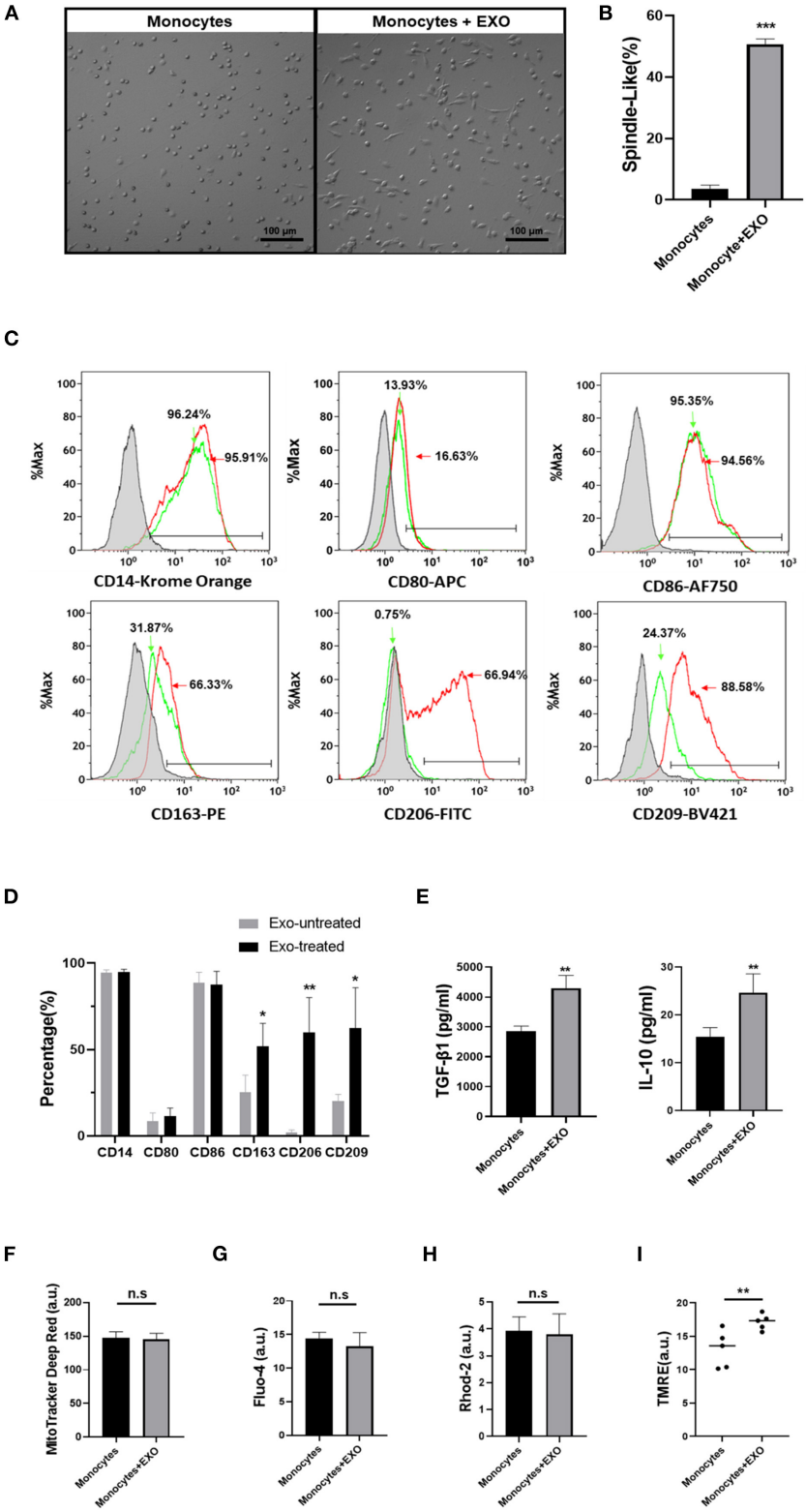

FIGURE 5 | Differentiation of monocytes into type 2 macrophage (M2) after *ex vivo* treatment with cord blood-derived stem cell (CB-SC)-derived exosomes. Monocytes were purified from peripheral blood mononuclear cell (PBMC) ($N = 3$) using $CD14^+$ microbeads (Miltenyi Biotec), with purity of $CD14^+$ cells >95%. **(A)** Morphology change of monocytes into the spindle-like cells after the treatment with CB-SC-derived exosomes ($N = 3$). Untreated monocytes served as control

(Continued)

FIGURE 5 | (left). **(B)** Quantify the percentage of spindle-like cells in the presence of treatment with CB-SC exosomes. The data were given as mean ± SD of two experiments with three donor-derived monocytes and six preparations of CB-SC-derived exosomes ($p < 0.001$). **(C)** Upregulate the levels of M2-assoaicte markers' expressions on monocytes/macrophages after the treatment with CB-SC-derived exosomes, such as CD163, CD206, and CD209 (red line). Untreated monocytes (green line) served as control. Isotype-matched immunoglobulin G (IgG) served as negative control. Data were collected from three donor-derived monocytes and three preparations of CB-SC-derived exosomes. **(D)** Modulate the levels of M2-assoaicte markers' expressions on monocytes/macrophages after the treatment with CB-SC-derived exosomes. The data were given as mean ± SD of three PBMC (N =3) treated with three preparations of CB-SC-derived exosomes ($N = 3$). **(E)** Increase in the levels of transforming growth factor (TGF)-ß1 and interleukin (IL)-10 after the treatment with CB-SC-derived exosomes ($N = 5$) in three donor-derived monocytes, shown by ELISA assay. **(F)** Flow cytometry failed to show the significant difference in mitochondrial mass between exosome-treated and untreated monocytes ($N = 3$, treated with five CB-SC-derived exosomes). **(G)** Flow cytometry analysis of cytoplasmic Ca^{2+} (Fluo-4) levels failed to show the significant differences between exosome-treated and untreated monocytes. **(H)** No difference in the levels of mitochondrial Ca^{2+} (Rhod-2) between the exosome-treated and untreated monocytes. **(I)** Improve the membrane potential of mitochondria in monocytes posttreatment with CB-SC-derived exosomes, as demonstrated by flow cytometry analysis after staining with tetramethylrhodamine, ethyl ester (TMRE). The data were represented from five experiments using five donor-derived monocytes in the presence or absence of the treatment with CB-SC-derived exosomes ($N = 5$) at 40 μg/ml. The monocytes were randomly assigned the treatment with single donor CB-SC-derived exosomes. The data **(F–H)** were given as mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$.

heterogeneity. Based on their profiles, macrophages are simply characterized with two subpopulations: type 1 macrophages (M1, proinflammation) and type 2 macrophages (M2, anti-inflammation) (22). Increasing clinical evidence and animal studies demonstrate the dysfunction of Mo/Mφ causing the pathogenesis of diabetes and other autoimmune diseases (17, 23–26). Notably, this study established that purified monocytes gave rise to cells with an M2 phenotype after the treatment with CB-SC-derived exosomes, displaying the elongated morphology and the expression of M2-associated markers (e.g., CD163, CD206, and CD209). However, the level of costimulating molecules CD80 and CD86 expressions failed to show the marked changes after the treatment with CB-SC-derived exosomes. This may be associated with our current protocol in which the purified $CD14^{+}$ monocytes were treated with CB-SC-derived exosomes in the presence of serum-free culture medium X-Vivo 15, without adding any other growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) or macrophage colony-stimulating factor (M-CSF). This was different from the conventional protocol that utilized cytokines such as M-CSF, IL-4, and/or IL-10 during the M2 differentiation (27). Both CD80 (28) and CD86 expressions are normally upregulated on the activated macrophages and served as a marker for M1 (28, 29). Owing to the plasticity of monocytes/macrophages, the phenotypes of M1/M2 were significantly affected by *ex vivo* culture conditions such as the serum-containing or serum-free culture media and cytokine stimulations (27). For instance, the expression of CD80 was substantially increased on the M1 in the presence of 10% fetal bovine serum (FBS) Roswell Park Memorial Institute (RPMI) compared to the serum-free X-Vivo 10 media, but without significant difference in the level of CD86 expression between M1 and M2 (27). Therefore, culture conditions need to be optimized and standardized for the M1/M2 study.

To elucidate the mechanisms by which signaling pathway contributed to the M2 differentiation, flow cytometry revealed that the mitochondrial membrane potential ($\Delta\psi m$) of monocytes was markedly increased after the treatment with CB-SC-derived exosomes, without affecting both intracellular and mitochondrial calcium concentrations. Since the differentiation to a M2 macrophage is strongly favored by an increase in oxidative phosphorylation (17), the upregulation of mitochondrial membrane potential by CB-SC-derived exosomes may thus lead to the differentiation to M2 macrophages. From this perspective, Calabria and colleagues reported that the mitochondrial membrane potential of neuronal cells was recovered by the treatment with exosomes isolated from adipose stem cells (30). The reason causing the upregulation of $\Delta\psi m$ by the interaction between exosomes and mitochondria need to be clarified in future studies. It is important to note that the differentiation of CB-SC-derived exosome-treated monocytes may be affected by patient's internal environment after being infused back during the SCE therapy. To this end, previous clinical data demonstrated that the percentage of $CD86^{+}CD14^{+}$ of monocytes was markedly reduced 4 weeks after the treatment with SCE therapy in type 2 diabetic subjects (7), even no change in the level of CD86 expression after *in vitro* treatment.

Based on the above-mentioned immune modulation potentials of CB-SC-derived exosomes, quantification of released exosomes from CB-SC cultures may provide a valuable biomarker for the Quality Control (QC) analysis of good manufacturing practice (GMP)-manufactured Stem Cell Educators before their clinical applications. However, the size of CB-SC-derived exosomes was too small at ∼100 nm, which cannot be directly detected by flow cytometry and visualized by optical microscopes. It will be challenging to perform the absolute quantification with current limited technologies. The conventional approach for the isolation of exosomes was achieved by ultracentrifugation, ultrafiltration, and/or precipitation, with a duration of 4–5 h. These isolated exosomes can be quantified using current commercial techniques such as asymmetrical-flow field flow fractionation (AF4) coupled with multidetection, nanoparticle tracking analysis (NTA), DLS, and surface plasmon resonance (SPR) (31). However, due to their sensitivity, specificity, cost, and time-consuming process, the isolation and quantification of exosomes would need to be standardized with the optimized protocol. It should be noted that we utilized the conventional approach for the isolation of CB-SC-derived exosomes in the current work that might have other vesicles in different proportions. In addition, different cord blood donor-derived CB-SC may release exosomes with variable contents of bioactive molecules, and *ex vivo* functional analysis will be necessary to parallel the exosome quantification for the QC analysis of SCE products.

Previous works demonstrated that CB-SC could give rise to three germ-layer-derived cells in the presence of different inducers (15, 20), and differentiate into functional insulin-producing cells after transplantation into the chemical streptozotocin (STZ)-induced diabetic mice, leading to the reduction in hyperglycemia (15). In addition, CB-SC expressed the ES cell markers such as the self-renewal-associated transcription factors OCT3/4 and NANOG (15). Exosomes released from CB-SC might carry the capability of tissue regeneration of parent cells in addition to the immune modulation capacity. To further optimize the clinical treatment protocol and improve the clinical efficacy of SCE therapy, detailed mechanisms need to be further investigated through *ex vivo* studies such as docking of exosomes on monocytes, trafficking of exosomes in the cytoplasm of monocytes, and polarizing the M2 differentiation by the exosome treatment, along with *in vivo* studies in the autoimmune-caused diabetic mouse models (32, 33). Specifically, the proteomic analysis through mass spectrometry-based proteome profiling and exosome RNA sequencing will be necessary to dissect the detailed molecular mechanisms underlying the long-lasting clinical efficacy of SCE therapy for the treatment of T1D and other autoimmune diseases.

The current *ex vivo* study provides a better understanding of how SCE therapy results in the anti-inflammatory clinical effects. CB-SC-derived exosomes preferably and quickly bounded to monocytes in 2–3 h. As mentioned above, during the coculture of CB-SC with patient's immune cells for clinical treatment during 8–9 h (4, 5, 7, 19), the SCE-treated monocytes may carry the CB-SC-derived exosomes back into the body, probably resulting in further M2 differentiation and induction of tolerance. Therefore, SCE therapy has the potential to revolutionize the treatment of diabetes and multiple autoimmune diseases through CB-SC-mediated immune modulation, without the safety and ethical concerns associated with conventional immune and/or stem-cell-based approaches.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

YZ supervised experiments and contributed to concepts, experimental design, data analysis, and interpretation, manuscript writing, and final approval of manuscript. WH performed most experiments and data analysis. XS, HY, and JS performed stem cell culture, flow cytometry, and TEM for exosomes.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00165/full#supplementary-material

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REVIEW
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Cell Versus Cytokine – Directed Therapies for Hemophagocytic Lymphohistiocytosis (HLH) in Inborn Errors of Immunity

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Hemophagocytic lymphohistiocytosis (HLH) is a heterogeneous hyperinflammatory syndrome with different pathways of pathogenesis resulting in similar clinical presentations. It is best defined and understood if presenting in the context of genetic immunodeficiencies associated with defects of lymphocyte cytotoxicity. In these "primary" forms of HLH, cellular and soluble immune effectors are relatively well characterized. While etoposide-based broad cell-directed therapies remain standard of care, more specific therapies targeting these effectors individually are increasingly available. Anti-CD52 as a cell-directed therapy and anti-IFN-gamma, IL-18BP, and JAK-inhibition as cytokine-directed therapies are expected to broaden the therapeutic options, but the precise role of these drugs in first-line and rescue treatment indications remains to be defined. A number of additional inborn errors of immunity are associated with episodes of immune activation fulfilling the clinical criteria of HLH. Impaired pathogen control is a key driver of hyperinflammation in some conditions, while others are characterized by a strong autoinflammatory component. This heterogeneity of disease-driving factors and the variable severity in disease progression in these conditions do not allow a simple adaptation of protocols established for "primary" HLH to HLH in the context of other inborn errors of immunity. Cytokine-directed therapies hold significant promise in these increasingly recognized disorders.

Keywords: hemophagocytic lymphohistiocytosis, inborn errors of immunity, pathogenesis, therapy, cytokine, inflammation, HSCT

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PRIMARY HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

Hemophagocytic lymphohistiocytosis (HLH) is a highly inflammatory syndrome with uncontrolled, excessive immune activation. HLH is the key manifestation in a range of autosomal-recessive genetic diseases defined as familiar forms of HLH (FHL). FHL includes FHL1 to FHL5 (OMIM #267700, #603553, #608898, #603552, and #613101) caused by defects in lymphocyte

Abbreviations: APC, antigen-presenting cell; ATG, antithymocyte globulin; CMV, cytomegalovirus; CNS, central nervous system; CSA, cyclosporine A; EBV, Epstein-Barr virus; FHL, familial hemophagocytotic lymphohistiocytosis; HLH, hemophagocytotic lymphohistiocytosis; HSCT, hematopoietic stem cell transplantation; JAK, janus kinase; MRI, magnetic resonance imaging; NK, natural killer cell; RIC, reduced-intensity-conditioning; VOD, veno-occlusive disease; XLP, X-linked lymphoproliferative syndrome.

cytotoxicity affecting perforin or proteins involved in the exocytosis of perforin-containing lytic granules (degranulation deficiencies) (**Table 1**). It was first described in 1952 as familial hemophagocytic reticulosis (1). In FHL2 patients with "null" mutations, the first manifestation of disease symptoms is in most cases observed in the first 6 months of life, but may already be present *in utero* or at birth (2). HLH tends to occur later in patients with other FHL variants (3, 4) and in patients with biallelic "hypomorphic" mutations and an initial HLH episode has been reported as late as 63 years of age (5). The incidence of FHL is estimated at 1:50,000–1:100,000 (6, 7). Some genetic immunodeficiency diseases associated with pigment dilution such as Griscelli syndrome type II (GS-II; OMIM # 607624) and Chediak-Higashi syndrome (CHS; OMIM #214500) are also caused by degranulation defects (8). The similar pathogenesis and the frequent occurrence of HLH in these conditions allow their classification as "primary" HLH (**Figure 1A**).

PATHOPHYSIOLOGICAL BASIS OF "PRIMARY" HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

FHL2-5, GS-II, and CHS all affect the cytotoxic granule-mediated cell death pathway (8, 9). Under physiological conditions, immune stimulation such as a viral infection leads to priming of cytotoxic T-lymphocytes by APC, followed by their activation and proliferation. These activated T cells and NK cells can recognize virus-infected target cells and subsequently eliminate these through polarized release of perforin- and granzyme-containing granules (10, 11). Entry of granzymes into target-cells by membrane-pores established by perforin activity mediates apoptotic cell death. Notably, this cytotoxic activity is also directed against APC, providing an important negative feedback-loop that limits T cell activation (12).

In "primary" HLH, deficient cytotoxic activity of CTL and NK cells impairs the timely elimination of APCs. Their persistence leads to continuous T-cell stimulation. Incessantly activated T-cells infiltrate tissues and release various pro-inflammatory mediators, in particular interferon-gamma, a potent macrophage-stimulating cytokine (13). Continuous macrophage activation, in turn, further fuels release of a broad range of inflammatory cytokines such as IL-1, IL-6, IL-18, and TNF-alpha (14–16) and leads to tissue infiltration of macrophages and hemophagocytosis. Since in the course of an immune response, T cells themselves can also become targets of the cytotoxic activity of NK cells and T cells, lack of this control mechanism may further impair immune homeostasis (17, 18). Clinical manifestations of HLH are mainly a result of tissue infiltration by T cells and macrophages and the accompanying excessive cytokine storm.

This model of "primary" HLH pathophysiology has mainly been established in key studies in cytotoxicity deficient mice that develop all clinical features used for the diagnosis of HLH in patients upon persistent wide-spread systemic infection with lymphocytic choriomeningitis virus (19). In most patients with "primary" HLH, no persistent systemic viral infection can be demonstrated (2), asking for a note of caution whether this model really explains all aspects of the human disease.

OTHER INBORN ERRORS OF IMMUNITY PREDISPOSING TO HLH: PATHOGENETIC HETEROGENEITY

In a group of additional inborn errors of immunity, HLH occurs less frequently, although it can still be the presenting clinical manifestation. In these diseases, HLH pathogenesis is variable and mostly different from that of "primary" disease (**Table 1**). A brief review of current understanding of pathogenesis of these diseases is relevant for the discussion of therapeutic approaches.

Two X-linked genetic diseases predispose to HLH predominantly in the context of EBV infection (20) (**Figure 1B**). XLP1 (OMIM #308240) is caused by defects in SAP, a small adaptor protein that regulates signaling in T and NK cells by binding to the SLAM family of signaling receptors (21). Many aspects of XLP1 pathogenesis can be explained by impaired T/NK-B cell interaction. As a consequence, affected patients frequently suffer from hypogammaglobulinemia and its infectious consequences due to impaired T cell help to B cells and lymphomas due to impaired control of malignant B cells (22). Cerebral vasculitis and aplastic anemia can also be life-threatening manifestations. Poor T/NK-cell mediated control of EBV-infected B cells, in part linked to impaired activation of 2B4 (a SLAM receptor) mediated cytotoxic function, is the basis of HLH, that develops in about 30% of XLP1 patients (23).

XLP2 (OMIM #300079) is caused by defects in XIAP, a protein with antiapoptotic functions, regulatory functions for autophagy and control functions for inflammasome activity (24, 25). It also modulates the NOD1/NOD2 pathways which contribute to intracellular sensing of bacterial infection. A link to lymphocyte cytotoxicity has not been established. Important clinical manifestations of XLP2 are early onset inflammatory bowel disease, splenomegaly and periodic fever (26). The pathogenesis of mostly EBV-induced HLH, which occurs in more than 30% of patients, is unclear. However, an autoinflammatory component due to dysregulated NLRP3 inflammasome activation is reflected by excessive levels of free serum IL-18 and is more prominent than in "primary" HLH (27). Notably, some, but not all biological activities of IL-18 are mediated by IFNγ (28). The frequency of HLH in XLP1 and XLP2 has led to their inclusion in the classification of "primary" HLH and the therapeutic principles of FHL have also been successfully used to treat HLH in XLP (20, 29). However, both XLP variants have a pathophysiology that is clearly different from "primary" HLH and this may offer different treatment options. This is particularly relevant for treatment of manifestations different from HLH in these conditions.

TIM3 deficiency (OMIM #618398) caused by *HAVCR2* mutations is another autosomal-recessive inborn error of immunity that predisposes to HLH in a particular context, i.e., in subcutaneous panniculitis T cell lymphoma (SPTCL) (30). TIM3 is an inhibitory molecule expressed mainly on T cells and NK cells, but also on myeloid cells. TIM3 mutations

TABLE 1 | Genetically determined forms of hemophagocytotic lymphohistiocytosis (HLH).

Primary HLH	Gene	Protein	Pathophysiology	Functional testing
Familial HLH (FHL)				
FHL-1	Unknown	Unknown		
FHL-2	PFR1	Perforin	Lack of perforin expression in lytic granules	Perforin expression
FHL-3	UNC13D	Munc13-4	Deficiency in fusion of lytic granule with plasma membrane	Degranulation
FHL-4	STX11	Syntaxin11	Deficiency in fusion of lytic granule with plasma membrane	Degranulation
FHL-5	STXBP2	Munc18-2	Deficiency in fusion of lytic granule with plasma membrane	Degranulation
Other immunodeficiency syndromes with defect in degranulation				
GS-II	RAB27A	Rab27a	Deficiency in docking of lytic granule to the plasma membrane	Degranulation hair microscopy
CHS	LYST	Lyst	Defect in maturation of vesicles into secretory cytotoxic granules	Degranulation hair microscopy
—				
Other inborn errors of immunity	**Gene**	**Protein**	**Pathophysiology**	**Functional testing**
Immunodeficiency syndromes with HLH as a frequent manifestation				
XLP-1	SH2D1A	SAP	Defective killing of EBV infected B-cells by CD8 and NK cells	SAP expression
XLP-2	BIRC4	XIAP	Impaired inhibition of inflammasome activity	XIAP expression L18MDP assay
TIM3 deficiency	HAVCR2	TIM3	Persistent T cell activation and increased production of inflammatory cytokines	TIM3 expression
Immunodeficiency syndromes with HLH as an occasional manifestation				
Chronic granulomatous disease (CGD)	CYBB, CYBA, NCF1, NCF2, NCF4	Components of NADPH oxidase	Excessive inflammatory responses due to altered inflammasome regulation by NADPH oxidase?	Oxidative Burst
(S)CID	>50 genes	various	Lack of pathogen control	Lymphocyte phenotyping
Wiskott-Aldrich syndrome	WAS	WASP	Lack of pathogen control Impaired cytoskeleton-inflammasome interaction?	WASP expression (FACS)
CD27 deficiency	CD27	CD27	Impaired co-stimulation of T cells Lack of EBV control	CD27 expression
ITK deficiency	ITK	ITK	Impaired TCR mediated signaling Lack of EBV control	ITK expression
IFNγ receptor deficiency	IFNGR1 IFNGR2	IFN-gamma receptor	Lack of pathogen control (mycobacteria, salmonella)	STAT1 phosphorylation
ALPS	FAS (het) FASLG	FAS FASLG	Defects in Fas ligand-mediated elimination of activated lymphocytes	TCR DNT Vitamin B12, soluble FasL
Autoinflammatory diseases with HLH as a frequent manifestation				
NLRC4 gain of function	NLRC4 (het)	NLRC4	Constitutive inflammasome activation IL-1β/IL-18 production	Genetic testing
CDC42 mutations	CDC42 (het)	CDC42	Impaired cytoskeleton-inflammasome interaction?	Genetic testing

causing aberrant protein folding and lack of surface expression lead to an autoinflammatory and autoimmune phenotype with hyperactivated myeloid cells producing high levels of IL-1 and IL-18 and uncontrolled CD8 T cell proliferation (31). This promotes SPTCL formation and its association with HLH.

Heterozygous NLRC4 gain-of-function mutations (OMIM #606831) lead to constitutive activation of the NLRC4 inflammasome resulting in enterocolitis and macrophage activation associated with a clinical picture of HLH. It is characterized by excessive levels of free IL-18 and IL-1beta (32, 33). Heterozygous mutations in CDC42 affecting amino acids 186, 188, or 192 also lead to a hyperinflammatory syndrome including neonatal cytopenias, hepatosplenomegaly, recurrent febrile episodes and urticaria-like rashes that can fulfill HLH criteria. This autoinflammatory disease is also characterized by very high levels of IL-18 and IL-1beta, suggesting dysregulated inflammasome function (34). The mutations are postulated to interfere with actin assembly, thus affecting signaling, cytoskeletal rearrangement and cell migration. All three conditions are characterized by a significant autoinflammatory disease component that calls for treatment approaches different from primary HLH (**Figure 1B**).

Finally, immune activation fulfilling the clinical criteria of HLH occasionally occurs in several additional primary

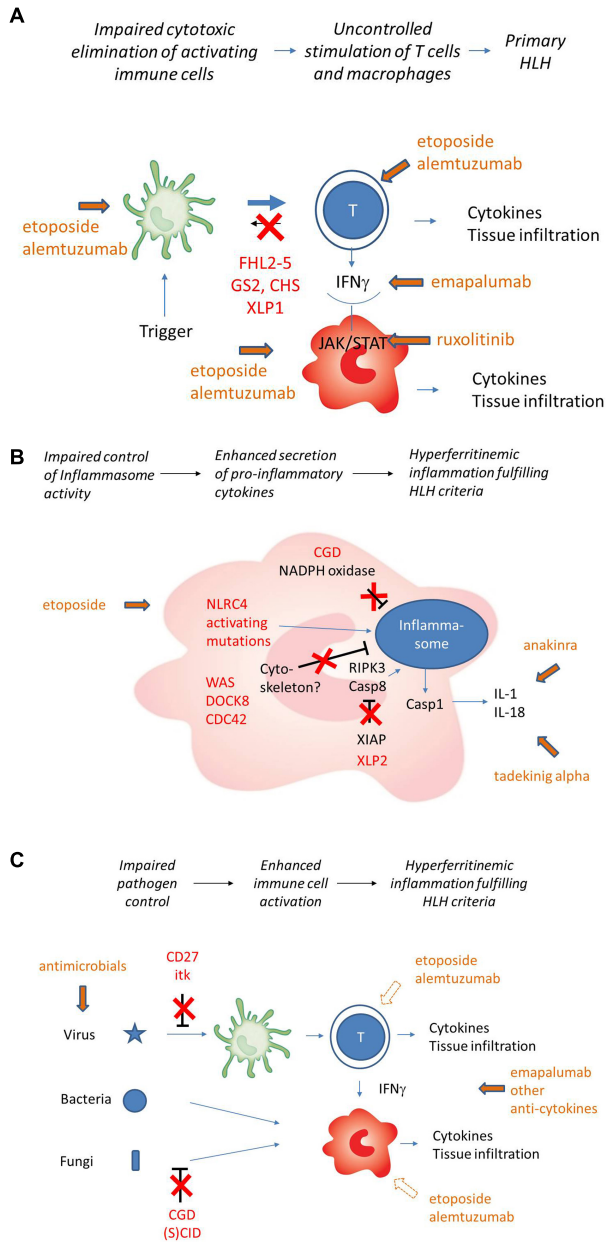

FIGURE 1 | (A) Pathogenesis of "primary" HLH (simplified). Impaired cytotoxicity (red) leads to uncontrolled T cell activation by APC. T cell secreted IFNg is the key driver of macrophage activation. Cellular and cytokine targets of therapy are indicated in green. **(B)** Pathogenesis of HLH in the context of impaired inflammasome homeostasis (simplified). Inborn errors of immunity shown (NLRC4, XIAP) or assumed (Cytoskeletal disorders, CGD) to be involved in inflammasome homeostasis are indicated in red. Cytokine targets of therapy are indicated in green. Macrophages are the key cells involved, T cells play a less prominent role. Cell-directed therapies are rarely used. **(C)** HLH pathogenesis in the context of inborn errors of immunity with impaired pathogen control (simplified). Inborn errors of immunity impairing virus control (mainly EBV) and/or bacterial/fungal control are indicated in red. Subsequent immune stimulation leads to hypersecretion of variable cytokines. Macrophage activation can occur in the absence of T cells, but T cells can be involved depending on the genetic defect and the trigger. Cell-directed therapies further impair pathogen control and should only be used in exceptional cases.

immunodeficiencies, including SCID, some combined immunodeficiencies such as Wiskott-Aldrich syndrome, CD27 deficiency and ITK deficiency, chronic granulomatous disease (CGD) and IFNγ receptor deficiency (35, 36) (**Figures 1B,C**). The examples of SCID and IFNγR deficiency illustrate that the clinical syndrome of HLH as defined by the HLH-2004 clinical criteria requires neither T cells nor IFNγ, illustrating that this form of HLH is different from "primary" HLH. In fact, the HLH-like immune activation in these diseases is in most cases due to impaired pathogen control and rather represents an infection-induced HLH. Additional factors such as altered inflammasome regulation by NADPH oxidase in CGD (37) and potentially impaired cytoskeleton – inflammasome cross-talk in patients with WAS, DOCK8 deficiency and CDC42 mutations likely also contribute (38–40). Overall, these examples illustrate that also in familial HLH cases, a careful characterization of the genetic disorder underlying HLH is required as it allows to choose treatment targeted at the specific pathogenesis.

THERAPEUTIC STRATEGIES

The heterogeneity in pathophysiology of "primary" HLH caused by cytotoxicity defects versus HLH associated with other inborn errors of immunity makes it obvious that there is no "one fits all" therapeutic strategy. Treatment must be targeted to the pathophysiology and results from treatment studies obtained in one group of diseases cannot simply be transferred to another. Therapeutic regimens in primary HLH are either directed at the immune cells involved, i.e., APC, T cells and macrophages, or at the cytokines secreted by these cells. The goal is to disrupt ongoing immune stimulation and to limit severe hyperinflammation and tissue damage. The implementation of broad cell-directed therapies was critical to improve survival in this life-threatening condition (41). However, more specific anti-cellular therapies and therapeutic targeting of particular key cytokines and their downstream effects are currently evaluated in clinical trials. In the absence of published data on several of these novel approaches, this review can only point out the therapeutic principles and indicate which trials to watch as they have the potential to impact on standard-of-care within the next 5 years.

Overall, the therapeutic approach to primary HLH can be divided into four main phases:

(1) Induction of remission.
(2) Control of triggers.
(3) Maintenance of remission and salvage therapy.
(4) Curing the underlying condition.

INDUCTION OF REMISSION

Timely treatment of HLH is essential for prognosis. Untreated patients with active "primary" HLH show a survival of approximately 2 months due to progressive organ failure (42). Delayed initiation of therapy increases the risk of neurological complications. In most cases, initial decisions must be made in the absence of a confirmed genetic diagnosis, but tests of protein

expression and degranulation are rapidly available and have high sensitivity and specificity for "primary" HLH (43–45). Important differential diagnosis requiring different treatment approaches such as malignancy or metabolic disease should be considered (46, 47). Leishmaniosis must be ruled out in all patients with a plausible risk (48).

TARGETING CELLS

For decades, first-line therapy for primary HLH has been centered on cell-oriented approaches. The widely used standard-of-care is based on the dexamethasone/etoposide-based HLH-94 and HLH-2004 studies. A consensus statement addressing various aspects of its use in detail has recently been published by the HLH Steering Committee of the Histiocyte Society (49).

Etoposide-Based Protocols

The HLH-94 protocol is based on immuno-chemotherapy including dexamethasone, etoposide and CSA to achieve remission of the hyperinflammatory state and to maintain remission until HSCT can be performed (41). Functionally, all agents target lymphocytes, macrophages and antigen presenting cells. The cytostatic agent etoposide induces cell death mainly in activated T cells (50), but also in macrophages and dendritic cells. The use of the calcineurin inhibitor CSA leads to an inhibition of the transcription factor NFAT (nuclear factor of activated T-cells) and thus to a reduced activation and proliferation of T cells. Steroids slow down inflammation by reducing cytokine secretion, but in addition, they have a moderate cytotoxic effect on activated T cells. It is recommended to treat patients with CNS involvement also with intrathecal methotrexate (51), although there is no clear evidence of benefit. The protocol has a 2-week intensive phase with dexamethasone and twice weekly administration of etoposide, followed by 6 weeks of weekly etoposide and steroid tapering. In this second phase, CSA is used to prevent reactivation (49). Rapid immunological testing followed by genetic confirmation of the underlying genetic disease is required in all patients and should provide the basis for HSCT within these 8 weeks (**Figure 2**). In the international multicenter registry-based HLH-2004 study, 5-year probability of survival for children with genetically verified familial HLH treated with this protocol was 59% (52). Dexamethasone/etoposide-based protocols have been successfully used in XLP or patients with TIM3 deficiency. It remains an ultimate choice also in other inborn errors of immunity, but the toxicity and immunosuppression associated with etoposide asks for more targeted therapies in these conditions.

Antithymocyte Globulin (ATG)

Antithymocyte globulin directly targets T cells and other lymphocytes, to a minor extent also granulocytes and monocytes (53). In a retrospective single-center analysis of 38 patients with familial hemophagocytosis, a protocol consisting of steroids, CSA and first-line ATG resulted in a higher initial remission rate compared to the HLH-94 protocol (active disease in 26% of patients versus 53%, after 2 months of therapy) but was associated with a higher percentage of relapses before HSCT (32% versus 13%) (54). In an attempt to combine advantages of both protocols, a trial combining ATG and etoposide has been performed (55), but the results have not yet been reported. There is no clear role for ATG beyond "primary" HLH.

Alemtuzumab (Anti-CD52)

More recently, the humanized monoclonal anti-CD52 antibody alemtuzumab has been used in patients with "primary" HLH (56). It is directed against the CD52 antigen (CAMPATH 1) which is a surface protein on mature lymphocytes and APCs. First promising results have been achieved when used in a bridging to transplant setting (57). Excellent initial results have been orally reported from a trial evaluating Alemtuzumab as first-line treatment for "primary" HLH (in combination with methyl-prednisolone and CSA) (58). The profound and long-lasting immune suppression and limits this drug to "primary" HLH, where induction of remission is rapidly followed by HSCT. The problem of viral (re-)activation is an important caveat when using alemtuzumab in XLP.

TARGETING CYTOKINES

In a disease associated with excessive production of a large number of cytokines, it is not self-evident that blockade of a single cytokine should have significant therapeutic effects. However, pivotal studies in mouse models of "primary" HLH have indicated that some key cytokines, in particular IFN-gamma, are drivers of the immune dysregulation (60) and that their neutralization can interrupt the inflammatory circle and restore immune homeostasis (52, 59). As a consequence, therapeutic approaches targeting IFN-gamma, its induction and its downstream effects have emerged as promising strategies that are at different stages of evaluation in clinical trials of primary HLH.

Interferon-Gamma

Emapalumab is a recombinant human monoclonal antibody against interferon gamma (60). It has received FDA approval in November 2018 for the treatment of pediatric and adult patients with primary HLH with refractory, recurrent, or progressive disease or intolerance to HLH therapy (see section salvage therapy). An international multicenter follow-up study to further assess the efficacy and safety of emapalumab is still ongoing (61). This trial will also provide data on its use as first-line therapy in "primary" HLH. Serum levels of CXCL9 emerge as an interesting biomarker for increased IFNγ activity (62) and may be particularly helpful when considering the use of emapalumab in first-line treatment of HLH in the context of other inborn errors of immunity. Notably, a patient with CDC42 mutation who did not respond to steroids, CSA, and anakinra was successfully treated with emapalumab (34).

JAK-Inhibition

Janus kinase inhibitors represent interesting therapeutic compounds in the context of HLH, since they not only inhibit

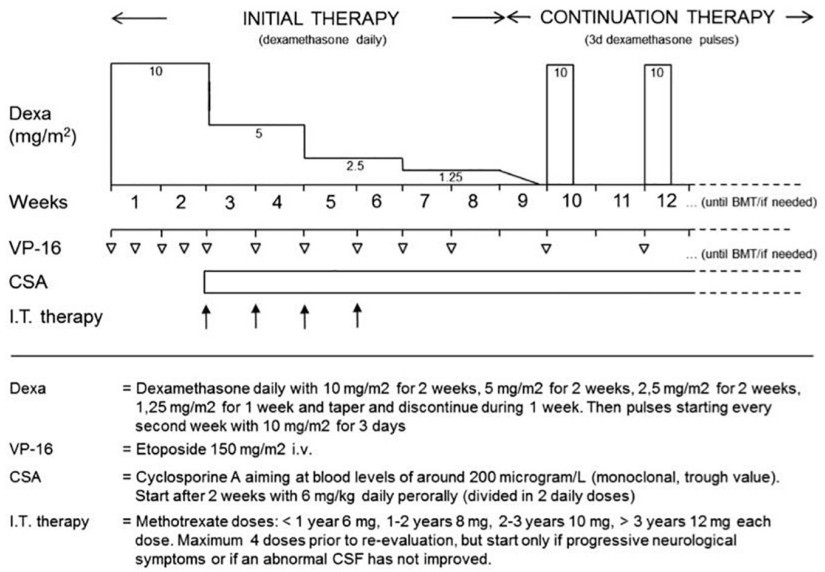

FIGURE 2 | 2018 consensus statements by the HLH Steering Committee of the Histiocyte Society recommending the use of HLH-94. The HLH-94 protocol is based on immunochemotherapy including dexamethasone, etoposide, and cyclosporine A (CSA). After an intensive phase of 2 weeks with high doses of dexamethasone and twice weekly administration of etoposide, dexamethasone is tapered until week nine. Cyclosporine A is used from week three onward to prevent reactivation. Intrathecal therapy with methotrexate is recommended in patients with CNS involvement. Immunological testing and genetic confirmation of the underlying genetic disease is required in all patients and should provide the basis for HSCT within 8 weeks.

signaling downstream of IFN gamma, but also of several other pro-inflammatory cytokines. In the mouse-model of LCMV-induced "primary" HLH, the disease manifestations, including CNS involvement, were reduced upon JAK1/2 blockade by ruxolitinib (63, 64). The successful individual use of ruxolitinib reported in single cases of secondary HLH (65–67) has resulted in its prospective evaluation for this indication (68). Preliminary results of a single-center phase 2 pilot study on the efficacy of ruxolitinib in secondary HLH demonstrate good tolerance to ruxolitinib in a small cohort of five patients (69). However, "primary" HLH is excluded in these studies. A trial investigating the benefit of JAK inhibition in first-line treatment of human "primary" HLH is in preparation. We are not aware of reports on the use of JAK inhibitors in HLH in the context of other inborn errors of immunity, but this is a plausible pathway to explore.

Targeting IL-18

IL-18 is released by activated macrophages and can induce IFN-gamma and other pro- inflammatory cytokines (28). Several reports have found elevated free IL-18 concentrations (i.e., IL-18 not bound to its binding protein IL-18BP) in the serum of patients with both "primary" and secondary HLH as well as in animal models and IL- 18 levels correlated with the presence of HLH-criteria and disease progression (70). In a murine model of "primary" HLH it was shown that treatment with IL-18BP can reduce severe organ damage, but does not improve survival (71). An ongoing multicenter, double-blind, placebo-controlled, randomized withdrawal trial evaluates efficacy and safety of IL-18BP (tadekinig alfa) in pediatric patients with NLRC4 associated hyperinflammation including HLH or XIAP deficiency, diseases, in which IL-18 levels are particularly elevated (73). While this treatment seems promising to attenuate the autoinflammatory manifestations of XIAP deficiency, it remains to be seen whether it also has a role in acute EBV-induced HLH in this disease.

IL-1, IL-6, and TNF Alpha Blockade

The pro-inflammatory cytokines elevated in "primary" HLH also include IL-1, IL-6, and TNF alpha, which can be targeted by monoclonal antibodies and other blocking agents. They have been used successfully in the context of secondary HLH (72, 74) and related conditions associated with a "cytokine storm" such as hyperinflammation associated with CAR-T cell therapy (75). Moreover, case reports have illustrated a partial effect of IL-1 blockade in patients with NLRC4 or CDC42 mutations (32, 34) and in patients with CGD (76). Anecdotal reports have reported efficacy of IL-6 blockade in manifestations of XIAP deficiency different from acute EBV-induced HLH (77). However, efficacy of IL-1, IL-6, or TNF alpha blockade in "primary" HLH has not been clearly documented, neither in first-line nor in rescue therapy.

CONTROL OF TRIGGERS

Infections should be diagnosed and treated aggressively in all forms of HLH. When active EBV infection is present,

rituximab (anti-CD20 antibody) can help controlling the immune stimulation by eliminating EBV infected B cells (78, 79). However, in some states of persistent EBV replication, EBV has been demonstrated in T or NK cells leading to resistance against rituximab treatment (80). Cell-targeted therapy results in significant immunosuppression, such that reverse isolation, aspergillus-effective antifungal and PCJ prophylaxis should be administered (81). Weekly monitoring for infection or reactivation of latent pathogens (EBV, CMV, adenovirus, aspergillus antigen) is recommended (49).

MONITORING TREATMENT RESPONSE

Monitoring response to therapy and detecting early signs of reactivation is crucial in patients with "primary" HLH (79, 82). The response of cytopenia is a sensitive parameter to judge treatment response (83). Since neutropenia frequently occurs treatment-related, thrombocytopenia is the more valuable parameter. Bone marrow puncture can be of some help in distinguishing between the activity of HLH and the myelotoxic side effect of therapy. Ferritin usually shows a significant decrease in the first days of successful treatment. However, complete normalization of ferritin can take weeks and can be even further delayed by the transfusion of erythrocytes (84). sCD25 is more dynamic, but it may still take a few days until a substantial decrease can be observed. In patients with initially low fibrinogen, this parameter can be used together with transaminases and coagulation studies to assess the treatment response (83). Other biomarkers for disease activity such as free IL-18 or CXCL9 are being explored. If more rapid turnaround times can be achieved, they might be valuable for guiding therapy in the future.

SALVAGE THERAPY IN REFRACTORY HLH

Early mortality of acute HLH remains a major concern. 25–50% of patients with acute "primary" HLH fail to achieve rapid and sustained initial remission after etoposide-based therapy. If cytopenia [in particular thrombocytopenia <40 G (G/L)] and ferritin and/or sCD25 fail to respond after 2 weeks, the risk for an adverse outcome increases, justifying consideration of alternative (salvage) therapy (49). There are no standard recommendations for the treatment of relapsing or refractory HLH. The salvage therapies published so far include alemtuzumab, anakinra, ATG, and regimens consisting of liposomal doxorubicin, etoposide, and dexamethasone (85). In an observational study reporting on treatment of 22 patients with refractory HLH with alemtuzumab, 86 percent of patients showed partial response, and 77 percent were able to receive HSCT (86). Notably, CMV and adenovirus viremia occurred in 23–32% of patients.

Emapalumab, a neutralizing antibody against INFγ, has recently been licensed as the first drug for the treatment patients with "primary" hemophagocytic lymphohistiocytosis (HLH) with refractory, recurrent or progressive disease or intolerance with conventional HLH therapy (87). The recommended starting dose is 1 mg/kg twice per week with dexamethasone as a background treatment, but doses can be increased up to 10 mg/kg based on clinical response (88). Due to the risk of serious infections (frequent during therapy of primary HLH patients and observed in 32% of patients in the trial) patients should receive prophylaxis for Herpes Zoster, Pneumocystis jirovecii, and fungal infections and should be monitored for tuberculosis, adenovirus, EBV and CMV.

The study included 27 patients with a mean age of 1 year (range: 0.1 to 13 years), with a "primary" HLH in 82% of patients. Patients had received various combinations of dexamethasone, etoposide, CSA, and anti-thymocyte globulin prior to emapalumab. Full response was defined as normalization of all, while partial response was defined as normalization of ≥3 HLH parameters and HLH improvement was defined as ≥3 HLH abnormalities improved by at least 50% from baseline. Twenty patients completed the 8-week study, while seven were prematurely withdrawn. Seventy percent (19/27) of patients proceeded to HSCT. The overall response rate was 63%, the median time to response was 8 days. A complete response was achieved in 7 patients, partial response in 8 patients and HLH improvement in 2 patients (88, 89). Since refractory primary HLH has a dismal prognosis, these data are encouraging. However, the exact place of this drug in the context of existing and emerging therapies remains to be defined.

DEFINITIVE THERAPY

Hematopoietic Stem Cell Transplantation (HSCT)

To prevent recurrences, allogenic stem cell transplantation should be carried out as soon as possible after achieving initial remission in the "primary" HLH (49, 90). It remains the only curative option. The timing of HSCT has to balance the risks between achieving full remission versus reactivation. Although active disease at conditioning remains a risk factor, full remission of all clinical symptoms is not required for successful HSCT. In particular, active neurological disease should prompt aggressive management including early HSCT (51).

Allogeneic HSCT is also the definitive treatment of choice for HLH in the context of several other inborn errors of immunity, including XLP1 or XLP2 and patients with TIM3 deficiency (91, 92). Furthermore, HSCT has been successfully performed in a patient with a CDC42 mutation (34). However, there has been no report of HSCT in NLRC4 deficiency, where it is unlikely to impact on IL-18 hypersecretion by intestinal epithelial cells (93). Careful and broad genetic and functional evaluation is therefore mandatory before proceeding to HSCT based on clinical grounds in rare cases of familial or recurrent HLH without detection of a genetic cause.

In cases with suspected "primary" HLH, donor search and pretransplantation diagnostics should be carried out promptly during the initial presentation. Bi-allelic mutations should be ruled out in potential related donors. In autosomal-recessive disease, heterozygous carriers are in most cases appropriate donors. In the X-linked conditions, skewed X-inactivation

should be excluded in potential female carrier donors (94). Conditioning regimes for HSCT in "primary" HLH have been discussed elsewhere.

Gene Therapy

For genetic diseases manifesting in hematopoietic cells, hematopoietic stem cell gene therapy is an important option (95). Preclinical murine studies in a perforin knock-out mouse showed a correction of the HLH phenotype after lentiviral gene therapy of autologous hematopoietic stem cells (96). However, high levels of expression were necessary to fully correct the HLH phenotype (97). Successful gene transfer into hematopoietic stem cells has also been demonstrated in a mouse model (98) and in patient T cells with MUNC13-4 deficiency (99). Furthermore, correction of cellular and humoral immune function was achieved by gene therapy in the murine model of XLP1 (100). Since these mice do not develop HLH, the question of whether the gene therapy can fully control the risk of HLH could not be addressed. These preclinical proof-of-concept studies show the therapeutic potential of gene therapy in "primary" HLH and it will be important to see them further investigated in clinical trials in the future.

SPECIAL SITUATIONS IN "PRIMARY" HLH

CNS Involvement and Isolated CNS-HLH

Central nervous system involvement is a common complication in "primary" HLH (30–73%) and leads to increased morbidity in long-term survivors (51, 101). Irritability, seizures, meningisms, focal deficits, or reduced level of consciousness are observed in active HLH. Diagnostic parameters of CNS involvement include variable combinations of elevated protein or cell count (>5 cells/μl), lymphocytic pleocytosis, activated monocytes and hemophagocytosis in the CSF. MRI brain morphology can demonstrate cerebral atrophy, diffuse white matter irregularities and multiple focal lesions (102–104). Delayed start of treatment for "primary" HLH increases the risk of neurological complications and is associated with worse CNS outcomes (105).

"Primary" HLH can also present as isolated CNS disease in the absence of any systemic manifestations. These occur particularly in older patients with hypomorphic mutations (106–110), most commonly in patients with FHL2 or Griscelli syndrome (111). Isolated CNS disease has also been documented in patients post-transplant with partial donor chimerism (112, 113). Systemic HLH-directed therapies can improve CNS-HLH unless irreversible damage has already occurred (51). Considering its value in other inflammatory brain diseases, alemtuzumab may provide an interesting option. In the further course, patients with isolated CNS-HLH are at risk for developing full-blown systemic HLH. Allogeneic HSCT is therefore also recommended in patients with isolated CNS disease (111).

Pre-emptive HSCT

Unless transplanted, all patients with "primary" HLH have a risk of developing life-threatening HLH at any time throughout their life. This risk has to be weighed against the risk of HSCT on an individual basis. In any case, genetic testing of family members, particularly of siblings should be offered promptly after diagnosis of the index case. In a recent analysis of 64 children with primary HLH (index cases), 32 asymptomatic carriers were identified. 16 of 22 asymptomatic carriers received pre-emptive transplantation, of which 15 are alive and in complete remission after 39 months of median follow-up. Eight-year probability of survival was significantly higher than that in index cases and survival in asymptomatic carriers receiving HSCT before disease activation was significantly higher than in those receiving HSCT after HLH activation (93% versus 64%) (114). Hence, most experts recommend pre-emptive HSCT for FHL unless mutations are very mild.

OUTLOOK

In the last few decades, significant progress has been made in understanding the genetic basis and pathogenesis of HLH in the context of inborn errors of immunity. This has set the stage for rapid diagnosis and a more targeted therapy of this serious clinical condition. The outcome of "primary" HLH has significantly improved with cell-targeted therapies. New cytokine-directed treatments will increase the therapeutic flexibility, but it remains to be seen whether they will show enough efficacy to fully replace this aggressive approach. In the emerging field of HLH associated with other inborn errors of immunity, established and novel cytokine-directed therapies are expected to become the treatment of choice.

AUTHOR CONTRIBUTIONS

OW wrote the manuscript. KW, KL, and SE contributed to manuscript revision and approved the submitted version.

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Increased Prevalence of *NLRP3* Q703K Variant Among Patients With Autoinflammatory Diseases: An International Multicentric Study

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Background: The *NLRP3* inflammasome has been recognized as one of the key components of innate immunity. Gain-of-function mutations in the exon 3 of *NLRP3* gene have been implicated in inflammatory diseases suggesting the presence of functionally important sites in this region. Q703K (c.2107C>A, p.Gln703Lys, also known in the literature as Q705K) is a common variant of *NLRP3*, that has been considered to be both clinically unremarkable or disease-causing with a reduced penetrance.

Objectives: We aimed to investigate the potential genetic impact of the *NLRP3* variant Q703K in patients with recurrent fever presenting with two autoinflammatory diseases: PFAPA (periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis) and CAPS (cryopyrin-associated periodic syndrome), as well as with undefined autoinflammatory disease (uAID).

Methods: This is an international multicentric observational retrospective study characterizing the clinical phenotype of patients presenting with recurrent fever suspected to be of auto-inflammatory origin and where the Q703K *NLRP3* variant was found. Monocytes of parents of 6 Q703K+ PFAPA patients were studied and levels of pro-inflammatory cytokines produced by monocytes of Q703K+ and Q703K- parents have been compared by ELISA.

Results: We report 42 patients with the Q703K *NLRP3* genetic variant: 21 were PFAPA patients, 6 had a CAPS phenotype, and 15 had an uAID.

The phenotypes of PFAPA, CAPS and uAID were quite similar between Q703K positive and negative patients with the exception of increased prevalence of pharyngitis in the Q703K positive CAPS population compared to the negative one. The *in vitro* production of IL-1β was not significantly different between Q703K+ and Q703K- monocytes from asymptomatic parents.

Conclusion: The evidence we report in our study shows an increased prevalence of *NLRP3* Q703K in patients with autoinflammatory diseases, suggesting an association between the Q703K variant and the risk of PFAPA, CAPS and uAID syndromes. However, we did not show a functional effect of this mutation on the inflammasome basal activity.

Keywords: Q703K, NLRP3, PFAPA, CAPS, autoinflammation, autoinflammatory diseases, recurrent fever

INTRODUCTION

Autoinflammatory diseases (AIDs) are clinically defined by repeating seemingly unprovoked attacks of multisystemic inflammation without underlying infection or autoantibody formation (1, 2). Monogenic origins have been established for some AIDs, including Familial Mediterranean Fever (FMF), Mevalonate Kinase Deficiency (MKD) also known as Hyperimmunoglobulinemia D and periodic fever syndrome (HIDS), TNF Receptor 1-Associated Syndrome (TRAPS) and Cryopyrin-Associated Periodic Syndrome (CAPS) (3). The pathophysiology of all these diseases is characterized by immune dysregulation due to enhanced IL-1β maturation, caused by direct or indirect inflammasome activation.

PFAPA (periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis) syndrome is considered to be an AID without defined genetic origin (2). It represents the most common cause of recurrent fever in children in European populations, and an annual incidence of 2.3 cases for 10'000 children per year has been recently reported (4, 5). It is characterized by regularly recurring episodes of high fever, accompanied by at least one of the three cardinal symptoms, including pharyngitis, cervical adenitis and aphthous stomatitis. Patients are relatively asymptomatic between attacks and show normal growth and development (6, 7). The diagnosis is based on clinical criteria (7, 8) and the exclusion of other causes of recurrent fever, such as infectious, autoimmune and malignant diseases. Generally the onset of disease dates before the age of 5 years and these febrile episodes usually resolve spontaneously before adulthood (9). The rapid response of the fever attacks to a single dose of corticosteroid, the absence of an infectious or autoimmune cause, and the dysregulation of interleukin (IL-)1β secretion during fever flares supports the hypothesis that PFAPA syndrome is an autoinflammatory disease (10, 11).

The NLRP3 inflammasome (formerly known as NALP3 or cryopyrin) has been recognized as one of the key components of innate immunity by sensing microbial ligands, endogenous danger signals and crystalline substances in the cytosol. Upon activation, the sensor protein NLRP3 assembles with the adaptor protein ASC and pro-caspase-1 to form the NLRP3 inflammasome (12). This interaction leads to the activation of caspase-1, which proteolytically processes pro-IL-1β and pro-IL-18 to form, respectively, active IL-1β and IL-18 (13). Gain-of-function mutations in exon 3 of the *NLRP3* gene have been implicated in hereditary auto-inflammatory diseases, grouped under Cryopyrin-associated periodic syndrome (CAPS), suggesting the presence of functionally-important sites in this region (14).

Interestingly, Q703K (rs35829419, c.2107C>A, p.Gln703Lys, also known in the literature as Q705K) is a rather common *NLRP3* variant in exon 3, of unclear pathogenic significance, that has variously been considered to be clinically unremarkable and a disease causing with reduced penetrance. However, it has been found to be associated with PFAPA syndrome, CAPS (15, 16) and other inflammatory diseases (17, 18). Moreover, its functional role in inflammasome activation is ambiguous, as it was demonstrated to lead to increased IL-1β secretion *in vitro* (19), but not in healthy and CAPS carriers (12, 20).

These genetic observations, together with the association of dysregulated levels of IL-1β, raised the question on the role of the Q703K *NLRP3* variant in auto-inflammation. We therefore screened children with recurrent fever suspected to be of autoinflammatory origin followed in JIR cohort and AID registry, for variants of genes involved in monogenic AID (genes: *NLRP3*, *MEFV*, *TNFRSF1A*, *MVK*) in order to investigate the prevalence of the Q703K variant in patients with autoinflammatory diseases and we characterized the phenotype in mutation-positive patients. Furthermore, we compared inflammasome basal activity in 6 healthy Q703K carriers with 6 healthy non-carriers; all these 12 healthy individuals were parents of 6 Q703K positive PFAPA patients.

MATERIALS AND METHODS

Study Population

All children clinically diagnosed with PFAPA (periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis), CAPS (Cryopyrin-associated periodic syndrome) or uAID (undefined autoinflammatory disease) attending the pediatric rheumatology consultation of Western Switzerland at the Lausanne University Hospital and the Geneva University Hospital, from November 2009 to November 2017, had a genetic analysis in the 4 major genes associated with monogenic periodic fevers: *MEFV, TNFRSF1A, MVK, NLRP3*. The data were collected from

the retrospective module of the JIR (Juvenile Inflammatory Rheumatism) cohort network. The JIR cohort is an international database which includes patients presenting an inflammatory rheumatism starting in childhood; only the center of Western Switzerland among the JIR network participated in this study. One more patient presenting with recurrent fevers and chronic glomerulonephritis followed in the adult immunology consultation was added in this data collection.

An additional and independent cohort of patients with recurrent fevers was identified from the AID-registry, which is part of the AID-Net (Network for autoinflammatory diseases), a research initiative funded by the German Federal Ministry of Education and Research (BMBF) und supported by the German Rheumatism Research Center (DRFZ) and the German society for pediatric rheumatology (GKJR) (21). Patient data was retrospectively documented between July 2009 and October 2017. All patients with a diagnosis of CAPS or PFAPA and availability of genetic analysis in the four fever genes either provided by the centers or analyzed in Muenster in samples of the Biobank.

Concerning the PFAPA patients, previously published diagnostic criteria (7) were applied. However, because PFAPA is not a well-defined disease and there are no confirmatory tests, the power of these criteria remains limited. New classification criteria, based on a consensus among a large panel of experts and confirmed on a large cohort of PFAPA patients (Eurofever), are currently under investigation and should bring soon some improvements in the classification of PFAPA (8). Consequently patients with disease onset after 5 years of age were included according to the published international multi-center cohort of 301 patients (22). Patients with all three constitutional symptoms were described as having "complete cluster"; otherwise "incomplete cluster" for those with one or two constitutional symptoms. All these children had normal growth and development and did not present any other symptoms suggesting an alternative diagnosis. CAPS diagnosis was suggested by the clinical presentation of patients according to the expert opinion and was confirmed by the new classification criteria (8) and/or genetical analysis.

The symptoms, treatment, response to treatment and family history of the patients with the Q703K *NLRP3* variant have been retrospectively extracted and described from both JIR and AID-Net cohorts. The phenotypes of Q703K-positive and Q703K-negative PFAPA, CAPS and uAID patients were compared, respectively.

In the second part of our study, six pairs of asymptomatic parents of Q703K positive children with PFAPA syndrome were also asked to participate in the study, to compare the inflammasome basal activity between Q703K positive and negative healthy parents.

All subjects received code numbers to guarantee anonymity. Approval for the study was obtained for JIR patients from the Cantonal Ethical Committee in Lausanne and in Geneva and for AID-net patients from the ethics committees and the data protection responsible at the Universities of Duisburg-Essen and Muenster, as well as the Medical Association Nordrhein in Duesseldorf, and the parents/caregivers gave their informed consent according to local ethical regulations.

Genetic Analysis

Genomic DNA was extracted using standard methods from peripheral mononuclear cells (PBMC). Mutation hotspots in the 4 major genes associated with monogenic periodic fevers were analyzed by using PCR and DNA sequencing. The regions analyzed were as follows: *MEFV* exons 2 and 10 (detects >95% of known pathogenic FMF mutations); *TNFRSF1A* exons 2,3,4, and 6 (detects close to 100% of pathogenic TRAPS mutations); *MVK* exons 9 and 11 (detects ∼70% of pathogenic HIDS); and *NLRP3* exon 3 (detects close to 100% of pathogenic CAPS mutations) (23).

Sample Preparation

Peripheral blood from the subjects was collected in S-Monovette tubes containing EDTA or, for the isolation of sera, clot activators. Anticoagulated blood was collected for DNA extraction and genetic analysis. For cytokine determinations (see below), sera were stored in sterile tubes at −80°C before analysis.

Monocyte Isolation and Stimulation-Cytokines Measurements

Anticoagulated blood was obtained from the asymptomatic parents of 6 unrelated Q703K-positive children with PFAPA (for each family: father, mother). Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque (Life Technologies). Monocytes were purified by magnetic-associated cell sorting (MACS Monocyte Isolation Kit-Miltenyi). Monocytes were stimulated overnight with ultrapure LPS (200 ng/mL) to activate inflammasome (24). Levels of IL-1β, TNF-a and IL-6 were measured in cell supernatants with ELISA (eBioscience).

Statistical Analysis

Differences between groups were analyzed by using a two-sample test of proportion with 95% confidence interval, the Wilcoxon signed-rank test, the two-tailed Fisher's exact test, the two-tailed Chi-square with Yates correction and the *t*-test. Statistically significant results are annotated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

RESULTS

Association of Q703K Variant With PFAPA, CAPS, and uAID Syndromes

The Q703K variant was observed in 21 out of 150 PFAPA patients (14%, 12/109 in JIR cohort, 9/41 in AID-Net cohort), in 6 out of 24 CAPS patients (25%, 1/8 in JIR cohort, 5/16 in AID-Net cohort) and in 15 out of 36 uAID patients (42%, 7/22 in JIR cohort, 8/11 in AID-Net cohort). All individuals tested positive for Q703K were heterozygous. We compared allele frequencies with data from the Genome Aggregation Database (gnomAD), a publicly available database containing genetic variations from over 10'000 human genomes and exomes, reporting an allelic frequency of 3.8 and 5.1% in general and European populations, respectively. Thus, Q703K showed a significantly increased variant's frequency ($P < 0.001$) in all three PFAPA, CAPS and uAID cohorts, suggesting an association of this variant with the risk of these syndromes (**Table 1**).

Clinical Characteristics of Patients Carrying the Q703K Variant

We found the *NLRP3* variant Q703K in 42 patients. Of these, 21 were PFAPA patients, 6 had a CAPS phenotype and 15 had an undefined auto-inflammatory disease (uAID). All 21 Q703K-positive PFAPA patients presented with at least one cardinal symptom and 7 had a complete cluster. One patient had neurological symptoms during fever flares (hypotonia, bulging fontanel, loss of contact, seizures). Seven patients had a positive family history of recurrent fever, recurrent pharyngitis or tonsillectomy and one patient had a family history of undefined rheumatic disease. All patients that have been treated with a single steroid dose during the flare responded within 12 h (11/11). Three patients were found to carry also other genetic variants in the *MEFV* gene.

Concerning the 6 Q703K-positive CAPS, 50% of them (3/6) showed a FCAS phenotype and 50% (3/6) a Muckle-Wells phenotype; no patient presented the neonatal form CINCA/NOMID. Two patients were found to carry also other genetic variants in *NLRP3* (D303N, G569A). Of 4 patients with known family history, only 1 was positive. Data for treatment were available in 5 patients; all of them were treated with anti-IL1 agents with a good response.

Clinical data are available in 12 up to 15 Q703K-positive uAID patients. The most frequent symptoms were recurrent fever (10/12), abdominal pain (5/12), oral aphthosis (3/12) and headache (2/12). Of five patients with known family history, two were positive. One patient presented severe proteinuria in the context of chronic glomerulonephritis. Data for treatment were available in 11 patients: 5 of them were treated with anti-IL-1 agents with good response in 4 of them; NSAIDS, steroids and colchicine have been used for the others.

It is important to state that JIR and AID-Net cohorts were assembled before the new classification criteria (8) have been published. For the prupose of our analyses, patients were pothoc re-classified accoring to recently published consensus criteria. Consequently, some patients have been treated with different diagnosis in "real life"; more precisely 12 CAPS patients (10 from AID-Net cohort and 2 from JIR cohort) turned to uAID and 7 CAPS patients (AID-Net cohort) to PFAPA diagnosis for the current analysis. Interestingly, the frequency of the Q703K allele according to the original diagnosis was the following: 11% (16/143) in PFAPA, 44% (19/43) in CAPS, and 3% (7/24) in uAID patients.

Comparison of Q703K-Positive and Q703K-Negative PFAPA Patients: Similar Phenotype in Both Groups

Comparisons of the clinical characteristics between the Q703K-positive and -negative PFAPA patients were performed in the JIR- and AID-Net cohorts, where all PFAPA patients underwent genetic analysis, and is shown in **Table 2**. There were no significant differences in the sex ratio, the prevalence of onset after the age of 5 years and the positivity of familial history. One patient in the Q703K-positive group had an atypical presentation with neurological symptoms; no patient

TABLE 1 | Q703K allele frequency in our PFAPA, CAPS and uAID patients compared to the healthy population from gnomAD genome and exome data.

Allele frequency observed in PFAPA (JIR & AID-Net cohorts)			Allele frequency observed in CAPS (JIR & AID-Net cohorts)			Allele frequency observed in uAID (JIR & AID-Net cohorts)		
14% (21/150)	Allele frequency in total population (gnomAD): 3.8% (10781/280716)	Allele frequency in European population (gnomAD): 5.1% (6584/129092)	**25% (6/24)**	Allele frequency in total population (gnomAD): 3.8% (10781/280716)	Allele frequency in European population (gnomAD): 5.1% (6584/129092)	**42% (15/36)**	Allele frequency in total population (gnomAD): 3.8% (10781/280716)	Allele frequency in European population (gnomAD): 5.1% (6584/129092)
	$P < 0.001$	$P < 0.001$		$P < 0.001$	$P < 0.001$		$P < 0.001$	P<0.001

TABLE 2 | Comparison table of clinical characteristics between Q703K-positive vs. -negative PFAPA patients.

	Q703K+ (n: 21)	Q703K- (n: 129)	*P*-value
Median age at onset (years.months)	2	1	
Median duration (days)	5*	4.25**	
Median interval (days)	30*	30**	
Sex ratio (M/F)	2/1 (14/7)	1/1 (66/63)	0.2402
Positive familial history	8/19	50/102	0.6249
Pharyngitis	17	107/126	0.7450
Oral aphthosis	11	65/120	1
Adenitis	16	78/128	0.2265
Abdominal Pain	13	66/118	0.6420
Complete cluster	7	40/116	1
Onset after 5 y.o.	3	16/127	0.7349
Good response to steroids	11/11 (100%)	101/105 (96%)	1

**data available in 16 patients.*
***data available in 127 patients.*

had neurological symptoms in the Q703K-negative group. The frequency of the other cardinal symptoms and complete clusters was similar in both groups. Almost all patients in both groups had a good response to steroids.

Comparison of Q703K-Positive and Q703K-Negative CAPS Patients: Higher Prevalence of Pharyngitis in the Q703K-Positive Group; Similar Disease Severity in Both Groups

Comparisons of the clinical characteristics between the Q703K-positive and -negative CAPS patients were performed in both JIR and AID-Net cohorts (**Table 3**). Phenotypes were quite similar in both groups. Except the higher prevalence of pharyngitis in the Q703K-positive group, there were no statistically significant differences in other clinical characteristics as recurrent fever, urticaria, arthralgia, myalgia, conjunctivitis, adenopathy, abdominal pain, neurologic symptoms, hearing loss. Furthermore, we did not observe significant differences in the disease severity, with the same prevalence of FCAS, MWS and CINCA/NOMID phenotypes in both groups as well as the same frequency of disease complications such as renal insufficiency, ocular complications, growth, and neurodevelopmental delay.

Comparison of Q703K-Positive and Q703K-Negative uAID Patients: No Statistically Significant Differences in the Phenotype of Patients

Comparisons of the clinical characteristics between the Q703K-positive and -negative uAID patients with complete datasets were performed in the JIR cohort and AID-Net cohort, where all uAID patients underwent genetic analysis, and is shown in **Table 4**. No statistically significant differences were found in the prevalence of clinical characteristics, disease complications, nor the positivity of familial history.

TABLE 3 | Comparison table of clinical characteristics between Q703K-positive vs. -negative CAPS patients.

	Q703K+ (n: 6)	Q703K- (n: 20)	*P*-value
Median age at onset (years.months)	0.7	0.1	
Median age at diagnosis	6.9	4.1	
Sex ratio (M/F)	2/1	2/1	1
Positive familial history*	1/4	9/16	0.5820
Recurrent Fever	3	4	0.2929
Pharyngitis	2	0	0.0462
Abdominal pain	1	2	1
Neurologic symptoms	2	7	1
Adenopathy	1	4	1
Urticaria	4	17	0.5581
Headache	3	2	0.0624
Myalgia	1	2	1
Arthralgia	4	12	1
Rash	1	1	0.4154
Diarrhea	1	0	0.2308
Conjunctivitis	2	5	1
Oral aphthosis	1	0	0.2308
Hearing loss	2	5	1
Disease Complications**	1	6	1
FCAS	3	4	0.2929
MWS	3	12	1
CINCA/NOMID	0	4	0.5425

**familial History is available in only 4 Q703K-positive and 16 negative CAPS patients.*
***disease complications include ocular complications, renal insufficiency, skeletal and joint deformities, growth and neurodevelopmental delay.*

No Role for Q703K in Monocyte-Derived IL-1β Secretion but Trend in Higher TNF-α Production

We compared the cytokine secretion between 6 Q703K-positive and 6 Q703K-negative healthy parents. As shown in **Figure 1**, the production of IL-1β, TNF-α or IL-6 were not significantly different between monocytes from Q703K positive and negative asymptomatic parents (Q703K positive: 4583.7 ± 2671.1, 3110 ± 2904.6, and 49043.7 ± 37257.9 pg/ml; Q703K negative: 3499.4 ± 2946.7, 935.6 ± 1259.4, and 45982 ± 18317.4 pg/ml, respectively). However, we observed higher levels of TNF-α production in Q703K-positive healthy parents, even if not significant (3110 ± 2904.6 vs. 935.6 ± 1259.4 pg/ml).

DISCUSSION

Q703K is an allelic variant of the *NLRP3* gene which pathogenic relevance and penetrance are both poorly understood. Our study supports the assumption of an association of the Q703K variant with a variety of autoinflammatory diseases. In particular, we demonstrate significantly increased variant frequencies in PFAPA, CAPS and uAID cohorts (14, 25, and 42%, respectively), compared to compiled gnomAD data (3–5%), suggesting an association between Q703K and these syndromes ($p < 0.001$).

In recent years, several studies have shown the implication of this variant in autoinflammatory conditions. In particular, Q703K was found in 7% of CAPS patients from the Eurofever Registry (25); Vitale et al. (15) also demonstrated that patients carrying Q703K may present with FCAS-like symptoms, and Insalaco et al. (26) described a new CAPS phenotype associated with the combination of Q703K with a second novel mutation in the *NLRP3* gene. Heterozygosity for Q703K was found in another 17 CAPS cases from Germany: 15 out of 17 cases were CAPS patients and 2 out of 17 were CAPS carriers, 1/17 additionally R92Q TRAPS mutation, 1/17 G569A CINCA variant and 1/17 S52N HIDS variant (27). On the other hand, Naseli et al. (28) found a milder CAPS phenotype in Q703K-positive patients without the typical cytokine pattern observed in typical CAPS patients, suggesting a weak clinical and functional effect of this variant. Furthermore, Kuemmerle-Deschner and her colleagues studied 45 patients with low penetrance *NLRP3* variants (Q703K, V198M, R488K) and found significantly more fever (76%) and gastrointestinal symptoms (73%) compared to CAPS patients with pathogenic *NLRP3* variants. Functional inflammasome testing identified an intermediate phenotype in low-penetrance *NLRP3* variants as compared to wild-type and pathogenetic *NLRP3* variants (20).

TABLE 4 | Comparison table of clinical characteristics between Q703K-positive vs. -negative uAID patients.

	Q703K+ (n: 12)	Q703K- (n: 5)	*P*-value
Median age at onset (years.months)	2.9	3.8	
Median age at diagnosis	5.3	5.8	
Sex ratio (M/F)	2.3/1	1/4	0.1189
Positive familial history	2/5	2/2	1
Recurrent Fever	10	3	0.5378
Pharyngitis	1	1	0.5147
Abdominal pain	5	2	1
Neurologic symptoms	0	0	1
Adenopathy	1	1	0.5147
Urticaria	1	3	0.0525
Headache	2	1	1
Myalgia	0	1	0.2941
Arthralgia	1	3	0.0525
Rash	1	0	1
Diarrhea	0	0	1
Conjunctivitis	0	0	1
Oral aphthosis	3	2	0.6
Disease Complications	1*	0	1

**severe proteinuria in the context of chronic glomerulonephritis.*

Our clinical analyses suggest a possible role of this variant in the clinical inflammatory phenotype of CAPS patients, as they present a statistically significant increased prevalence of pharyngitis. Moreover, it is important to state that Q703K positive CAPS patients have comparable disease severity and complications with those presenting with typical pathogenic *NLRP3* mutations. Interestingly, when analyzing the JIR cohort's PFAPA patients separately, we observe statistically significant increased gastrointestinal symptoms (abdominal pain) in Q703K positive patients compared to the negative

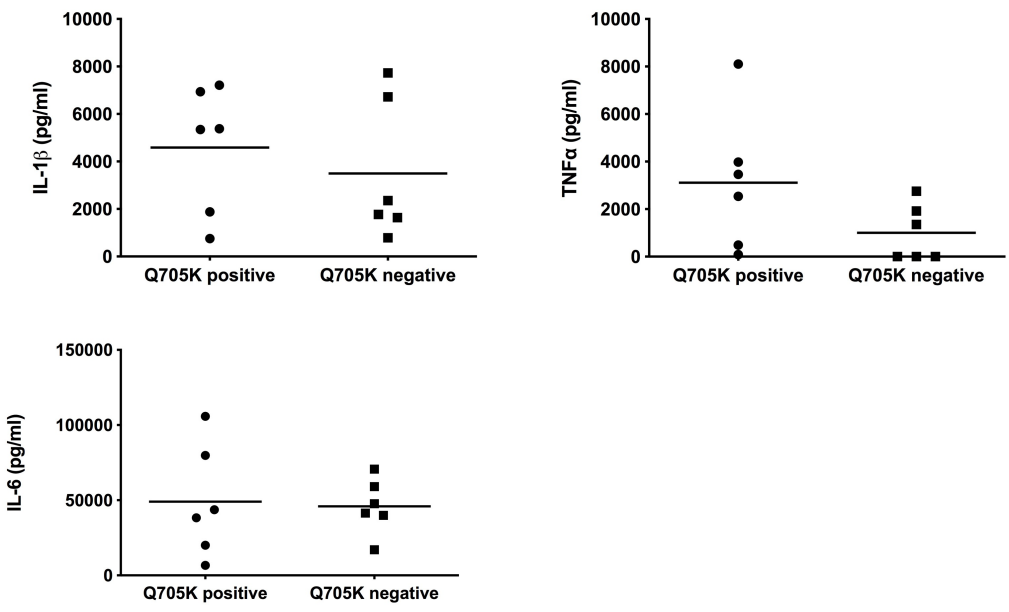

FIGURE 1 | Cytokine secretion by monocytes isolated from PFAPA families. Human PBMC-derived monocytes were cultured in three independent wells and stimulated overnight with 200 ng/ml of LPS. IL1β, TNF-alpha, and IL-6 were measured in supernatants by ELISA. Each dot (mean of values from triplicate monocyte cultures) represents a parent (mother or father) of a PFAPA patient.

ones (**Supplementary Table 1**), in line with the study of Kuemmerle-Deschner et al. (20). On the other hand, we describe atypical neurologic manifestations in one Q703K PFAPA patient, supporting previous publications that might suggest a tropism of this variant to the central nervous system (29, 30).

Analyzing clinical data most of the Q703K-positive patients in the JIR cohort had a diagnosis of PFAPA, while Q703K-patients from the AID-net cohort had higher rate of CAPS diagnosis. Clinical phenotypes of both diseases are similar, not well-defined because of a number of symptoms typically found in both PFAPA as well as CAPS patients. This difference mirrors the uncertainty of clinicians when laboratories report low-penetrance variants. There is certainly a bias for clinicians to skew a patient's diagnosis into a CAPS-direction if an *NLRP3* variant is detected, and it depends on personal experience and expertise which diagnosis is made. This is also influenced by previous PFAPA-diagnostic criteria that excluded patients with genetic findings in any of the 4 genes responsible for the most common hereditary fever syndromes. A further limitation of our study is the lack of whole exome sequencing in our patient populations, not allowing to exclude modifiers in further genes. Summarizing results of our present cohort study, we suggest considering Q703K *NLRP3* variant as a relevant variant in genetic testing, as it can be associated with PFAPA, CAPS and uAID phenotypes. Moreover, patients carrying this variant probably have a significantly higher risk to develop autoinflammatory disease, but details concerning incidence, severity and prognosis require further studies before this patient group can be integrated into or distinguished from current diagnostic entities. Furthermore, it would be interesting to validate this observation in larger patients' cohorts and in patients with other autoinflammatory diseases classified as FMF or TRAPS to assess whether this finding is specific to PFAPA, CAPS and uAID patients or if it is a more general observation.

In the second part of our study, we aimed to investigate whether Q703K is a gain-of-function variant by comparing the cytokine production between Q703K-positive and Q703K-negative healthy parents. In contrast to the findings of Verma et al. (16, 19) suggesting that Q703K is a gain-of-function alteration leading to an overactive NLRP3 inflammasome, we did not find significant difference in cytokine production between Q703K carriers and non-carriers. However, this is in line with other previous studies (12, 31); in particular Kuemmerle-Deschner et al. showed as well that there was no significant increase of Caspase-1 or Interleukin 1 in Q703K-patients (20). Our hypothesis is that the Q703K variant may be a low-penetrance gain-of-function mutation which might need the presence of further genetic variants to induce auto-inflammation. This is supported by the results of Sahdo et al. (12) showing that carriers of both *NLRP3* Q703K and *CARD8* C10X had higher cytokine levels compared to controls, but carriers of isolated C10X or Q703K variants had similar plasma levels of IL-1β to non-carriers, suggesting a potential synergic effect of different genetic variants in inflammasome activation. Moreover, the combination of Q703K with *CARD8* C10X has been found to correlate with increased caspase-1 activity and IL-1β secretion in patients with CAPS-like symptoms, as well as with dysregulated apoptosis (16, 32). These combined polymorphisms have also been implicated in severe chronic inflammatory diseases, like rheumatoid arthritis and Crohn's disease (17, 18).

In conclusion, the evidence we report in our study supports an association between *NLRP3* Q703K and the risk of autoinflammatory syndromes, as indicated by the significantly increased prevalence of this variant in our PFAPA, CAPS and uAID populations. However, we did not find a functional effect of this mutation as the cytokine secretion was similar in healthy Q703K-positive and -negative individuals. We hypothesize that this mutation might act synergistically with other genetic variants or epigenetic alterations by inducing excessive inflammasome activation and autoinflammation. Lastly, the functional effect of the *NLRP3* Q703K alone is probably not sufficient to induce severe autoinflammatory presentations but contributes to more attenuated phenotypes.

KEY MESSAGES

- The *NLRP3* Q703K variant is associated with various autoinflammatory diseases (PFAPA syndrome, CAPS and undefined AID). However, the correlation between the genotype and the phenotype is unclear and the diagnosis depends on the clinical presentation.
- The effect of Q703K variant remains unclear. However, given its increased prevalence among patients with autoinflammatory diseases, some contribution to the inflammatory phenotype cannot be ruled out.
- The Q703K is not sufficient to induce autoinflammation *per se*, but might be a reduced-penetrance mutation that could have a synergistic effect with other mutations.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Cantonal Ethical Committee in Lausanne and in Geneva, Ethics committees of Duisburg-Essen and Muenster, as well as the Medical Association Nordrhein in Duesseldorf. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

KT, HW, NB, DF, and MH were involved in the conception and design of the study. KT, HW, DF, MH, VH, AV, EL, GHo, J-PH, KP-B, RB, AJ, and GHe organized the databases. KT performed the statistical analysis. NB performed the experiments. MM, IM, and NF performed the genetic studies. KT, HW, EL, NB, DF, and MH analyzed the data. KT wrote the first draft of the manuscript. HW and NB wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00877/full#supplementary-material

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frontiers in Immunology

REVIEW
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The Use of Interleukine-1 Inhibitors in Familial Mediterranean Fever Patients: A Narrative Review

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Purpose: Familial Mediterranean fever (FMF) is the most common monogenic auto-inflammatory disease characterized by recurrent attacks of fever and serositis. It is associated with mutation in pyrin inflammasome leading to interleukin-1 (IL-1) over secretion. Although colchicine is the first line treatment in FMF, 5–10% of patients are reported in literature as non-responders. Colchicine is not always well-tolerated due either to its direct toxicity or to co-morbidities that preclude the administration of its proper dosage. For these patients an alternative or additional treatment to colchicine is necessary. This literature review reports the published data regarding the use of IL-1 inhibitors in Familial Mediterranean Fever.

Results: There is no uniform definition of colchicine resistance, but the different studies of treatment with IL-1 inhibitors provide evidence of IL-1 pathogenic role in colchicine-resistant FMF. IL-1 inhibition is an efficacious option for controlling and preventing flares –at least at the short term- in FMF patients who are insufficiently controlled with colchicine alone. Although canakinumab is the only approved drug in Europe for colchicine resistant FMF treatment, experience with anakinra is also substantial. In the absence of comparative studies both treatments seem to be an equal option for the management of these patients. Overall the safety profile of IL-1 inhibitors seems not different in FMF patients than in the other diseases and can be considered as globally safe. The main side effects are local injection site reactions and infections.

Conclusion: IL-1 inhibitors have the potential to improve patient outcome even in FMF patients with co-morbidities or severe complications in whom inflammation control is difficult to achieve with colchicine alone. Nevertheless, current data are limited and further evaluation of long-term efficacy and safety of IL-1 inhibitors are necessary, in order to provide robust evidence in this domain.

Keywords: familial mediterranean fever, anakinra, canakinumab, interleukine-1, colchicine, autoinflammation, amyloidosis

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KEY MESSAGES

- There is no uniform definition of colchicine resistance. A standardized evaluation of adherence to colchicine treatment is mandatory before considering IL-1 inhibitors in FMF patients
- Although canakinumab is the only drug approved in Europe for the treatment of colchicine resistant FMF, experience with anakinra is more substantial in the literature.
- There are no comparative effectiveness assessment studies for canakinumab vs. anakinra. Both treatments seem to be an option for the management of colchicine resistant or intolerant FMF patients.
- The use of anakinra in pre-attack prodromal period ("on-demand treatment") may be a reasonable approach for alleviating symptoms of an impending attack in patients with insufficient response to the maximum tolerated dosage of colchicine and low risk of amyloidosis.
- The use of IL1 inhibitors as a maintenance therapy in patients unresponsive or intolerant to colchicine is globally safe and effective.

INTRODUCTION

Familial Mediterranean fever (FMF) is the most frequent monogenic auto-inflammatory disease. FMF is characterized by self-limited episodes fever associated to polyserositis and raised inflammatory markers (1). The disease is mostly seen among particular ethnic groups such as patients with a Middle Eastern ancestry or originating from the Mediterranean basin (2). Autosomal recessive mutations in the *MEFV* gene are responsible for the symptoms in FMF (3, 4). Although its pathogenesis is not fully understood, pyrin is a crucial player in the regulation of innate immunity and FMF-associated missense mutations induce an uncontrolled IL-1 release (5).

Amyloid deposition and the development of end-stage renal disease are the most severe complications of FMF. Since 1972, colchicine is the cornerstone of treatment for FMF patients. To date, only the daily intake of colchicine has proven its effectiveness on the long-term in preventing or improving inflammatory attacks, but also in decreasing the frequency of secondary amyloidosis (6–8).

Nevertheless, cases of unresponsiveness to colchicine have been reported, although this situation remains rare, probably <10 % of FMF patients (9–11). In addition, colchicine treatment is not always well-tolerated due either to direct colchicine toxicity or to co-morbidities that preclude the administration of the proper colchicine dosage. For these patients an alternative or additional treatment to colchicine is necessary. IL-1 inhibitors are the first candidates given the involvement of IL-1 in pathophysiology of the inflammatory attacks. Four biologic drugs blocking IL-1 are currently available. Of them, anakinra, and canakinumab have been approved for clinical use in Europe, whereas the soluble decoy IL-1-receptor, rilonacept, and the human-engineered monoclonal anti-IL-1, gevokizumab, are not authorized in European countries.

However, the precise indications for initiating IL-1 blocking agents in FMF patients are still unclear and poorly codified. Given the cost of these biological agents and their potential risk of side effects (mainly infections), their use needs still to be defined.

The objective of this article is to review the current knowledge about the use of IL-1 inhibitors in FMF, with the aim of defining the indications and the place of these more recent products in the therapeutic arsenal of the disease.

Abbreviations: AAA, amyloid type A protein amyloidosis formerly known as secondary amyloidosis; FMF, familial Mediterranean Fever; IL-1, interleukine 1; *MEFV* gene, mediterranean fever gene; NSAIDs, non-steroidal anti-inflammatory drugs; TNF, tumor necrosis factor.

METHODS

Literature Search Strategy

A literature search on the use of IL-1 inhibitors and FMF was conducted from 1947 until 2019 using the Medline, Embase, and Cochrane databases using the following terms: "anakinra," "canakinumab," "IL-1 inhibitor," "Interleukin 1 Receptor Antagonist Protein," "IL-1 blockade" and "familial Mediterranean fever." The terms were combined as both key words and MeSH terms. We excluded articles about rilonacept and gevokizumab, as both agents are not authorized in European countries.

Additional articles were retrieved by checking manually the references of the recovered articles and the "related articles" function on Pub-Med (www.pubmed.gov) were also assessed for possible inclusions. Only articles published in English or French before September 2019 have been included to this review.

Data Assessment

All four coauthors read and approved the retrieved articles. We extracted data of the selected articles using predefined scoring forms and classification tables that enabled us to analyze the published data in five different domains: 1/ indications for IL-1 inhibitors in FMF, 2/ efficacy of IL-1 inhibitors in FMF, 3/ comparison of anakinra vs. canakinumab in FMF, 4/ comparison of maintenance vs. on-demand treatment in FMF and 5/ safety of IL-1 inhibitors in FMF.

RESULTS

Sixty one studies or case reports or series concerning 811 patients were identified: 30 case reports or case series with 5 or less patients, 29 case series or open studies with more than 5 patients and 2 randomized studies. Five hundred and seventy one patients (70.4%) originated from the Middle East, 140 (17.2%) from Europe, 99 (12.2%) from international studies or registries and 1 patient from the USA. The retrieved articles are detailed in **Table 1**.

Anakinra was the main IL-1 inhibitor used ($n = 496$, 61.2%), rarely prescribed as an "on-demand" treatment (20/496, 4.0%). Two hundred and thirty-four (28.9%) patients were treated with canakinumab and 81(10.0%) with both IL-1 inhibitors, starting in all patients except 2 with anakinra before switching to canakinumab. The **Supplementary Figure** represents the

TABLE 1 | References of the articles described in the literature review.

Bibliography number	First author	Number of FMF patients	Number of patients treated with Anakinra	Number of patients treated with Canakinumab	Number of patients treated with both	Median age	Description of patients with AA amyloidosis
Chae et al. (12)	Chae	1	1				yes
Vitale et al. (13)	Vitale	32	32				
Vitale et al. (14)	Vitale	6		6			
Belkhir et al. (15)	Belkhir	1	1				yes
Kuijk et al. (16)	Kuijk	1	1				
Gattringer et al. (17)	Gattringer	2	2				
Roldan et al. (18)	Roldan	1	1				
Mitroulis et al. (19)	Mitroulis	1	1				
Calligaris et al. (20)	Calligaris	1	1				
Moser et al. (21)	Moser	1	1				yes
Hennig et al. (22)	Hennig	1	1				yes
Bilginer et al. (23)	Bilginer	1	1				yes
Petropoulou et al. (24)	Petropoulou	1	1				
Meinzer et al. (25)	Meinzer	1		1			
	Meinzer	5	5			12	
	Meinzer	1			1		
Ozen et al. (26)	Ozen	5	5			16	
Alpay et al. (27)	Alpay	1	1				yes
Stankovic Stojanovic et al. (28)	Stankovic	4	4			27	yes
Hacihamdioglu et al. (29)	Hacihamdioglu	1			1		
Estublier et al. (30)	Estublier	1	1				
Soriano et al. (31)	Soriano	1	1				
Ter Haar et al. (32)	Ter Haar	3	3				
Celebi et al. (33)	Celebi	1	1				yes
Mercan et al. (34)	Mercan	2	2				
Brik et al. (35)	Brik	7		7		9,5	
Başaran et al. (36)	Basaran	4	4			17	
	Basaran	4			4	12	
Ugurlu et al. (37)	Ugurlu (poster)	19			19		yes
Gül et al. (38)	Gül	9		9		22	
Cetin et al. (39)	Cetin	12	12			31	yes
	Cetin	8		8		18	yes
Eroglu et al. (40)	Eroglu	5	5			13	yes
	Eroglu	3		3		13	
	Eroglu	6			6	13	
Sevillano et al. (41)	Sevillano	1	1				yes
Alpa et al. (42)	Alpa	1			1		
Rossi-Semerano et al. (43)	Rossi	10	10			21	
	Rossi	1		1		21	
	Rossi	3			3	21	
Özçakar et al. (44)	Ozçakar	3		3		19	
	Ozçakar	10	10			14	yes
Sozeri et al. (45)	Sozeri	1		1			yes
Kucuksahin et al. (46)	Kucuksahin	24	24				
	Kucuksahin	2		2		29	yes
Laskari et al. (47)	Laskari	9		9		23	
	Laskari	5			5	43	
Ben-Zvi et al. (48)	Ben-Zvi	25	25				
Georgin-Lavialle et al. (49)	Georgin-Lavialle	1	1				

(Continued)

TABLE 1 | Continued

Bibliography number	First author	Number of FMF patients	Number of patients treated with Anakinra	Number of patients treated with Canakinumab	Number of patients treated with both	Median age	Description of patients with AA amyloidosis
Abbara et al. (50)	Abbara	1	1				yes
Ozen et al. (51)	Ozen	20	20				
	Ozen	13		13			
Pecher et al. (52)	Pecher	13	13			31	
Barut et al. (53)	Barut	16	4	12			
Trabulus et al. (54)	Trabulus	9		9		33	yes
De Benedetti et al. (55)	De Benedetti	63		63			
Özçakar et al. (56)	Ozçakar	1	1				yes
	Ozçakar	4			4	23	yes
Jesenak et al. (57)	Jesenak	1			1		
Yildirim et al. (58)	Yildirim	3		3		57	yes
Yazilitaş et al. (59)	Yazilitas	11		11		14	yes
Ergezen et al. (60)	Ergezen (poster)	48	48				
Kohler et al. (61)	Köhler	29	29				yes
	Köhler	2			2		yes
Babaoglu et al. (62)	Babaoglu	15	15			34	
Gülez et al. (63)	Gülez	12		12		16, 5	yes
	Gülez	3			3	10	
Varan et al. (64)	Varan	33	33				yes
	Varan	11		11			yes
Varan et al. (65)	Varan	10	10				yes
	Varan	7			7	21	yes
Akar et al. (66)	Akar	133	133				yes
	Akar	19		19			yes
	Akar	18			18		yes
	Akar	2			2		yes
Berdeli et al. (67)	Berdeli	22		22		13, 8	
Hasbal et al. (68)	Hasbal	1	1				yes
Sargin et al. (69)	Sargin	14	14			41	
Eren Akarcan et al. (70)	Eren Arkacan	9		9		14, 3	
Kisla Ekinci et al. (71)	Kisla Ekinci	14	14			11	yes
Sendogan et al. (72)	Sendogan	4			4		yes
	TOTAL	**811**	**496**	**234**	**81**		

prescription of IL-1 inhibitors in FMF since the first description in 2006.

We were unable to identify the patients who had been described more than once in the literature: first in case reports, than in case series and/or in retrospective studies. That is the reason why we were unable to assess bias or outcome assessments.

DISCUSSION

Indications for IL1 Inhibition in FMF Patients

Colchicine Resistance

The main indication for the prescription of IL-1 inhibitors is colchicine resistance. However, the meaning of "colchicine resistance" has evolved over the past decade, and a consensus definition remains elusive. The criteria for insufficient response to colchicine are highly variable in the different studies. In many studies, no specific criteria are given to determine whether a patient is resistant or not; the indication to treat with IL-1 inhibition is often made only on the notion of "frequent attacks" despite colchicine treatment. Other studies give a more precise definition of colchicine resistance, but again the different definitions are highly variable. The highest agreement for colchicine resistance is the persistent elevation of acute phase reactants between the attacks (12, 15–20, 23, 24, 26–29, 33, 34, 36, 38–40, 44, 46, 49, 50, 52, 55, 57–59, 61, 63–65, 73). No consensus exists about attack frequency: some authors define colchicine resistance in patients who experience more than 1

typical inflammatory attack per 3 months (38, 53), while others refer to colchicine resistance if there are more than 2 typical attacks per trimester (46) and still others if the patient has monthly attacks (35, 40, 48, 51, 52, 55, 59, 62–65). None of the studies considers that attack frequency may vary with age and therefore the probable necessity to define this parameter differently in adults and children. Some studies take also into consideration the severity of inflammatory episodes in their definition of colchicine resistance (2, 4, 5, 7–11, 17–20, 27, 28, 34, 35, 38, 40, 44, 48, 49, 52, 53, 55, 57, 58, 73) but rarely give a precise definition for this item. Only very few studies include the notions of quality of life assessment or school or work attendance (4, 7–12, 18, 19, 27, 34, 53, 61, 73) into the definition of colchicine resistance, but without defining what is tolerable as absence from work or school or quality of life.

The maximum dosage of colchicine is usually set at 2 mg/day (27, 35, 38, 46, 54, 55, 66), but may change in the different studies, especially in children where doses vary with age in whom the standard dose is sometimes defined as 1.2 mg/m^2/day (53). The standard and the most accepted minimal dose before considering resistance in the literature is the "maximum tolerated dose" (74).

The last factor of lack of precision is the difficulty to determine incompliant patients since there is no reliable and practical detection method to estimate active colchicine levels. Only one study had a standardized methodology to verify colchicine compliance by counting the remaining tablets (40). However, compliance with colchicine treatment appears to be low overall (10, 75, 76), which explains why the EULAR recommendations note that lack of compliance should be considered in all patients who do not respond adequately to colchicine (54).

Colchicine Intolerance/Toxicity

Therapeutic oral doses of colchicine in patients without hepatic or renal failure have few side effects and are generally well-tolerated. The most common side-effects are gastrointestinal, including diarrhea, vomiting and nausea. Gastrointestinal toxicity is dose dependent and may improve by diminishing lactose intake (77), or lowering the colchicine dose. Rarer acute adverse effects include myopathy, rhabdomyolysis, and myelosuppression (78). A colchicine neuromyopathy may occur with chronic daily use, particularly in patients whose dose has not been appropriately adjusted for renal disease (79–81). Of note fatalities during therapeutic use have been reported only in patients with chronic renal insufficiency taking unadjusted doses of colchicine or when colchicine has been given intravenously, or combined with CYP3A4 inhibitors (82–84). Due to all these side effects, the intolerance or toxicity of colchicine is therefore a possible indication for the treatment with IL-1 inhibitors.

The main reason for starting IL-1 antagonist in the different studies was the poor digestive tolerance of colchicine (12, 27, 46–48, 85), but in the various studies it was not specified whether means to increase digestive tolerance had been implemented before considering IL-1 inhibition. The second reason for IL-1 inhibition was neuromyopathy (15, 25, 31, 42, 47, 51, 58, 62, 68), occurring exclusively in patients with a risk factor such as renal disease or drug interactions. Myelotoxicity (24, 47) and hepatotoxicity (46, 58) was responsible for the initiation of an IL-1 inhibitor only on a very ad hoc basis. From these studies it appears that the intolerance or toxicity of colchicine is only exceptionally responsible for the need to initiate IL-1 blockade. This is particularly true in children who only exceptionally display co-morbidities that may decrease colchicine tolerance.

TABLE 2 | Check list before considering colchicine resistance.

☐	Verify that the diagnosis of FMF is accurate
☐	Verify that reported symptoms are related to inflammation (Check inflammatory markers during symptoms)
☐	Eliminate common causes of fever and pain (infection, leukemia, …)
☐	Question the patient about personal, social or psychological problems that may be triggers for inflammatory attacks - Propose behavioral approaches for stress management
☐	Ensure compliance at full dose for 3–6 months If the maximum dose is not reached, increase gradually the colchicine dosage by 0.5 mg (0.25 mg before the age of 5) every 3 months
☐	Ensure colchicine tolerance by - Dietary modifications (limit lactose intake) - Splitting the total daily dose - Associating antidiarrheal and spasmolytic agents to colchicine
☐	In patients with a sudden deterioration of FMF despite full compliance of colchicine, look for other causes of inflammation: - Inflammatory rheumatism, vasculitis - Mild myeloid hemopathy (in the elderly) - Chronic peritonitis or peritoneal mesothelioma (in the elderly)
☐	Document prospectively the attack recurrence for 3–6 months in order to confirm the number of reported inflammatory episodes

In view of the many aspects regarding colchicine treatment, it seems essential to extensively evaluate all these domains before considering colchicine resistance or intolerance. In **Table 2**, we propose a checklist to help the physician to assess the FMF patient before planning alternative or additional treatment plans to colchicine.

Severe Complications or Associated Co-morbidities

Secondary amyloidosis

Amyloidosis is characterized by deposition of misfolded insoluble proteins in various organs and tissues. It is a life-threatening progressive disease unless underlying causes are treated early before irreversible organ damage occurs. AA Amyloidosis (AAA) is the most severe complication of FMF (86, 87). There is no cure for amyloidosis but it is preventable by suppression of inflammation. Hence, it is crucial to control inflammation in patient with preexisting AAA (88). As such, IL-1 inhibitors are good candidates in AAA FMF patients suffering from persistent inflammatory symptoms despite the regular use of colchicine at maximal dose.

At present, we were able to identify 160 patients who received IL-1 inhibitors for FMF and AA amyloidosis; they were mainly treated with anakinra compared to canakinumab, due to anakinra's short half-life. The exact number of FMF patients treated for AAA is not available because most series include FMF patients with and without AAA, and the details of data specific to patients with AA amyloidosis were not available, even in supplementary data.

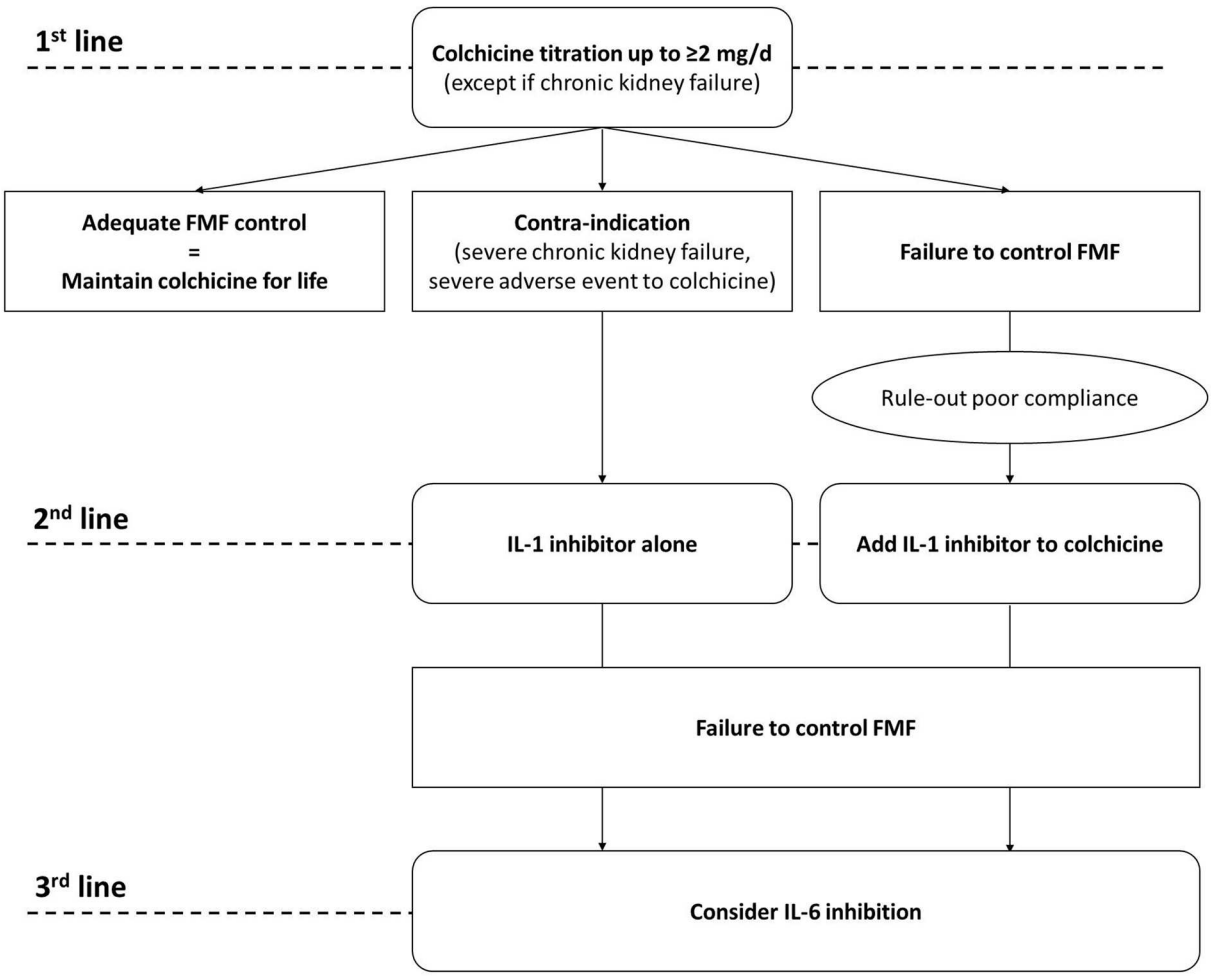

FIGURE 1 | Proposal of a therapeutic algorithm for AA amyloidosis in patients with FMF.

When the data was available ($n = 56$), the foremost reasons for initiating an IL-1 inhibitor were in decreasing order of frequency: colchicine resistance ($n = 24$) (21, 22, 27, 28, 33, 39, 40, 44–46, 54, 56, 58, 72), AA amyloidosis and severe renal failure ($n = 14$) (12, 15, 41, 59, 71, 72), AA amyloidosis onset during the course of FMF ($n = 13$) (15, 23, 28, 31, 50, 72), colchicine intolerance ($n = 7$) (12, 15, 46, 58, 68, 72). Unfortunately, the exact reasons were not always specified in AAA patients from FMF series. Altogether, the main reason for IL-1 inhibitor prescription among patients with AAA secondary to FMF was severe kidney failure hindering the adjustment of the colchicine dosage necessary to normalize inflammatory markers. The second main reason was colchicine resistance defined by the persistence of raised inflammatory markers despite the regular daily intake of the maximum tolerated dose of colchicine, which was not always specified.

Anakinra, was mainly prescribed at 100 mg/day except for patients with end stage renal failure who received 100 mg of anakinra 3 times a week, each dialysis day. Canakinumab was mostly prescribed at 150 mg/month and was not chosen in case of end stage renal failure. Considering the recent prescription of IL-1 inhibitors in AAA secondary to FMF, there is not much hindsight to assess tolerance and efficacy in the long term but in the short term, the tolerance and efficacy seem correct. However, it is important to note that in the absence of renal failure, and if AAA reveals FMF, the treatment of choice remains colchicine (9). In case of AAA in FMF patients, colchicine should be tried first, starting at 1 or 1.5 mg/day and increased gradually in steps of 0.5 mg every 3 months during at least 6 months with a close monitoring of inflammatory markers and proteinuria, before starting IL-1 inhibitors. Indeed, in patients who are virgin of any colchicine treatment, the onset of AAA is first and foremost a sign of colchicine absence.

Figure 1 proposes an algorithm for the decision to initiate an IL-1 inhibitor in FMF patients with AAA.

Associated co-morbidities

Unresponsiveness to colchicine—even if taken properly—may be due to associated inflammatory diseases. Most reports concern FMF associated ankylosing spondylitis with a good response to IL-1 inhibitors (29, 40, 46, 49, 63, 64) even if older studies showed also efficacy with TNF blocking agents (89, 90).

Other inflammatory conditions associated to FMF, such as Behçet's disease (23, 40), inflammatory bowel disease (27, 28, 33, 64), protracted febrile myositis (25, 30, 34, 40, 63), hidradenitis suppurativa (50) and Henloch-Schönlein purpura (25) also responded well to IL-1 inhibitors.

Finally, IL-1 inhibitors were described to be effective among FMF children with failure to thrive (36, 45) or children with an important need of corticosteroids (70); unfortunately the inflammatory status between attacks was not specified in these children.

In the light of these studies, patients with FMF previously well-controlled with colchicine alone, need a careful assessment for inflammatory comorbidities appearing later in the course of the disease. Indeed, the therapeutic implication of distinguishing this subgroup from the "true" colchicine resistance may be major: one can hypothesize that IL-1 inhibitors may be tapered and possibly withdrawn once the associated disease is controlled.

Efficacy

To evaluate the efficacy of IL-1 inhibitors, phase 2 studies (in which the patient is his own control) and placebo-controlled studies provide the best information.

At our knowledge only one randomized, double-blind, placebo-controlled trial with anakinra in FMF patients who were colchicine resistant was published (48). In this study 25 patients were enrolled and randomized (12 in the treatment group and 13 in the placebo group). All the patients received maximum tolerated doses of colchicine during the whole study period. Seven patients discontinued the study, all of whom were from the placebo group, due to treatment failure or to side effects. Complete response was achieved in 7 patients of the treatment group whereas the 5 remaining patients had a partial response. For all the patients of the treatment group, anakinra improved furthermore life quality. In this specific study, anakinra combined to colchicine also decreased he number of joints attacks. These findings may support a role for dual therapy with colchicine especially in patients with FMF articular complications.

For canakinumab a first open-label pilot trial was conducted in 7 children who experienced at least 1 investigator-confirmed FMF attack per month (35). The median 28-day time-adjusted attack rate decreased from 2.7 to 0.3 in this study and the proportion of days that participants were experiencing an attack decreased from 24.2 to 3.6%. Furthermore, serum acute phase reactants levels normalized during the treatment period and health-related quality of life improved in all patients.

A second small open-label pilot trial with canakinumab in patients with monthly attacks, showed that monthly injections prevented attacks in 8/9 patients and reduced the frequency of attacks in the remaining patient (38). Furthermore, serum acute phase reactants levels (C-reactive protein and serum amyloid A protein) remained low throughout the treatment period in all the patients. Significant improvement was also observed in both physical and mental component scores.

These preliminary results in colchicine resistant FMF patients could be confirmed in a placebo-controlled phase 3 study with an injection every 4 weeks, in which 61% of 31 patients treated with canakinumab (150 mg or 2 mg/kg in children) vs. 6% of 32 patients in the placebo group had a complete response and did not experience any flare of the disease for 40 weeks while treated (55). This proportion increased to 71% of patients if the blinded dose in non-complete responders was increased to 300 mg (or 4 mg/kg in children) every 4 weeks. A dosing interval of every 8 weeks was enough to maintain complete disease control in 46% of patients with colchicine resistant FMF. In patients who did not have a complete control of the disease, the mean attack frequency decreased from more than 30 to <2 per year and remaining attacks seemed less severe.

Overall these studies provided evidence of the pathogenic role of IL-1 in colchicine-resistant familial Mediterranean fever. They also showed that IL-1 inhibition is an efficacious option for controlling and preventing flares –at least at the short term- in these patients.

Anakinra vs. Canakinumab

Although canakinumab is the only drug approved in Europe for the treatment of colchicine resistant FMF, in literature, experience with anakinra is also significant. Up to now, there are no comparative effectiveness assessment studies for canakinumab vs. anakinra. The preference of one treatment over the other can therefore be based only on indirect data. Significant reasons to prescribe anakinra rather than canakinumab are the price and/or reimbursement conditions of the drugs, explaining probably partially why the experience in the literature with anakinra is more substantial than with canakinumab. By analyzing studies in which patients have switched from one treatment to another, it seems that the main reason for switching is the ease of administration of canakinumab and/or the loss of compliance to anakinra after longer periods of use (36, 43, 56, 61, 64, 66, 72). A second reason for switching from anakinra to canakinumab is the occurrence of injection site reactions or other side effects with anakinra (such as urticaria or the rise of liver enzymes) which seem less prominent when taking canakinumab (40, 42, 43).

Interestingly the loss of efficacy of the first line IL-1 inhibitor can also be a reason for switching from one IL-1 inhibitor to the other. An inadequate or partial response has principally be described with anakinra (mainly after an initial good response and a secondary recurrence of symptoms), followed by a better response with canakinumab (29, 36, 40, 43, 47, 57, 64, 66, 72). However, in none of these reports the adherence to daily injections has been assessed. Moreover, that the worsening of the clinical picture with IL-1 antagonist could be secondary to other potential non-FMF conditions was only exceptionally discussed. An inadequate response with canakinumab has also been described in 2 reports (36, 66); both reports relate that canakinumab treatment was changed to anakinra for clinical and/or laboratory worsening, with a good response. These observations raise the question of whether the efficacy of IL-1 inhibitors is sustainable over the long term.

At present and in the absence of comparative studies both treatments seem to be an equal option for the management of colchicine resistant or intolerant FMF patients.

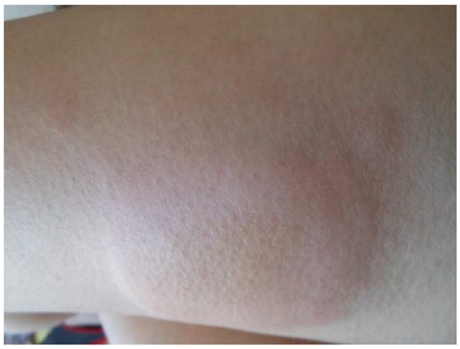

FIGURE 2 | Example of an injection site reaction with anakinra.

Maintenance Therapy vs. on Demand Treatment

The particularity of FMF is that the disease evolves by flare-ups. Usually, the only treatment offered are NSAIDs (naproxen, diclofenac, indomethacin, etc) that may alleviate symptoms during attacks but which are rarely completely effective (91). It therefore seems quite logical to offer intermittent treatment with IL-1 inhibitors to patients who continue to have attacks despite proper colchicine treatment. Paradoxically, this attitude has been little investigated. We identified simply 3 publications, concerning 20 patients treated with anakinra only during flares of the disease (19, 43, 92). The most interesting data is described in a retrospective study of Babaoglu and co-authors who investigated retrospectively The Gazi FMF cohort (92). The cohort is made up of 689 FMF patients of whom 78 patients were treated with IL-1 inhibitors among those 15 were treated with an on-demand anakinra protocol. Patient reporting attack severity, duration, frequency, absenteeism were significantly improved when receiving an on demand treatment with anakinra. Furthermore, prophylactic on-demand use of anakinra in patients with prominent triggers seemed also successful. All the patients continued the maximum tolerated dosage of colchicine and none had persistent inflammation before starting the on-demand protocol. The authors conclude that the use of anakinra during the prodromal period would be a reasonable approach for halting or alleviating symptoms of an impending attack allowing patients to diminish the loss of workdays and to improve the quality of life. Another advantage of this approach would be reducing cost and adverse effects of continued use of IL-1 inhibitors in selected patients with marked prodromes or triggers and low risk of amyloidosis.

Safety

In the different studies, the safety of LL-1 inhibition seemed generally good, at least at the short term. Only one case series reported a death following a treatment with canakinumab (59). The patient had end stage renal disease and severe multiorgan amyloidosis and died due to sepsis and peritonitis 1 year after cessation of IL-1 inhibition treatment. One opportunistic infection (fungal pneumonia) was reported in one patient receiving canakinumab (66). No malignancies were reported in any of the studies or case series.

The main reported side effect were local injection site reactions (17, 20, 25, 36, 40, 42, 43, 46, 48, 55, 57, 66, 69). Nevertheless, this side effect seems far more frequent in patients treated with anakinra than with canakinumab. **Figure 2** shows an illustrative example of such a local site reaction. Up to now severe (43, 60, 66) or mild anaphylactic reactions (16, 40) were described only with anakinra.

The second most reported side effects were infectious complications, in a probably equivalent manner regardless of the type of IL-1 inhibitor used. The infections concerned chiefly the upper and lower respiratory tract (28, 35, 38, 48, 55) and were sometimes considered to be severe (12, 17, 39, 40, 56, 59, 66). Cutaneous infections (55, 63, 66) or viral infections of the herpes simplex group (54, 66) were also reported.

Anakinra seems to be more often responsible for cases of leucopenia (28, 37, 40, 62, 66), whereas headache (considered occasionally to be severe) seems to be observed more frequently with canakinumab (25, 38, 48, 55, 57).

Overall the safety profile of IL-1 inhibitors seems not different in FMF patients than in the other diseases including cryopyrin associated periodic syndrome, rheumatoid arthritis, adult-onset Still's disease and systemic-onset juvenile idiopathic arthritis. However, longer studies on FMF and post-marketing real-life experience are needed to verify the persistence of the relatively good IL-1 inhibitor tolerance on the long term.

CONCLUSION

The results from the present review suggest that IL-1 inhibitors are good candidates for colchicine resistant and/or intolerant FMF patients. IL-1 inhibitors have the potential to improve patient outcome even in patients with co-morbidities or severe complications in whom inflammation control is difficult to achieve with colchicine alone. Nevertheless, current data are limited and further evaluation of long-term efficacy and safety of IL-1 inhibitors are necessary, in order to provide robust evidence in this domain.

AUTHOR CONTRIBUTIONS

VH conceived and wrote the review. CV and AF performed the literature search. VH and SG-L approved and validated the selected articles and checked the data assessment. All authors agreed to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00971/full#supplementary-material

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REVIEW
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Management of Mevalonate Kinase Deficiency: A Pediatric Perspective

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Background: Mevalonate kinase deficiency (MKD) is an inborn error of metabolism leading to a syndrome characterized by recurrent inflammation. This clinically manifests itself as fever and can be accompanied by gastrointestinal symptoms, oral ulcers, cervical lymphadenopathy, and skin rash.

Methods: We searched Pubmed, Embase, Cochrane, and CINAHL for relevant articles. All articles were screened by both authors. Relevant articles were included in this review.

Results: The interleukin-1 antagonist canakinumab is the only well-studied and effective treatment for MKD patients with 35% of patients reaching complete remission in a large randomized controlled trial. Other therapeutic options include glucocorticoids and the IL-1 antagonist anakinra, although the level of evidence for these treatments is weaker. If patients fail to these treatments, the biologicals etanercept or tocilizumab can be used. Mildly affected patients might benefit from cheaper, less invasive treatments such as paracetamol and NSAIDs.

Conclusion: Canakinumab is the only evidence-based treatment for mevalonate kinase deficiency. However, the costs limit availability for many patients. Cheaper and more readily available options include glucocorticoids, anakinra, etanercept, and tocilizumab, although there is limited evidence supporting these treatments.

Keywords: mevalonate, autoinflammatory, hyperimmunoglobulinemia D syndrome, Interleukin-1, canakinumab, stem cell transplantation

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INTRODUCTION

Mevalonate kinase deficiency is an autosomal recessive inborn error of metabolism. Bi-allelic mutations in the gene mevalonate kinase (*MVK*) lead to a reduced activity of the mevalonate kinase enzyme. This enzyme catalyzes the conversion of mevalonic acid to phosphomevalonic acid, an early step in isoprenoid biosynthesis. The defect leads to accumulation of its substrate, mevalonic acid, as well as to a shortage of isoprenoid end-products. Ultimately, these changes give rise to a syndrome, characterized by severe, more or less spontaneous recurrent inflammation. Patients suffer from episodic high-grade fever, that may be accompanied by oral ulcers, cervical lymphadenopathy, nausea, vomiting, diarrhea, skin rashes or arthritis. Complications include macrophage activation syndrome and ultimately AA-amyloidosis. Severely affected individuals may present with central nervous system damage or retinal degeneration, the pathogenesis of which is poorly understood. The spectrum of severity in mevalonate kinase deficiency is wide. The extremely rare, most severe phenotype is known as mevalonic aciduria (MA), whereas the more common, purely inflammatory phenotype had been known as the hyperimmunoglobulinemia-D

periodic fever syndrome (HIDS) due to the elevated levels of serum immunoglobulin D in some of these patients (1). Understanding of the chain of events leading from the metabolic defect to the inflammatory phenotype is patchy. It is very likely that a shortage of certain isoprenoids, notably geranylgeranyl pyrophosphate leads to excessive release of interleukin-1β (IL-1β) (2). This appears to be mediated, at least in part, by inactivation of the small GTPase RhoA, and the subsequent activation of the Mediterranean fever (*MEFV*) gene product, pyrin (3). Pyrin, in turn, forms an inflammasome resulting in caspase-1 mediated IL-1β release. Since *MEFV* expression is limited to phagocytes, these cells are believed to be the source of inflammatory cytokines in mevalonate kinase deficiency. IL-1β exerts its pro-inflammatory effects through binding to the IL-1 receptor (2). All steps in this process are, at least in theory, amenable to therapeutic intervention. In addition to shortage of non-sterol isoprenoids, other potential mechanisms have been proposed, such as excess mevalonic acid, reduced 25-hydroxycholesterol and there are indications that cytokines other than IL-1β, such as gamma-interferon, TNF-α and IL-6 play an important role (4). In this paper we will address possible approaches and discuss the evidence for those that have been studied in clinical practice.

METHODS

We searched PubMed, EmBase, Cochrane and CINAHL for English language articles using the following search strategy: "mevalonate kinase deficiency" OR "hyperimmunoglobulinaemia d" OR "hyper igd" OR hids OR mvk OR "mevalonic aciduria") AND (predniso* OR canakinumab OR anakinra OR therapy OR treatment OR rilonacept OR glucocorticoid* OR non-steroidal OR acetaminophen OR paracetamol OR colchicine OR simvastatin OR tocilizumab OR etanercept OR adalimumab OR infliximab. All articles were screened on title and abstract by both authors. After screening of title and abstract remaining relevant articles were screened on full-text. In addition, we reviewed the references of the retrieved papers for any missing sources. We did not exclude preclinical studies in order to address potential therapeutics avenues that have not (yet) reached clinical practice. In the analysis we rated studies according to the level of evidence provided (5).

RESULTS

The search on Pubmed, Embase and Cochrane yielded 379 articles. After title and abstract screening 37 articles remained for full-text screening. Twelve articles were included for this review (**Figure 1**). We will discuss the findings along the pathophysiological chain from the genetic defect to the clinical symptoms.

Gene Therapy

In theory, mevalonate kinase deficiency could be cured by repairing or replacing one of the mutant alleles in affected tissues. This would recapitulate the situation in heterozygous carriers, who are known to be clinically unaffected. However, to date, there have been no studies reporting such an approach, whether clinical or pre-clinical.

Enzyme Replacement

Mevalonate kinase is a cytoplasmic enzyme. In contrast to lysosomal enzymes, exogenous replacement cannot restore the protein in its natural compartment. If excess mevalonate were responsible for the clinical features of the disorder, this would not matter very much. However, it is more likely that shortage of isoprenoid end products is to blame. No studies on enzyme replacement have been performed.

Replacing the Affected Cells

Mevalonate kinase is a ubiquitous enzyme. However, the inflammatory phenotype most likely results from the expression of this defect in phagocytes. Therefore, replacing mutant myeloid cells by genetically normal ones by allogeneic stem cell transplantation, might be curative. No controlled studies have been published on this approach. Yet, several case reports have been published, some with encouraging results.

The first report described a 3-year old with mevalonic aciduria not responding to treatment with anakinra and etanercept. Allogeneic bone-marrow transplantation from an HLA-identical sister was performed. Follow-up after 15 months showed complete remission with normal inflammatory parameters, although the patients still had mild neurological manifestations (persistent atactic gait) (6). An report from the UK has described the same approach in an 8-year old boy with MA. This patient experienced severe graft-vs.-host disease after cessation of cyclosporine (due to inadequate chimerism) and suffered from several viral infections. All these problems resolved and at follow-up after 1 year the patient was in complete remission regarding febrile episodes (7). However, not all patients benefited from stem cell transplantation. A Turkish case-report described a boy with severe mevalonic aciduria leading to ascites and respiratory distress. After treatment with glucocorticoids and canakinumab had failed, an allogeneic bone marrow transplantation from an HLA-identical sister was performed at 138 days after birth. Although ascites regressed, the patient died due to septicemia 3.5 months after transplantation (8).

Altering Isoprenoid Biochemistry

One small, randomized placebo controlled double blind cross-over trial with six patients studied reduction of the accumulating substrate mevalonate by means of oral statin treatment (9). There was a modest, but significant improvement in the treatment group with 5 of 6 patients reporting improvement while using simvastatin (level of evidence 1B). However, the largest retrospective series to date did not confirm these promising results, with statins failing in 11 of 15 patients. Three of these patients even mentioned worsening of their disease, while only four patients reported some benefit (level of evidence 3) (1).

Increasing geranyl-geranyl-pyrophosphate levels is a logical approach, which might be achieved by exogenous supplementation of geranyl-geranyl-pyrophosphate itself or its precursors or by skewing the metabolic flow in the mevalonate pathway toward geranyl-geranyl-pyrophosphate by blocking

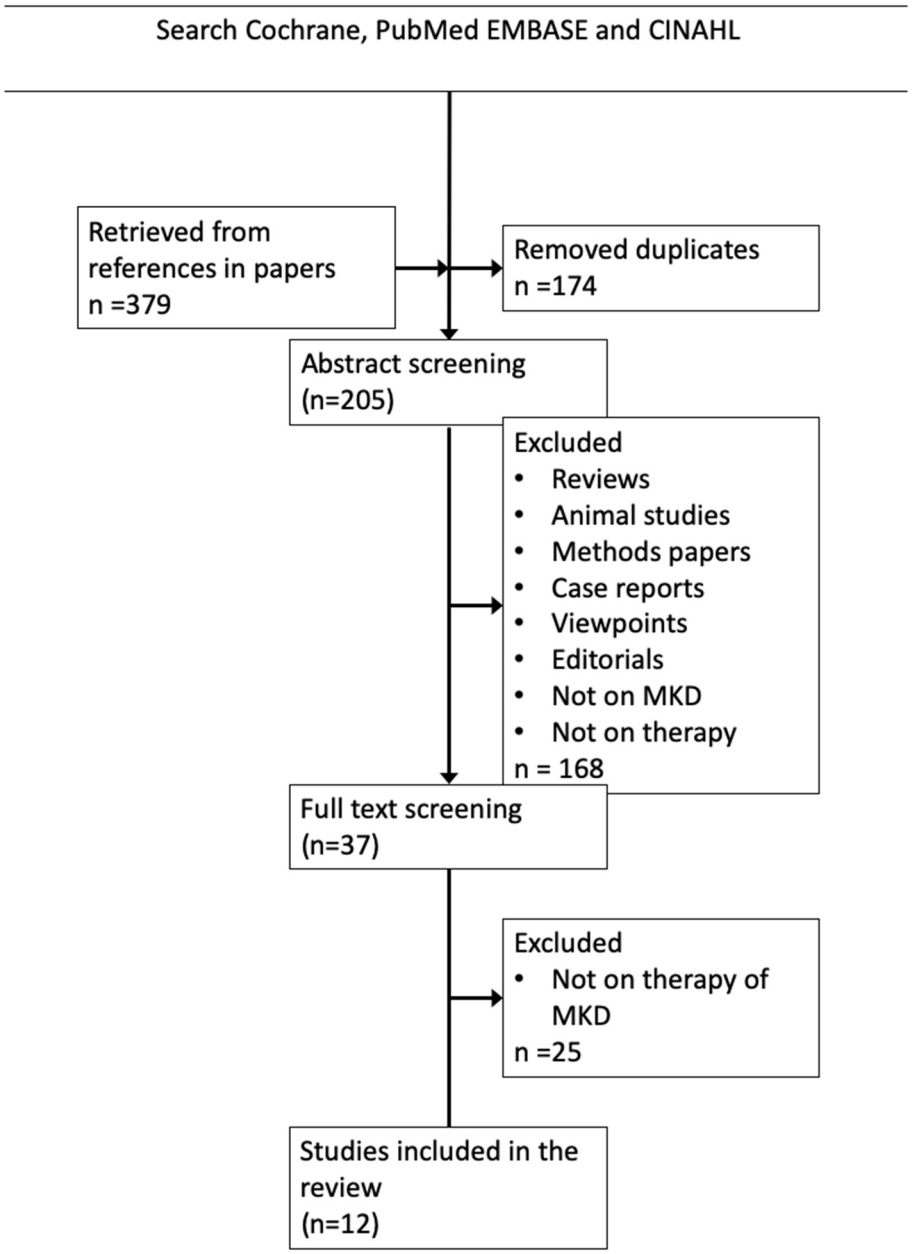

FIGURE 1 | Literature search strategy and results. The Boolean search string in PubMed, Cochrane EMBASE and CINAHL was (((((((“mevalonic aciduria”) OR MVK) OR HIDS) OR “Hyperimmunoglobulinaemia D syndrome”) OR “Hyperimmunoglobulinemia D syndrome”) OR “mevalonate kinase deficiency”)) AND ((((((((((((acetaminophen) OR paracetamol) OR non-steroidal) OR simvastatin) OR glucocorticoid) OR predniso*) OR rilonacept) OR colchicine) OR canakinumab) OR anakinra) OR therapy)) OR treatment).

the enzyme squalene synthase. Both approaches have been investigated, but only in preclinical studies.

Inhibiting the Pyrin Inflammasome

The pyrin inflammasome is central to the pathogenesis of another autoinflammatory disease, familial Mediterranean fever (FMF). Colchicine, which is an effective drug in FMF has not been formally studied in mevalonate kinase deficiency. Early reports noted its lack of efficacy (10). Retrospective data from the Eurofever registry indicate that colchicine may provide limited benefit in a minority of patients, but the evidence is weak (level of evidence 3) (1). Other inflammasomes, such as that containing NLRP3, might be involved in which case there would be a role for specific NLRP3-inhibtors. One pre-clinical study has investigated the link between the NLRP3 inflammasome and MKD in PBMC’s from a mildly affected MKD patient. Stimulation of these cells with the NLRP3 specific stimulant nigericin led to much greater IL-1β production compared to PBMC’s of either parent.

When blocking the NLRP3 inflammasome by MCC950, this response was abolished. This suggests that blocking the NLRP3 inflammasome, might hold potential for the treatment of MKD (11). Four other NLRP3 inhibitors have been recently identified to be pharmacological inhibitors of the NLRP3 inflammasome. These inhibitors, CY-09, OLT1177, Tranilast, and Ordinonin have not been studied in a MKD or a model thereof. Two of these inhibitors (OLT1177 and Tranilast) appear to be relatively safe in humans who were treated for other conditions. However, further studies are needed to investigate the safety and therapeutic potential for MKD (12).

Blocking Interleukin-1β

Since IL-1β plays an important role in the inflammatory phenotype of MKD, the IL-1β blocking agents anakinra and canakinumab have been employed in a substantial number of patients with success. The IL-1 antagonist rilonacept has not been described as a treatment for MKD.

A prospective, observational study including 11 MKD patients described the usage of anakinra (13). Patients with a more severe phenotype were assigned to continuous treatment, whereas patients with a milder phenotype were free to choose between continuous or on-demand treatment (children <16 years received 1.6 mg/kg/day, to be raised to a maximum of 2 mg/kg/day; adults received 100 mg/day, to be raised to a maximum of 200 mg/day in case of failure). This study showed that treatment was accompanied by shortening of fever attacks, lower CRP levels and reduction of symptoms, but did not lead to reduced frequency of fever episodes. This treatment appeared most effective when started within 24 h after the start of a fever episode. A disadvantage reported by patients using continuous treatment is the need of daily painful injections. Adverse events included local injection site reactions and upper respiratory tract infections ($n = 2$) (level of evidence 2B). Deshayes et al. described 10 adult patients receiving anakinra continuously (100 mg/day). Twenty percent failed to this treatment, while 50% responded partially. Another 30% had a complete response to anakinra. Though, two of the three patients with a complete response showed a loss of efficacy within 6 months after start of the treatment. Further, 60% suffered from injection site reactions, while 20% experienced respiratory infections (level of evidence 3) (14). The retrospective Eurofever registry reported continuous treatment with anakinra in 19 patients. Thirteen patients responded partially, while three patients had a complete response. Another three patients failed to this treatment. Eight patients used anakinra during attacks only, with three patients having a complete response and five a partial response (level of evidence 3) (1). Due to the retrospective design of this study, information about the dose was lacking. Failure to treatment might have been caused by inadequate dosing of anakinra.

Although anakinra has been beneficial for many patients, the long-term acting IL-1 antagonist canakinumab has been far better studied. In a large, randomized controlled trial (The CLUSTER trial) De Benedetti et al. investigated the efficacy of canakinumab in periodic fever patients, including 72 MKD patients (15). The included patients were ≥2 years old and had ≥6 attacks per year. This study showed that treatment with canakinumab, 150 mg (or 2 mg/kg in children <40 kg) every 4 weeks, leads to complete remission in 35% of patients after 16 weeks, compared to 6% in patients receiving placebo. Dose increase to 300 mg (or 4 mg/kg in children <40 kg) every 4 weeks further increased the effect to 57%. In a minority of patients, the dose could be subsequently reduced to 150 mg (or 2 mg/kg in children <40 kg) every 8 weeks. Patients who did not achieve complete remission still had a significant reduction in attack frequency. Patients receiving canakinumab also scored better on a physician's global assessment score compared to placebo and had a significant reduction of C-reactive protein levels. During this trial, there were no reported deaths or opportunistic infections such as tuberculosis. Seven serious adverse events were reported while using canakinumab (three cases of pneumonia and one case of pharyngitis, laryngitis, gastroenteritis and conjunctivitis) (level of evidence 1B). Another study described seven patients being treated with canakinumab. Four of them had a partial response, while three had a complete response. Two patients who responded partially had failed to respond to anakinra earlier (level of evidence 3). This long acting treatment only requires monthly injections, which is a major advantage for patients compared to daily injections when using anakinra. However, the cost of this drug (≥$70,000/year) preclude its use by many patients. In comparison, anakinra costs ∼$35–40 for a single dose of 150 mg (annual costs depend on continuous or on-demand use, but in Western countries canakinumab is ∼7–10 times more expensive).

Blocking Other Cytokines

Since TNF-α and IL-6 are thought to contribute to the pathophysiology of MKD, antagonists of these cytokines have been used to treat patients.

TNF blockade with etanercept has been beneficial in a number of patients. Etanercept has been described in a retrospective case series in 27 patients, with 16 patients reporting some benefit and 11 patients not responding (16). Two of these 16 patients reached complete remission (level of evidence 3). Another retrospective study described 8 patients assigned to etanercept, with 7 of them achieving complete remission on this treatment (level of evidence 3) (1). However, another retrospective study reported failure of etanercept in 5 out of 9 patients (level of evidence 3). Given the superior evidence for IL-1 blockade, etanercept is nowadays not a first-choice option, but mostly used to treat patients who have no access to or no response on IL-1 blockade (level of evidence 3).

Tocilizumab is not widely used to treat patients with MKD. However, it has been used successfully in several patients who did not respond to treatment with other biologicals or glucocorticoids. Treatment with tocilizumab has been described in a case of a 13-year old girl after failing to anakinra and etanercept. On this treatment her disease went in remission, although infections had been an initial concern (level of evidence 4) (17). Another case report has mentioned a 32-year old woman failing treatment with simvastatin, NSAIDs and anakinra leading to hospitalization 11 times in 1 year (18). Treatment with tocilizumab was started along with methylprednisolone, since treatment with tocilizumab was experimental. This treatment was followed by remission, which enabled tapering

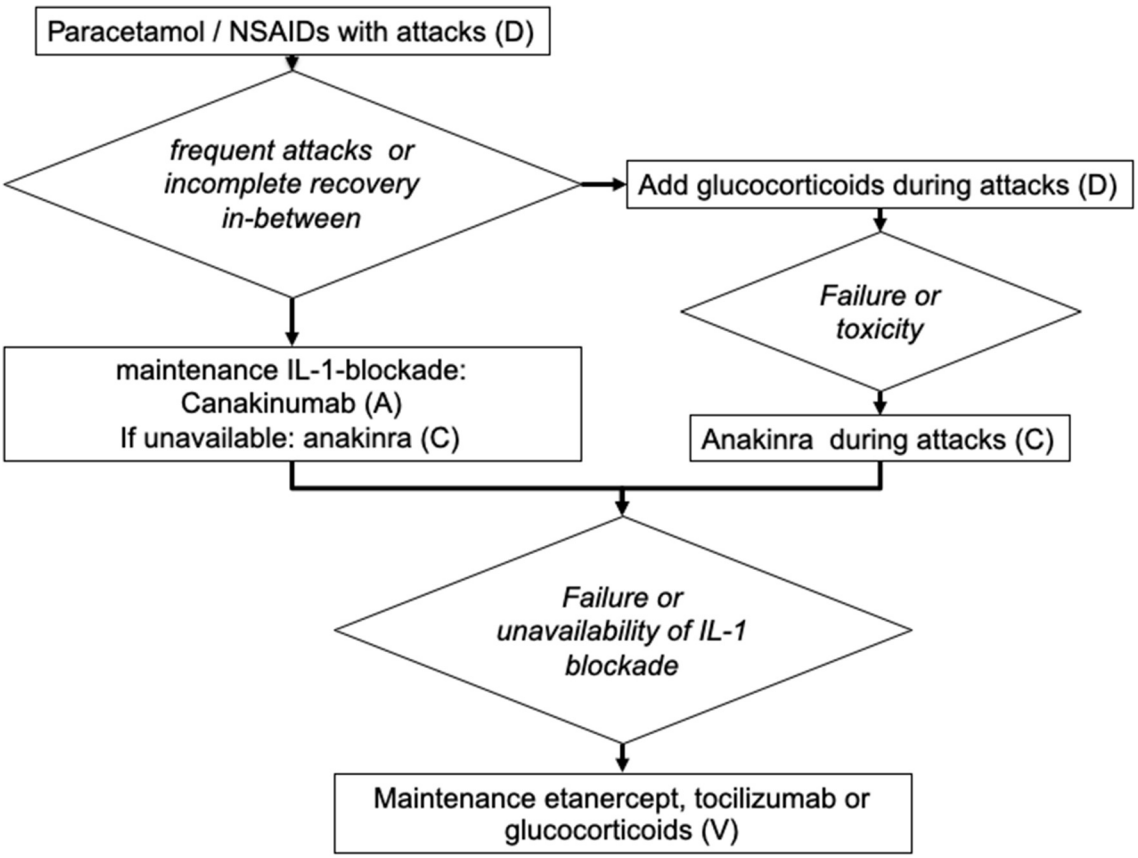

FIGURE 2 | Suggested algorithm for the treatment of patients with mevalonate kinase deficiency (adapted for the treatment of children). The capital letters (A–D) indicate the strength of recommendation (5).

of methylprednisolone. Further, a retrospective study described two MKD patients with a complete response to tocilizumab. Prior to this treatment they failed to anakinra and/or etanercept. One of this patients underwent kidney transplantation due to AA-amyloidosis. After this transplantation he developed EBS septicemia, which led to halving of the tocilizumab dose until the septicemia resolved (19).

Non-specific Inhibition of Inflammation

Treatment with paracetamol and non-steroidal anti-inflammatory drugs (NSAIDs) can relieve symptoms during inflammatory attacks, but does not lead to remission. Although evidence for this approach is lacking, symptomatic treatment of disease flares this way is standard practice and may be sufficient to control mild infrequently relapsing disease. Glucocorticoids also reduce symptoms in MKD patients, especially when given in high dose at the start of an attack (20).

Other Therapies

In severely affected patients with ongoing inflammation, the disease can lead to AA-amyloidosis and ultimately kidney failure. The Eurofever cohort reported five patients with AA-amyloidosis (in a cohort of 114 patients). Four of these patients underwent kidney transplantation due to end-stage kidney failure, while one patient died due to the complications of dialysis (1). Therapy with biologicals can be continued in the work-up period toward kidney transplantation when patients are undergoing hemodialysis.

RECOMMENDATIONS

Given the dearth of evidence, any recommendation will ultimately be based on expert opinion and can be of limited strength only. The recommendations below are given by pediatricians.

Symptomatic relief during attacks, though never formally studied, is provided by non-steroidal anti-inflammatory agents and paracetamol (evidence level 5, strength of recommendation D). The low costs of these drugs enable treatment for a large group of mildly affected patients with a limited number of fever attacks.

The only truly evidence-based therapy in mevalonate kinase deficiency is IL-1 blockade with canakinumab (evidence level 1b). It is the treatment of choice in patients with frequent disease flares (strength of recommendation A). However, canakinumab is very costly and hence not available to many patients. Moreover, the study design had excluded young (<2 year old) patients

and those with infrequent disease flares limiting the evidence to older and more severely affected individuals. Anakinra (evidence level 3) is a rational alternative as it is less expensive, but many patients don't have access to it either or do not tolerate the daily painful injections (strength of recommendation C). However, pain at the injection site can be reduced by local application of ice packs before or by hydrocortisone cream after injection. In patients in whom IL1 antagonists are not tolerated or ineffective, maintenance therapy with tocilizumab or etanercept may be attempted (evidence level 4, strength of recommendation D). In patients with uncontrollable diseases who have no access to or do not respond to biologicals, empirical treatment with high dose glucocorticoids may be attempted.

Continuous cytokine blockade is not warranted in patients with infrequent attacks who recover fully in-between. In such patients we advise treatment on demand to abort attacks. The level of evidence supporting any of these strategies is low (strength of recommendation D).

We have summarized these recommendations in a tentative flow diagram (**Figure 2**).

None of these approaches addresses the basic molecular defect. Future avenues might do so. These include gene therapy and supplementing of deficient isoprenoid metabolites. They hold promise, however uncertain, of respectively, cure or disease control by oral medication.

CONCLUSIONS

There is strong evidence for the effectiveness of canakinumab in mevalonate kinase deficiency. The design of the CLUSTER study excluded the most severely affected patients (those with intractable disease before the age of 2 years) and mildly affected patients. The latter may benefit from cheaper, less invasive measures, such as NSAIDS or glucocorticoids. When treatment with canakinumab is unavailable, anakinra is a rational alternative. When IL-1 blockade fails or is unavailable, treatment with other biologicals, such as etanercept or tocilizumab, can prove to be successful. Only in the severest of cases should allogeneic stem cell transplantation be considered.

AUTHOR CONTRIBUTIONS

The search strategy and draft of the article were done by JJ and JF.

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Gene–Dose Effect of *MEFV* Gain-of-Function Mutations Determines *ex vivo* Neutrophil Activation in Familial Mediterranean Fever

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Familial Mediterranean fever (FMF) is caused by mutations within the Mediterranean fever (*MEFV*) gene. Disease severity depends on genotype and gene dose with most serious clinical courses observed in patients with M694V homozygosity. Neutrophils are thought to play an important role in the initiation and perpetuation of inflammatory processes in FMF, but little is known about the specific characteristics of these cells in FMF patients. To further characterize neutrophilic inflammatory responses in FMF and to delineate gene–dose effects on a cellular level, we analyzed cytokine production and activation levels of isolated neutrophils derived from patients and subjects with distinct *MEFV* genotypes, as well as healthy and disease controls. Serum levels of interleukin-18 (IL-18) (median 11,485 pg/ml), S100A12 (median 9,726 ng/ml), and caspase-1 (median 394 pg/ml) were significantly increased in patients with homozygous M694V mutations. Spontaneous release of S100A12, caspase-1, proteinase 3, and myeloperoxidase (MPO) was restricted to *ex vivo* cultured neutrophils derived from patients with two pathogenic *MEFV* mutations. IL-18 secretion was highest in patients with two mutations but also increased in neutrophils from healthy heterozygous MEFV mutation carriers, exhibiting an *ex vivo* gene–dose effect, which was formerly described by us in patients' serum. CD62L (l-selectin) was spontaneously shed from the surface of *ex vivo* cultured neutrophils [median of geometric mean fluorescence intensity (gMFI) after 5 h: 28.8% of the initial level]. While neutrophils derived from healthy heterozygous mutation carriers again showed a gene–dose effect (median gMFI: 67.1%), healthy and disease controls had significant lower shedding rates (median gMFI: 83.6 and 82.9%, respectively).

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Co-culture with colchicine and/or stimulation with adenosine triphosphate (ATP) and lipopolysaccharide (LPS) led to a significant increase in receptor shedding. Neutrophils were not prevented from spontaneous shedding by blocking IL-1 or the NLRP3 inflammasome. In summary, the data demonstrate that *ex vivo* cultured neutrophils derived from FMF patients display a unique phenotype with spontaneous release of high amounts of IL-18, S100A12, MPO, caspase-1, and proteinase 3 and spontaneous activation as demonstrated by the loss of CD62L. Neutrophilic activation seems to be independent from IL-1 activation and displays a gene–dose effect that may be responsible for genotype-dependent phenotypes.

Keywords: autoinflammation, familial mediterranean fever, Neutrophil, S100A12, IL-18

INTRODUCTION

The prototypic autoinflammatory disease familial Mediterranean fever (FMF) is caused by pyrin-encoding *MEFV* (Mediterranean fever) gene mutations (1, 2). Recurrent self-limiting acute flares of inflammatory disease with involvement of serosal membranes and fever are key characteristics of FMF (3). Within FMF, there is wide clinical and genetic heterogeneity, but the most common mutation Met694Val (M694V) is associated with the most severe clinical phenotype in a homozygous state. FMF patients homozygous for M694V present with more joint and skin involvement, higher acute phase reactants during a clinically inactive disease, a higher rate of secondary amyloidosis, and a higher colchicine dose requirement compared to patients with other genotypes (4–6). Neutrophils are the main tissue-infiltrating cells during FMF attacks and therefore the most likely responsible for a large proportion of the observed inflammatory symptoms (7). RNA analysis of isolated short-time cultured neutrophils from patients with FMF revealed an altered spontaneous gene expression profile, for example, caspase-1, c-FOS, TLR2, and MMP9, when compared to control neutrophils (8).

Self-activation of the pyrin inflammasome and subsequent enhanced maturation of interleukin-1β (IL-1β) is of central importance in the pathophysiology of FMF (9). Elevated IL-1β secretion has been described from monocytes and neutrophils derived from FMF patients and has been identified as the main cytokine driving disease pathology in an FMF mouse model (9–11). During inflammatory attacks, neutrophils from FMF patients release neutrophil extracellular traps (NET) containing IL-1β (12, 13). Nevertheless, measuring IL-1β levels in serum from patients is hardly possible (14), and a constitutive pyrin inflammasome activation in patient macrophages *ex vivo* has not been described (15).

In a previous study, we reported unstimulated neutrophils from homozygous M694V patients to spontaneously release higher levels of the IL-1 family cytokine IL-18, caspase-1, and myeloid cell-derived S100A12 compared to neutrophils from healthy controls (HCs) *in vitro* (11). In addition, highly elevated serum levels of these proteins can be detected in the serum of FMF patients and were shown to differentiate clinical status and genotype (11).

These results raised the hypothesis that neutrophils carrying *MEFV* mutations do exhibit a highly characteristic activation status. In order to further decipher the neutrophilic inflammatory response in FMF and to delineate the gene–dose effect of *MEFV* mutations, we analyzed the spontaneous and induced cytokine secretion by neutrophils derived from patients and controls. Furthermore, the activation state of neutrophils was determined by measuring the density of surface molecules. With these analyses, we addressed the following objectives: (i) the spontaneous marker release and change of surface marker expression are restricted to neutrophils derived from patients with FMF, (ii) the amount of spontaneous neutrophilic activation depends on a gene–dose effect, and (iii) the spontaneous release of inflammatory markers is restricted to a specific set of proteins. For these reasons, we included the analysis of neutrophils derived from patients with other chronic active inflammatory disorders, for example, Crohn's disease, rheumatic diseases, cystic fibrosis, autoinflammatory diseases, and immunodeficiencies with chronic inflammation, as well as acute infections.

PATIENTS AND METHODS

Patients and Control Groups

HCs ($n = 9$, mean age 42 years), healthy heterozygous *MEFV* carriers ($n = 6$, mean age 45 years), and patients with FMF and two pathogenic mutations ($n = 12$, mean age 19 years) or other diverse inflammatory diseases [infections $n = 6$, cystic fibrosis $n = 5$, Crohn's disease $n = 4$, rheumatic diseases $n = 4$, tumor necrosis factor receptor-associated periodic syndrome (TRAPS) $n = 2$, and immunodeficiencies with chronic inflammation $n = 2$, mean age of all 41 years] were recruited at the Children's Hospital and the Clinic for Pneumology and Infectious Diseases (both Charité Berlin) as well as the Immanuel Hospital Berlin Buch. For patients' characteristics, see **Table 1**, **Table S1**. Clinical status was assessed by a standardized questionnaire. In patients with chronic inflammatory diseases other than cystic fibrosis, infections within the last 2 weeks prior to blood sampling were excluded. In Crohn's disease, disease activity was assessed by use of the Harvey–Bradshaw index, which captures general well-being, abdominal pain, number of liquid stools per day, abdominal mass, and complications (mild 5–7, moderate 8–16, and severe >16) (17). In patients with cystic fibrosis, severity of

TABLE 1 | Characteristics of the patients in the core study cohort.

	Controls	Asymptomatic heterozygous M694V carriers	Patients with two mutations within *MEFV* other than M694V homozygosity	Homozygous M694V FMF patients	Other inflammatory diseases (total)	Infections	Cystic fibrosis	Crohn's disease	Rheumatic diseases	TRAPS	Immunodeficiencies
Patients, no	9	6	7	5	23	6	5	4	4	2	2
No. male/no. female	4/5	3/3	4/3	4 /1	19/4	6/0	4/1	4/0	2/2	1/1	2/0
Age at inclusion, mean (range) years	42 (27–61)	45 (40–51)	19 (15–29)	19 (15–25)	41 (10–82)	59 (37–82)	32 (19–47)	23 (16–41)	54 (19–74)	26 (10–41)	41 (24–53)
Mean severity (SD)	n.a.	n.a.	Attack frequency/in last 12 months: 0	Attack frequency/in last 12 months: 3 (6)	n.a.	n.a.	Bilton score: 4 (0.71)	Harvey–bradshaw score: 6.75 (1.50)	DAS 28: 4.3 (0.50)	n.a.	n.a.
Genotype	n.a.	6 × heterozygous M694V	2 × M694V/V726A, 2 × M694V/M680I, 1 × M694V/V726A/E148Q, 1 × M694V/A744S, 1 × M680I homozygous	M694V homozygous	n.a.	n.a.	1 × F508del and 1717-1 G>A, 4 × F508del/ F508del	n.a.	n.a.	2 × heterozygous T50M	1 × gp91phox
Inflammation markers in serum											
CRP, median (range), mg/L	1.0 (<0.3–6.0)	3 (0.8–5.8)	3 (0.3–8)	82.1 (1.3–112.5)	18.4 (0.3–182.8)	84.9 (39.6–182.8)	18.4 (8.2–66.1)	14.9 (2.9–55.5)	9.5 (3.0–50.7)	1.0 (0.3–1.9)	5.2 (1.5–8.9)
S100A12, median (range), ng/ml	362 (55–966)	456 (236–570)	710 (354.6–43,154)	9,725 (1,267–12,864)	325 (73–1,189), $n = 8$	$n = 0$	568 (375–761), $n = 2$	236, $n = 1$	471 (276–1,189), $n = 3$	95 (73–116)	$n = 0$
Caspase-1, median (range), pg/ml	158 (53–254)	263 (140–275)	207 (107–424)	394 (265–1,552)	157 (59–108)	154 (75–800)	157 (103–302)	119 (64–257)	251 (165–31)	62 (59–64)	113 (65–161)
IL-18, median (range), pg/ml	189 (106–243)	442 (245–651)	2,699 (571–7,322)	11,485 (4,054–18,028)	309 (138–15,221)	397 (139–1,350)	261 (138–398)	209 (192–493)	5,163 (758–15,221)	338 (306–370)	381 (227–535)

CRP, C-reactive protein; IL-18, interleukin-18.

Cutoff for inflammation markers: CRP <5 mg/l; S100A12 <140 ng/mg. IL-18 in healthy controls is 169 pg/ml [117, 243] (16). In case of incomplete data on individual serum marker concentration, the number of analyzed samples is indicated.

pulmonary exacerbation was assessed by changes in (1) sputum volume or color, (2) cough, (3) malaise and/or fatigue, (4) weight loss, (5) decrease in FEV_1 $\geq$10% or radiographic changes, and (6) dyspnea (maximal count 6) (18). In patients with rheumatoid arthritis, disease activity was measured by disease activity score 28 (DAS 28), which summarizes (1) number of tender joints (0–28), (2) number of swollen joints (0–28), (3) C-reactive protein (CRP) (mg/L), and (4) subjective evaluation of disease activity by the patient (0–100 visual analog scale). Patients who received >5 mg/day prednisolone equivalent were excluded.

Ethical Approval

This study was approved by the ethical commission of the Charité – Universitätsmedizin Berlin (Ref: EA2/033/09). Written informed consent was obtained from all HCs, patients, and/or their parents or legal guardians.

In vitro Analyses

Neutrophils were isolated by a two-density centrifugation using Percoll (GE Healthcare, Freiburg, Germany) within 30–60 min after blood drawing (for the experimental approach, see **Figure S1**). Cells were counted, and purity was determined by cell-counting flow cytometry (Sysmex). An additional serum sample was aliquoted, immediately frozen, and stored at −80°C for later analysis of inflammatory mediators. Neutrophils (5×10^6 cells/ml) were left untreated or stimulated for 5 h with phorbol myristate acetate (PMA) (10 nM; Sigma-Aldrich, Munich, Germany) or lipopolysaccharide (LPS) (10 ng/ml LPS-RS Ultrapure; InvivoGen), with or without the addition of colchicine (5 μg/ml; Sigma) at time 0 and with or without the addition of adenosine triphosphate (ATP) disodium salt (Sigma-Aldrich) at 3.5 h. Cells were harvested after 30 min and 1, 2, 3, 4, or 5 h, respectively. In HC, heterozygous *MEFV* mutation carriers and FMF patients cell viability were determined by microscopy after Trypan blue staining and flow cytometry after propidium iodide and annexin staining (Becton-Dickinson. Heidelberg, Germany).

Expressions of S100A12, IL-18, and IL-1β were analyzed in neutrophils derived from a previously described cohort of HCs and FMF patients with active disease (numbering in **Table S1**: controls 1.10–1.13, patients 2.14–2.19, data shown in **Figure S4**) (11).

After stimulation, RNA was isolated from 5×10^6 neutrophils according to the user manual ["Total RNA Isolation" (Macherey-Nagel) and reverse transcribed into cDNA "RevertAid H minus First Strand cDNA Synthesis Kit" (Fermentas)]. RT-PCR was performed with the ABI PRISM 7900HT Sequence Detection System after adding primers, SYBR FAST qPCR and SYBR Green by KAPA Biosystems.

Measurement cycle threshold (Ct) in comparison to housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) und ribosomal protein L (RPL) was analyzed (ΔCt). The following primers were used: GAPDH 236 forward 5′-GCA AAT TCC ATG GCA CCG T-3′, GAPDH 339 reverse 5′-GCC CCA CTT GAT TTT GGA GG-3′, RPL 13A 277 forward 5′-AGG TAT GCT GCC CCA CAA AAC-3′, RPL 13A 418 reverse 5′-TGT AGG CTT CAG ACG CAC GAC-3′, IL-1β forward: 5′-GCG GCC AGG ATA TAA CTG ACT TC-3′, IL-1β reverse 5′-TCC ACA TTC AGC ACA GGA CTC TC-3′, IL-18 forward 5′-TTC AAC TCT CTC CTG TGA GAA CA-3′, IL-18 reverse 5′-ATG TCC TGG GAC ACT TCT CTG-3′, S100A12 reverse 5′-TGT TTG CAA GCT CCT TTG TAA GC-3′, and S100A12 73 forward 5′-CAA AAC TTG AAG AGC ATC TGG AGG-3′.

Analysis of Inflammatory Mediators

ELISAs following the manufacturers' standard protocols were performed in patients' and control serum for S100A12 (Circulex, Nagano, Japan), IL-18 (human IL-18 ELISA kit, MBL, Woburn, USA), and caspase-1 (Human Caspase-1/ICE Immunoassay, R&D, Abingdon, UK).

Cytokines or cytokine receptor antagonists (IL-1β, IL-6, IL-8, IL-10, IL-18, IL-1Ra, and TNFα), neutrophilic granula proteins (Proteinase 3, MPO), and chemokines [MCP-1, MIP-1α (CCL-3), MIP-1β (CCL-4), and MIP-3α (CCL-20)] in culture supernatants (SNs) were quantified by multiplexed bead array assays (ProcartaPlex, Thermo Fisher, Waltham, MA, USA; R&D Systems, Minneapolis, MN, USA) according to the manufacturers' instructions. S100A12 was detected by a combination of in-house monoclonal anti-S100A12 antibodies (19) translated to the MagPlex microsphere platform (Luminex, Hertogenbosch, The Netherlands) (20). Data acquisition was performed on a MagPix instrument (Merck Millipore) using xPONENT v4.2 software (Luminex). Data were analyzed using ProcartaPlex Analyst software (v1.0; eBioscience).

Flow Cytometry

Fluorescence-activated cell sorting (FACS) analysis (Canto, FACS Diva software) was performed by the use of CD45-PE-Cy5, CD11b-APC, CD16-PC7, and CD62L-FITC antibodies and isotype staining by use of mIgG1-PE and mIgG1-APC. mIgG1-FITC and CD62L-FITC were purchased from Becton Dickinson; all other antibodies were purchased from Beckman Coulter. In the neutrophil-enriched cell population, granulocytes were positively distinguished from cell debris, and lymphocytes by positive staining with CD45 and high side scatter. Eosinophilic granulocytes were differentiated by the expression of CD16. To identify activation, neutrophils were stained with CD11b and CD62L (**Figure S2**). The gate for the isotype control was set to exclude 99% of the total population.

Statistical Analysis

Data were analyzed with GraphPad Prism software (Version 8.0 for Mac OS X, GraphPad Software, La Jolla, CA, USA), and tests applied as indicated in figure legends. Significance of differences in serum levels of inflammatory mediators were analyzed by Kruskal–Wallis followed by Dunn's multi-comparison test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$, and $p \leq 0.05$ were considered statistically significant.

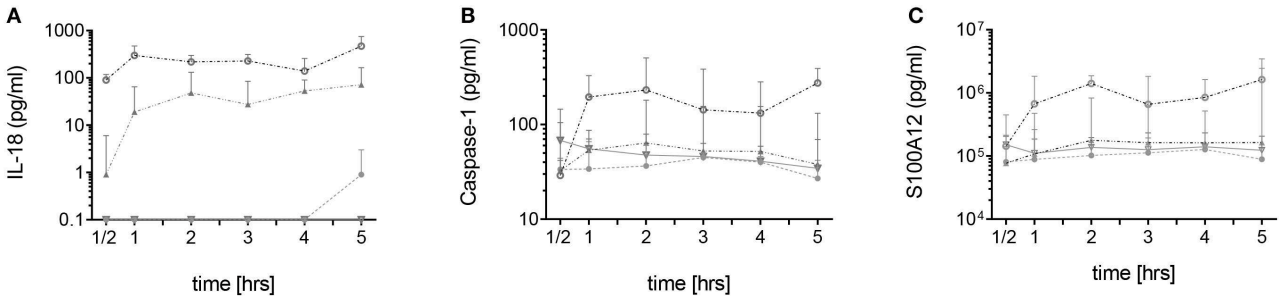

FIGURE 1 | Spontaneous secretion of IL-18, caspase-1, and S100A12 by neutrophils derived from *MEFV* mutation carriers and controls. Isolated neutrophils from healthy controls (filled circles, $n = 5$–7), healthy heterozygous *MEFV* mutation carriers (filled triangles, $n = 6$), FMF patients with compound heterozygous or homozygous mutations (open circles, $n = 9$), and patients with other chronic inflammatory and infectious diseases (open triangles, $n = 7$–22) were cultured without stimulation. Secretion of IL-18 **(A)**, caspase-1 **(B)**, and S100A12 **(C)** was assessed by ELISA in the supernatants at indicated time points. Note that values for S100A12 are represented on a logarithmic scale and in pg/ml. Values are given as median and interquartile ranges.

RESULTS

Serum Levels of S100A12, IL-18, and Caspase-1 Detect Inflammation in FMF

Although most of the FMF patients investigated in this cohort were well-controlled by continuous colchicine therapy (**Table S1**), homozygous M694V mutation carriers showed a significant increase of CRP [median 82.1 mg/L (range 1.3–112.5 mg/L), $p < 0.05$] compared to patients with other mutations or HCs (**Table 1**, **Table S1**). It was shown previously that IL-18 and S100A12 are especially sensitive to detect subclinical inflammation in patients with FMF (11, 21). In this independent cohort, serum levels of IL-18, S100A12, and, interestingly, caspase-1, a marker for inflammasome activation, were also significantly increased in patients with homozygous M694V mutations compared to controls [IL-18: median 11,485 pg/ml (4,054–18,028), $p < 0.0001$, S100A12: median 9,726 ng/ml (1,267–12,864), $p < 0.01$; caspase-1: median 394 pg/ml (265–1,552), $p < 0.01$]. Furthermore, IL-18 levels were significantly increased in patients with two mutations other than M694V homozygosity compared to healthy subjects [median 2,699 pg/ml (571–7,322), $p < 0.01$].

These observations confirmed that IL-18 and S100A12 as well as caspase-1 are increased in FMF and prompted us to analyze their secretion pattern in *ex vivo* isolated neutrophils in more detail.

FMF Neutrophils Spontaneously Release S100A12, IL-18, and Caspase-1

Therefore, we extended our analyses in this patient cohort to *ex vivo* studies of neutrophils as a prominent source of IL-18, caspase-1, and S100A12 secretion (11). For this reason, we determined the kinetics of spontaneous protein secretion and compared findings to neutrophils derived from healthy heterozygous *MEFV* mutation carriers, as well as patients with active infections and other inflammatory diseases.

After cell preparation, neutrophils were enriched to a mean of 90.6% (SD 9.3%). The amount of monocytes, a potential contaminating source of proinflammatory cytokines, ranged at a mean percentage of 0.8% (SD 1.3%). Between the different diseases, no differences in cell distribution was observed (**Table S2**). The addition of ATP, LPS, or colchicine did not decrease cell viability as measured by Trypan blue staining as well as by flow cytometry after propidium bromide and annexin staining (**Tables S3, S4**).

In neutrophils derived from FMF patients, IL-18, caspase-1, and S100A12 were rapidly secreted during the first 60 min of culture. For IL-18, a gene/dose-dependent secretion was observed with the highest levels secreted by cells derived from patients with two pathogenic *MEFV* mutations followed by secretory activity of cells derived from healthy heterozygous carriers (**Figures 1A–C**). As previously described, no spontaneous increased IL-1β secretion was observed (**Figure 2**, **Figure S3B**). In neutrophils derived from patients with acute infections and other active inflammatory diseases, secretion of these inflammatory markers did not differ from HCs (**Figure 2**, **Figure S3B**).

As previously demonstrated by single ELISA (11) and confirmed in this cohort, the spontaneous secretion of S100A12 and IL-18 by neutrophils from FMF patients cannot be further enhanced by *in vitro* cell stimulation but can be reduced by the addition of colchicine (**Figure 2**, **Figure S3B**). To elucidate whether this spontaneous release is specific for these particular mediators and for neutrophils from FMF patients, we analyzed the secretion of various cytokines, chemokines, and granular proteins in neutrophils derived from patients with different inflammatory conditions (**Figure 2**, **Figures S3A,B**). IL-18, S100A12, proteinase-3, and MPO are the only proteins that were spontaneously secreted at high levels by neutrophils from FMF patients, and further stimulation with PMA did not increase protein concentration in the SN. Addition of colchicine resulted in decreased IL-18, S100A12, and proteinase-3 secretion into culture SN. In contrast, blocking the IL-1 signaling pathway or the activation of NLRP3 by the addition of Anakinra or MCC950, respectively, did not alter the secretion of these proteins (data not shown). Furthermore, no highly increased and/or specific spontaneous protein release from neutrophils derived

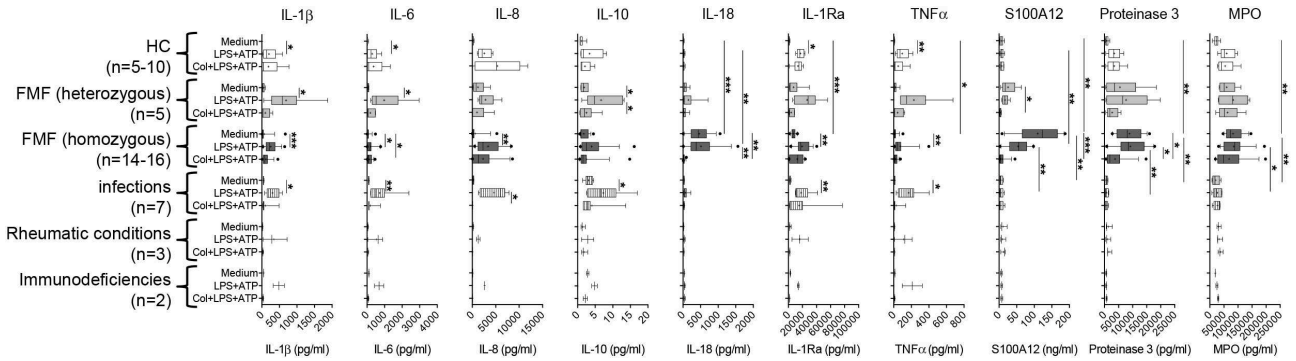

FIGURE 2 | Spontaneous and induced secretion of various mediators by neutrophils derived from *MEFV* mutation carriers and patients with other inflammatory diseases. Isolated neutrophils derived from controls and from patients with indicated diseases were cultured without stimulation or were stimulated with 10 ng/ml of lipopolysaccharide (LPS) for 5 h and 1 mM adenosine triphosphate (ATP) for 90 min. Part of the cells was cultured with 5 μg/ml colchicine added at time 0. Concentrations of interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-18, IL-1RA, TNFα, S100A12, proteinase 3, and MPO were quantified by multiplexed bead array assay (D). Data were analyzed by Kruskal–Wallis followed by Dunn's multi-comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Note that different assay systems were used in **Figure 1** compared to **Figure 2** explaining potential differences in concentrations.

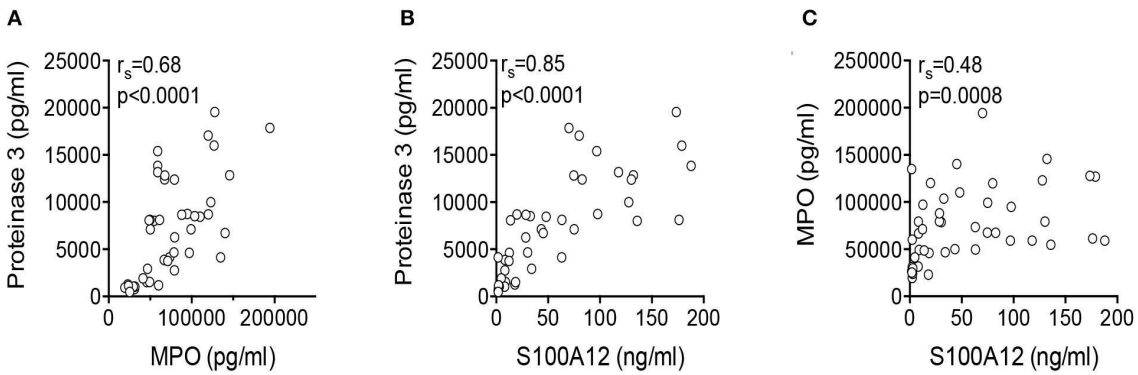

FIGURE 3 | Correlation of secreted protein concentrations. Correlations of proteinase 3 and MPO **(A)**, S100A12 and proteinase 3 **(B)**, and S100A12 and MPO **(C)** in the supernatant of unstimulated neutrophils derived from FMF patients were analyzed by Spearman rank correlation.

from disease controls was observed. Additionally, unstimulated neutrophils from patients with FMF and other inflammatory diseases did not release significant concentrations of chemokines, for example, MCP-1, MIP-1α (CCL-3), MIP-1β (CCL-4), and MIP-3α (CCL-20).

Interestingly, spontaneous S100A12 secretion correlated with the amount of measured proteinase 3 ($r_s = 0.85$, $p < 0.0001$) and MPO ($r_s = 0.48$, $p = 0.0008$) (**Figure 3**).

Although we observed a marked spontaneous release of S100A12 and IL-18 from FMF neutrophils, cell stimulation only increased transcription of IL-1β but did not alter transcription levels of S100A12 and IL-18 (**Figure S4**).

Taken together, the spontaneous release of S100A12, IL-18, MPO, and proteinase 3 is restricted to neutrophils derived from FMF patients and, in the case of IL-18 when analyzed by ELISA, also increased in healthy mutation carriers.

FMF Neutrophils Reveal Spontaneous Loss of Surface-Bound CD62L

To further characterize spontaneous neutrophilic activation, expression of surface markers on neutrophils derived from the different patient groups was measured by means of flow cytometry. CD62L, a molecule responsible for endothelial attachment and transmigration into affected tissues indicating cell activation if shed from the cell surface, was rapidly shed from the surface of neutrophils derived from FMF patients during the first 2 h of culture and to a lesser extent during the following 3 h. After 5 h, the median of the geometric mean fluorescence intensity (gMFI) reached 28.8% (range 11.4–65.4) of the initial value (**Figure 4A**). No difference in the level of CD62L shedding was observed when comparing FMF patients with increased CRP to those with CRP values within the normal range (data not shown). In neutrophils derived from healthy mutation carriers, CD62L expression declined more constantly

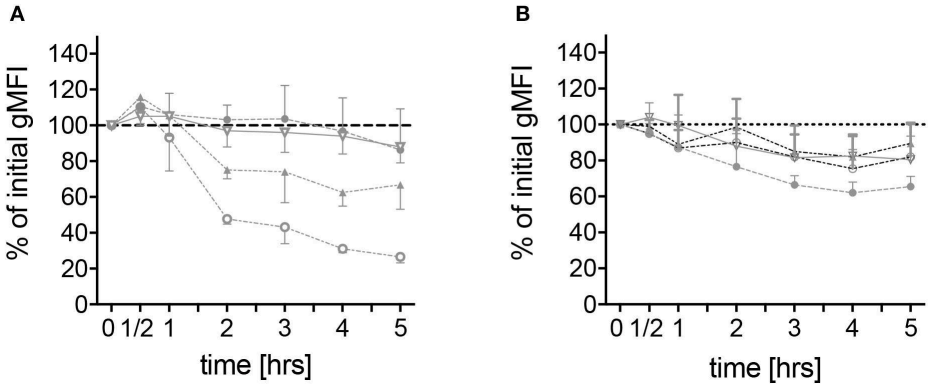

FIGURE 4 | Spontaneous alteration of CD62L and CD11b expressions on neutrophils derived from *MEFV* mutation carriers and controls. Isolated neutrophils from healthy controls (filled circles, $n = 6$), healthy heterozygous *MEFV* mutation carriers (filled triangles, $n = 6$), FMF patients with compound heterozygous or homozygous mutations (open circles, $n = 10$), and patients with chronic inflammatory as well as infectious diseases (open triangles, $n = 24$) were cultured without stimulation. Geometric mean fluorescence intensity of CD62L **(A)** and CD11b **(B)** expression was measured at the indicated time points by flow cytometry. Values are given as mean and interquartile ranges.

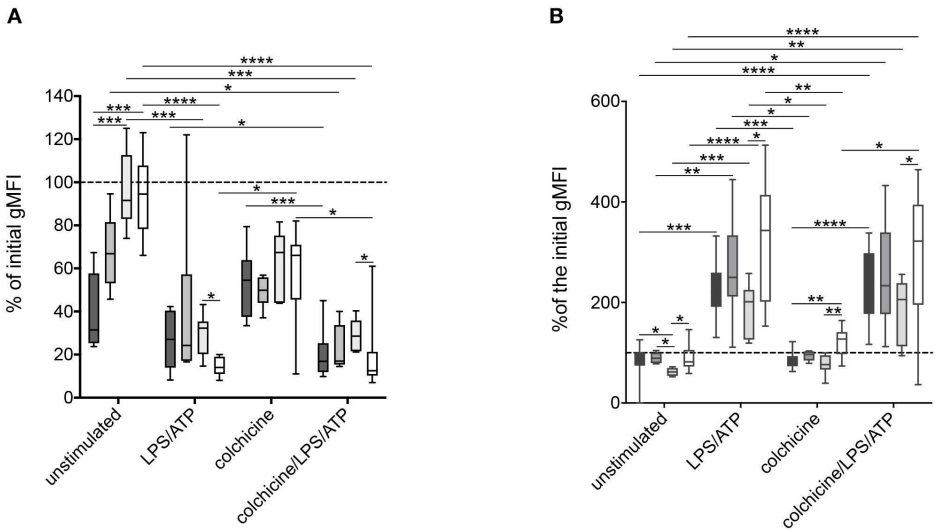

FIGURE 5 | Alteration of surface markers after stimulation of neutrophils derived from *MEFV* mutation carriers and patients with other inflammatory diseases. Isolated neutrophils from FMF patients ($n = 10$, dark gray), heterozygous mutation carriers ($n = 6$, middle dark gray), controls ($n = 7$, light dark gray), and patients with other inflammatory diseases ($n = 11$, white) were cultured as described in **Figure 2**. After 5 h, CD62L **(A)** and CD11b **(B)** expressions were analyzed. Box-and-whisker plots depict 5th–95th percentiles. Significance was analyzed by Kruskal–Wallis followed by Dunn's multi-comparison test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

over time, reaching a final median gMFI of 67.1% (range 45.7–94.6). Neutrophils from patients with infections and other active inflammatory diseases and HCs exhibited only slight CD62L shedding [median gMFI 82.9% (range 73.9–112.6) and 83.6% (61–136.0), respectively; **Figure 4A**, **Figure S5**].

Of note, one heterozygous FMF patient with persisting symptoms (one to two attacks per month despite regular colchicine intake, patient 2.13 in **Table S1**) demonstrated a rapid shedding of CD62L comparable to the effects observed in homozygous FMF patients and different from heterozygous healthy mutation carriers (gMFI 18.5%), indicating a possible link to disease activity beyond genotype effects.

Co-incubation with colchicine and/or stimulation with ATP and LPS led to a significant increase of CD62L shedding in all analyzed patient groups (**Figure 5A**). The addition of the IL-1 receptor antagonist anakinra and the NLRP3-inhibiting compound MCC950 did not alter this stimulation-dependent CD62L shedding (**Figure S6**).

As a member of the MAC-1 complex, CD11b plays a role in neutrophil recruitment and can be used as a marker of neutrophil activation (22). No difference in CD11b expression

on unstimulated neutrophils from patients with different diseases and *MEFV* mutation carriers was observed (**Figure 4B**). Stimulation led to an increase of CD11b expression with no differences between the disease groups. In contrast to the expression of CD62L, the sole addition of colchicine did not alter CD11b levels (**Figure 5B**).

These observations demonstrate again a spontaneous gene/dose-dependent activation of *MEFV* mutated neutrophils, which does not depend on either autocrine IL-1 action or on induction of the NLRP3 inflammasome.

DISCUSSION

In this study, we confirm that IL-18 and S100A12 can sensitively detect inflammation in FMF. Furthermore, we affirm the spontaneous hypersecretion of these proteins together with caspase-1 by analyzing patients with well-controlled FMF and demonstrate that this secretion occurs rapidly within the first 2 h of culture. Since the effects of a spontaneous mediator release were not observed in neutrophils derived from patients with other highly active inflammatory diseases such as infections, Crohn's disease, rheumatic and autoinflammatory diseases, cystic fibrosis, and immunodeficiencies with chronic inflammation, these effects seem to be FMF specific. The effect of spontaneous mediator release was observed in both active (11) and mostly well-controlled (this cohort) colchicine-treated FMF patients. This indicates that therapeutically applied colchicine does not control the *ex vivo* analyzed spontaneous activation of neutrophils irrespective of the clinical phenotype. These observations are supported by a previous work demonstrating increased transcription of selected genes by short-time-cultured neutrophils derived from colchicine-treated FMF patients (8).

Many clinical observations in FMF indicate a genotype–phenotype correlation with most severe diseases observed in the presence of homozygosity of the highly pathogenic M694V mutation and with milder diseases in patients harboring other mutations or being heterozygous mutation carriers (6). Analysis demonstrating gene/dose-dependent IL-1β secretion by stimulated monocytes from FMF patients with different genotypes might in part explain these observations (10, 15). Furthermore, levels of neutrophil-derived proteins in patients' serum correlated with the underlying *MEFV* genotypes (11). This is in line with the clinical observation that S100A12 is particularly sensitive in detecting subclinical inflammation in healthy heterozygous mutation carriers (21). Additionally, neutrophils derived from patients with poorly controlled FMF spontaneously secrete high levels of S100A12 and IL-18 (11).

Both IL-1β and IL-18 exhibit proinflammatory effects, the latter one in most instances through the induction of interferon-γ (23). So far, mainly monocytes were used to study aberrant cytokine secretion in cells derived from FMF patients: long-term stimulation with LPS for 18 h—a condition which induces canonical (caspase-1-dependent) as well as non-canonical (caspase-4/5- and caspase-8-dependent) inflammasomes (24, 25) induced a strong IL-1β production in monocytes derived from FMF patients (10). Similarly, IL-18 and IL-1β secretion was enhanced in monocytes from patients with FMF when treated with the pyrin-specific stimulus *Clostridium difficile* toxin B (TcdB) as a second signal for a short time (15). The role of IL-1β in the pathogenesis of FMF has now clearly been demonstrated by the successful application of IL-1-blocking biological agents in FMF patients (26, 27). IL-1β and IL-18 as well as the S100A12 molecules lack a specific signal sequence and are therefore secreted by an ER/Golgi-independent pathway referred to as "unconventional secretion" (28, 29) or via gasdermin D (GASDMD)-mediated processes (30).

Caspase-1 processes the intracellularly located pro-IL1β/IL-18 into active cytokines (31). In macrophages, the inflammatory caspase-1, caspase-4, caspase-5, and caspase-11 have the capacity to induce GASDMD-dependent osmotic cell lysis, named pyroptosis, through the formation of large oligomeric membrane pores (32). In murine *MEFV*$^{V726A/V726A}$ macrophage IL-1β release, pyroptosis, and autoinflammatory symptoms seem to depend entirely on GASDMD activity (33). In FMF, this process is solely controlled by dephosphorylation of pyrin through the inhibition of protein kinases PKN1/2 (34). Additionally, emerging evidence suggests that neutrophilic activation can also lead to caspase-1- and GASDMD-dependent IL-1β and IL-18 maturation and secretion without concomitant lytic cell death (35, 36). In the present study, spontaneous S100A12 and IL-18 secretion correlated with inflammasome activity as measured by an increased caspase-1 secretion *in vivo* and *ex vivo* without the occurrence of significant cell death after 5 h of culture with or without stimulation. This observation suggests a differentially regulated IL-18 and S100A12 hypersecretion in neutrophils from FMF patients involving the mutated pyrin inflammasome, as well as GASDMD. In contrast to studies performed in *ex vivo* stimulated murine neutrophils (35, 36), IL-1β was only marginally elevated in the SNs of neutrophils derived from FMF patients, suggesting monocytes rather than neutrophils are the major source of soluble IL-1β in patients with FMF (10). But still, neutrophils still contribute to IL-1β-mediated inflammation during acute FMF attacks by the release of NET-associated IL-1β driven by mechanisms of autophagy (12, 13).

Activation of neutrophils is a complex and not fully understood process involving many different receptors, for example, G-protein-coupled receptors, Fc receptors recognizing Ig-opsonized pathogens and immunocomplexes, adhesion receptors, cytokine receptors, and innate immune receptors recognizing damage-associated molecular patterns. Engagement of these receptors led to neutrophil migration, differential gene expression, reactive oxygen species (ROS) production, and exocytosis of intracellular granules and vesicles (37). The degranulation in neutrophils is a tightly controlled process characterized by a microtubule-dependent granule transport toward the cell surface and a subsequent fusion of the organelle membrane with the cell membrane (38). Azurophil granules contain myeloperoxidase and proteinase-3 (38), two proteins

which have been found abundantly in the SN of unstimulated neutrophils from FMF patients. This observation suggests a spontaneous *MEFV*-dependent degranulation of *ex vivo* cultured neutrophils from patients with FMF. Although the concentrations of these molecules correlate strongly with those of the S100A12, it seems unlikely that they are secreted together since S100A12 does not appear to be stored in granules.

The selectin CD62L (L-selectin) and the integrin Mac1 ($\alpha_M\beta_2$; CD11b/CD18) are involved in neutrophil attachment, rolling, and stable tethering on endothelial cells and thus play a crucial role in transmigration of neutrophils from the blood into affected tissues (39). A disintegrin and metalloproteinase 17 (ADAM17) is a type 1 transmembrane protein with a sheddase activity for the membrane-bound CD62L. In contrast to other ADAM family members, the activity of ADAM17 is greatly enhanced by cell activation, for example, via the recognition of PAMPs or TNFα, leading to a loss of membrane-bound CD62L within minutes (40, 41). In addition, neutrophil degranulation is associated with increased protein kinase-C-dependent integration and activation of ADAM17 into the cell surface membrane (40, 42). Thus, the spontaneous *ex vivo* loss of CD62L expression on the surface of neutrophils can be explained by their activation and/or by the exocytosis of granula. Of note, at the time of cell isolation, CD62L expression was not altered in neutrophils from patient with FMF compared to controls in the present study or in published data (43), indicating that the shedding process is greatly enhanced by the culture conditions that may mitigate *in vivo* regulation of CD62L surface expression.

Our study has several limitations: due to the elaborative cell preparation process, only a limited number of patients were analyzed; thus, differences between genotypes other than M694V homozygosity might have been missed. Furthermore, no patients during acute flare were analyzed. In order to translate the observed pathophysiological alterations as a marker for disease management, a simpler protocol, for example, the analysis of whole blood, must be established. In order to establish a mechanistic explanation for our observations further functional studies on neutrophils derived from FMF patients have to be performed.

In summary, our data indicate that the differential secretion of inflammatory mediators such as IL-18 and S100A12 by neutrophils with mutations in the *MEFV* gene plays an important role in the pathophysiological processes in FMF. Our *ex vivo* studies of neutrophils detected a highly inflammatory phenotype that depends on a gene–dose–response relationship. A more detailed knowledge about the role of neutrophils in the pathophysiology of FMF may contribute to the development of specific markers for functional characterization of *MEFV* variants, as well as therapy control, and thus improve patient management.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethikkommission der Charité – Universitätsmedizin Berlin Campus Charité Mitte, Charitéplatz 1, 10117 Berlin Geländeadresse: Virchowweg 10. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

IS, JF, BO, NU, SW, and CK performed most experiments. SH, HB, RK, PE, and ES recruited patients. MM, DF, CK, HW, and TK planned and supervised the study and wrote the manuscript. All authors approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00716/full#supplementary-material

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Human Autoinflammatory Diseases Mediated by NLRP3-, Pyrin-, NLRP1-, and NLRC4-Inflammasome Dysregulation Updates on Diagnosis, Treatment, and the Respective Roles of IL-1 and IL-18

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Recent research has led to novel findings in inflammasome biology and genetics that altered the diagnosis and management of patients with autoinflammatory syndromes caused by NLRP3-, Pyrin-, NLRP1-, and NLRC4-inflammasomes and spurred the development of novel treatments. The use of next-generation sequencing in clinical practice allows for rapid diagnosis and the detection of somatic mutations that cause autoinflammatory diseases. Clinical differences in patients with NLRP3, pyrin, and NLRP1 inflammasomopathies, and the constitutive elevation of unbound free serum IL-18 that predisposes to the development of macrophage activation syndrome (MAS) in patients with gain-of function mutations in *NLRC4* led to the screening and the characterization of novel diseases presenting with constitutively elevated serum IL-18 levels, and start to unravel the biology of "high IL-18 states" that translate into the use of biomarkers that improve diagnosis and monitoring of disease activity and investigations of treatments that target IL-18 and IFN-gamma which promise to improve the management and outcome of these conditions. Lastly, advances in structural modeling by cryo-electron microscopy (cryo-EM) of gasdermin, and of NLRP3- and NLRC4-inflammasome assembly, and the characterization of post-translational modifications (PTM) that regulate inflammasome activation, coupled with high-throughput screening (HTS) of libraries of inflammasome-inhibiting compounds, promise a new generation of treatments for patients with inflammasome-mediated diseases.

Keywords: inflammasome, autoinflammatory diseases, NLRP3, pyrin, NLRC4, NLRP1, GSDMD

INTRODUCTION

Genetically-defined autoinflammatory diseases present with systemic and organ-specific inflammation caused by Mendelian defects in critical innate immune pathways (1, 2). The discovery that gain-of-function (GOF) mutations in NOD-like receptors (NLR) that form IL-1β activating inflammasomes cause systemic autoinflammatory diseases led to the successful repurposing of targeted anti-cytokine treatments that block IL-1 signaling and spearheaded precision medicine in autoinflammation.

Recent insights into the structure and function of the four inflammasomes, NLRP3-, pyrin-, NLRC4, and NLRP1, that so far have been associated with human disease, revealed differences in assembly, and their downstream function. These discoveries shed light on pathomechanisms that may cause the phenotypic differences between the inflammasome-mediated diseases. In particular, the unique association of the NLRC4 inflammasome with extremely high serum IL-18 levels and of the NLRP1 inflammasome with keratinocyte differentiation defects point to the differential roles of the respective inflammasomes in hematopoietic vs. non-hematopoietic cells and tissues.

Although the autoinflammatory diseases spectrum continues to expand and now includes syndromes caused by type-I IFN, IL-17, TNF, and IL-6 dysregulation (1, 2), this review focuses on recent updates on inflammasome biology gained by insights into the structure, post-translational modifications and differences in IL-18 cleavage that spur the development of inflammasome-specific targeted treatments. Advances in genetic diagnoses using next generation sequencing together with the emergence of novel treatment targets, promise to benefit conditions with inflammasome-amplified inflammation beyond autoinflammatory syndromes, that include malignancies, metabolic, vascular, and neurodegenerative diseases.

UPDATES ON NLRP3 AND NAIP/NLRC4 INFLAMMASOME ACTIVATION AND IL-1 RELEASE

Inflammasomes are intracellular sensors that regulate host defense, cell homeostasis, and cell death. Upon activation they recruit and activate caspase-1, which cleaves the proinflammatory cytokines pro-IL-1β, pro-IL-18, and gasdermin-D (GSDMD) (**Figure 1**). Cryo-electron microscopy (Cryo-EM) provided stunning models by deconvoluting the structure of the gasdermin pore and the assembly of the disc-like structures that initiate the assembly of the NLRP3 and NLRC4 inflammasomes that shed light on the enigma of IL-1 (and IL-18) release from activated monocytes (3) and on the mechanism of pyroptotic cell death (4, 5).

Cryo-EM Structure of Gasdermin-D, NLRP3 and NLRC4, and Novel Insights Into IL-1 Release

High-resolution 3D-evaluations of the molecular structure of gasdermin- D, a key regulator of pyroptosis (4) revealed that 27 cleaved N-terminal fragments of murine GSDMA3 (6) or human GSDMD (7) assemble a 27-multimeric ring that forms a pore which gets inserted into the cell membrane and serves to release cleaved IL-1β and IL-18 from a respective cell (8–11) (**Figure 1B**, left panel). If the inflammasome-activating signal is weak, the endosomal sorting complexes required for transport (ESCRT) system (12) can repair and close the pores (13, 14). A strong activating signal that exceeds the ESCRT repair capacity causes leakage of cell content through the pores that results in pyroptotic cell death (15, 16). In contrast to caspases-3, -6, and -7 mediated apoptosis (17, 18) which is not immunogenic, pyroptotic cells leak immunogenic contents and illustrate the importance of tight regulation of gasdermin-D cleavage through enzymatically active caspase-1.

In fact, the conventional view of regulation of caspase-1 activity was challenged in a recent study examining caspase-1 activation. In contrast to the model that caspase-1 tetramers, composed of two p20 and two p10 subunits (p20/p10), are activated through auto-processing (19), the authors demonstrate that full-length p46 and transient p33/p10 caspase complexes are enzymatically active and that auto-processing of p33/p10 generates inactive p20/p10 subunits (20). While therapies that block caspase-1 have failed, knocking out GSDMD in murine models of CAPS, and FMF prevented disease development (21, 22), suggesting GSDMD as a potential treatment target.

In late 2001/2002, the NLRP3 inflammasome was the first cytoplasmic sensor discovered to be linked to human disease (23), but only recently cryo-EM has solved the structure that provided insights into mechanisms that ignite its assembly. In the cryo-EM model, NEK7, a mitotic kinase that had been identified as critical in activating the NLRP3 inflammasome (24–26), was bound to the LRR domain. cryo-EM models suggest that it binds adjacent NLRP3 monomers and licenses self-nucleation through formation of an 11 or 12-subunit disc-like complex (endecamer or dodecamer) (27) (**Figure 1B**, middle panel) which assembles ASC and caspase-1 filaments that cleave IL-1b (not shown).

Cryo-EM of the (NAIP)-NLRC4 inflammasome illustrates a different activation mechanism. Microbial flagellin and components of the bacterial Type III injection system bind to NAIP, which undergoes a conformational change that enables binding of NAIP to an NLRC4 protomer and exposes a "catalytic" oligomerization interface that recruits another NLRC4. Self-oligomerization continues until 10-11 NLRC4 protomers and one NAIP form a disc that can recruit caspase-1 (3) (**Figure 1B**, right panel). NLRC4 can also oligomerize by helical assembly and form filaments similar to ASC and caspase-1 (28, 29). These oligomerization processes allow for rapid signal amplification during inflammasome activation. The biological roles of the disc-like vs. filament assembly are currently not understood.

Activation of the NLRP3 Inflammasome and Characterization of Post-translational Modification (PTM) Sites That Regulate Inflammasome Activation and Provide Novel Treatment Targets

Over the last 17 years studies of the NLRP3 inflammasome activation revealed complex signaling pathways that lead to inflammasome assembly (**Figure 1C**). To summarize current insights, the canonical activation of the NLRP3 inflammasome requires two signals. The first signal is mediated by a range of "triggers" (i.e., DAMPS and PAMPS, PRPs, TLRs, NOD2, TNFR1) that cause NF-kB mediated upregulation of *IL1B* transcription (30, 31). The second signal leads to caspase-1 activation and is triggered by mechanisms that cause potassium-efflux including through P2X7 channel activation, exposure to pore-forming ionophores, lysosomal damage, activation of the

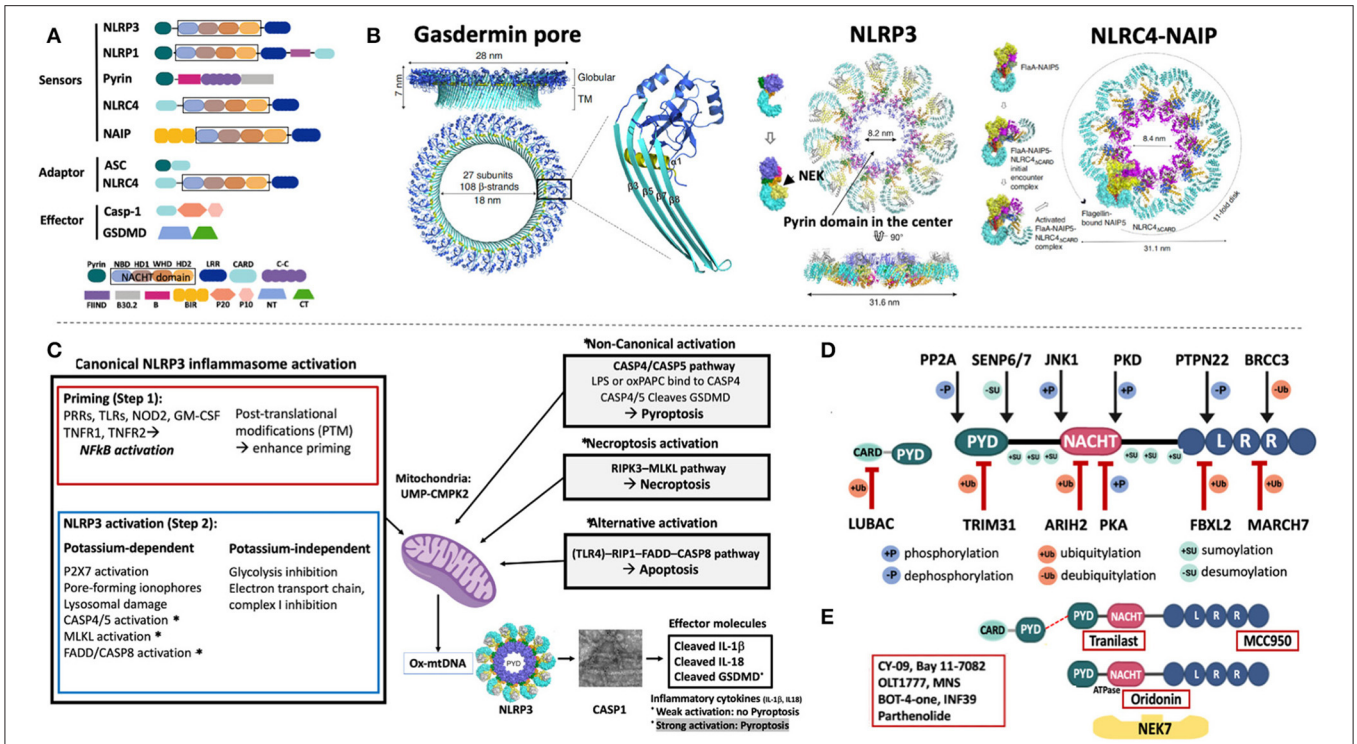

FIGURE 1 | Domain structure and activation of inflammasomes that cause human diseases. **(A)** The inflammasome sensors are tripartite proteins with an amino-terminal PYRIN (PYD), CARD, or BIR domain, a nucleotide-binding NACHT domain, and a carboxy-terminal leucine-rich repeat (LRR) domain. Intracellular sensors, including NLRP3, NLRP1, Pyrin, and NLRC4/NAIP, oligomerize upon stimulation and recruit and activate pro-Caspase-1 (pro-Casp-1), which cleaves proinflammatory cytokines (pro-IL-1β, pro-IL-18, not shown), and gasdermin-D (GSDMD). All sensors except for pyrin, have a "NACHT domain" that includes an NBD, an HD1, a WHD1 and a HD2 domain (shown in black box). **(B)** The 3D cryo-EM structure of the gasdermin D pore is shown (left panel); 27 cleaved N-terminal gasdermin fragments assemble a 27-multimeric ring, the gasdermin pore. The cryo-EM structures of NLRP3 (middle panel) and NLRC4-NAIP (right panel) demonstrate assembly of 11 or 12 NLRP3 or NLRC4/NAIP monomers through self-oligomerization into a disc-like structure. NLRP3 binds to NIMA-related Kinase 7, NEK7; NLRC4 binds to NAIP, which is a sensor of microbial flagellin, and of components of the bacterial Type III injection system. **(C)** Inflammasome activation. *Canonical NLRP3 inflammasome activation* requires a first or "priming" step which encompasses pattern recognition receptor/cytokine induced transcriptional upregulation of pro-*IL1B* and genes of some NLRP3 inflammasome components. The second step that leads to NLRP3 activation can be K+ efflux-dependent or independent and eventually leads to mitochondrial stress and the production of oxidized mitochondrial DNA (Ox-mtDNA); its production is controlled by the rate-limiting enzyme UMP-CMPK2. *Non-canonical inflammasome activation* is triggered by caspase-4/5 in humans (and caspase-11 in mice) that cleave GSDMD but not the pro-inflammatory cytokines and induces pyroptosis without priming step 1. Furthermore, activation of the RIPK3-MLKL pathway mediates necroptosis and alternative activation through FADD-Caspase-8 induces apoptosis and triggers inflammatory cytokine release through NLRP3 activation. One hypothesis to reconcile how different NLRP3 activating signals activate the inflammasome is through the common generation of mitochondrial distress and the release of Ox-mtDNA. **(D)** Post translational modifications of NLRP3 and ASC control inflammasome activation and have become targets for drug development. In resting macrophages, the LRR domain of NLRP3 is ubiquitylated. Deubiquitylation by the deubiquitinating enzyme (DUB) BRCC3, and dephosphorylation by protein tyrosine phosphatase, PTPN22 promote NLRP3 oligomerization while the E3 ubiquitin ligases, MARCH7, and FBXL2, ubiquitinate the NLRP3 LRR domain to inhibit NLRP3 inflammasome activation. The NACHT domain is modified by phosphorylation and dephosphorylation at serine residues, p.S194 and p.S293 by JNK1, and PKD, respectively, which activate, while phosphorylation or ubiquitylation at sites modified by PKA and ARIH2, respectively, inactivate the NLRP3 inflammasome. Modifications of the PYD domain at a Lys48-linked ubiquitylation site by the E3 ubiquitin ligase, TRIM31, cause proteasomal degradation of NLRP3 whereas dephosphorylation at p.S5 by PP2A and desumoylation by SENP6/SENP7 promote NLRP3–ASC, NLRP3 PYD–PYD interactions and inflammasome activation. Six conserved sumoylation loci keep NLRP3 in a resting state; desumoylation by SENP6/7 promotes NLRP3 activation. **(E)** Presumed drug-NLRP3 interaction sites are depicted. The MCC950 mechanism of action is unknown, while Tranilast, a tryptophan analog binds to the NACHT domain and inhibits NACHT-NACHT interaction between NLRP3 monomers. Oridonin binds to the NACHT domain and blocks NLRP3 and NEK7 interaction. A group of direct NLRP3 inhibitors including OLT1177 (Dapansutrile), a β-sulfonyl nitrile compound, block the NACHT ATPase activity. Residue numbers refer to human protein (ENST00000336119). **(A,B)**: B, Pyrin B-box; B30.2, Pyrin B30.2 domain; BIR, Baculovirus IAP-repeats; CARD, Caspase Recruitment Domain; Casp-1, Caspase 1; C-C, coiled-coiled domain; CT, C- terminal domain of gasdermin; FIIND, Function to Find Domain; HD1, Helical Domain 1; HD2, Helical Domain 2; LRR, Leucine Rich Repeat; NACHT, NAIP/C2TA/HET-E/TP1; NBD, nucleotide-binding domain; NT, N-terminal domain of gasdermin; PYD, pyrin domain; P20, protein 20; P10, protein 10; WHD, Winged Helix Domain. **(C)**: CASP1, caspase-1; CASP4/5, caspase-4/5; CASP8, caspase-8; FADD, Fas-Associated protein with Death Domain; GM-CSF, Granulocyte-monocyte colony stimulating factor; GSDMD, Gasdermin D; LPS, Lipopolysaccharide; MLKL, mixed-lineage kinase domain-like protein; NFkB, nuclear factor-kB; NOD2, nucleotide-binding oligomerization domain-containing protein 2; oxPAPC, oxidized phospholipid 1-palmitoyl-2-arachidonoyl-sn-glycerol-3-phosphorylcholine; P2X7, purinoceptor 7; PRR, Pattern recognition receptor; RIP1, receptor-interacting protein 1; RIPK3, receptor interacting protein kinase 3; TLR, Toll-like receptor; TNFR1, tumor receptor factor receptor 1; TNFR2, tumor receptor factor receptor 2; UMP-CMPK2, Cytidine Monophosphate Kinase 2. **(D,E)**: ARIH2, Ariadne homolog 2; BRCC3, BRCA1/BRCA2-containing complex subunit 3; FBXL2, F- box/LRR- repeat protein 2; JNK1, c-Jun N-terminal kinase 1; MARCH7, membrane-associated RING finger protein 7; NEK7, NIMA related kinase 7; *PKA*, protein kinase A; PKD, protein kinase D; *PP2A*, protein phosphatase 2A; *PTPN22*, protein tyrosine phosphatase, non-receptor type 22; SENP6/SENP7, Sentrin/SUMO-specific protease 6/7; TRIM31, tripartite motif containing protein 31.

non-canonical caspase 4/5 pathway, necroptosis by the RIPK3-MLKL pathway (32–34), and activation through the alternative RIP1-FADD-CASP8 pathway which are described in more detail in the figure legend of **Figure 1C** (35). Potassium-independent inflammasome activation is mediated by inhibition of the oxidative transport chain and glycolysis (36, 37) (**Figure 1C**) that ultimately causes release of oxidized mitochondrial DNA (ox-mtDNA) that can directly bind and activate NLRP3 (38). The enzymatic activation of the mitochondrial deoxyribonucleotide kinase, UMP-CMPK2, that can synthesize ox-mtDNA (39) in a rate-limiting step, and has recently been characterized. UMP-CMPK2 can be activated in a TLR–MyD88/TRIF–IRF1 dependent manner, which has become a target for drug development. Lastly, cell compartment disruption can activate the inflammasome by disassembly of the trans-Golgi network which serves as scaffold for NLRP3 (20). This process can be induced by mitochondria-associated membrane (MAM) localization to Golgi membranes, which causes diacylglycerol accumulation and the recruitment of protein kinase D, a phosphorylase that phosphorylates NLRP3 and triggers its release from MAMs and subsequent oligomerization (40). Mulitple other PTMs that modify NLRP3 inflammasome activation illustrate the complex regulation of the NLRP3 inflammasome and are described in **Figure 1D**.

To summarize, ubiquitination of PTM sites in the LRR domain in resting macrophages, inactivate the NLRP3 inflammasome, whereas deubiquitylation, and dephosphorylation of respective LRR sites activate it. Ubiquitylation, phosphorylation and sumoylation sites in the NACHT and PYD domain further modify NLRP3 function. Six conserved sumoylated loci (p.K88, p.K133, p.K204, p.K552, p.K652, p.K689) keep NLRP3 in a resting state (41). Mutations in the SUMO conjugation motifs, p.R137H, p.P651S, p.E690K, and p.E692K, cause CAPS and in p. D90Y, p.E206G, and R556X, an unspecified autoinflammatory syndrome thus suggesting that sumoylation defects may cause disease by lowering the NLRP3 activation threshold. The role of PTM in regulating NLRP3 inflammasome activation triggered the characterization of enzymes that may be subject to inhibition by small molecules and inspired high throughput screening (HTS) of chemical compound libraries for small-molecules that inhibit the activation of the NLRP3 inflammasome.

So far, three small molecule compounds have been identified that have been or are currently tested in clinical trials (**Figure 1E**), although their exact mechanisms of action remain elusive. 1. Tranilast is used in an ongoing single arm prospective study in CAPS (NCT03923140), its effect on pharmacokinetics and pharmacodynamics when co-administered with febuxostat in patients gout and hyperuricemia (NCT00995618) have also been evaluated (42). 2. OLT1177 (Dapansutrile) showed efficacy when topically applied as gel in a randomized, double-blind controlled study in osteoarthritis (NCT02104050). In an open-label, proof-of-concept, phase 2a trial, dapansutrile reduced target joint pain in a dose-dependent manner in adult patients with acute gout flares (EudraCT 2016-000943-14) (43). It is currently tested in a phase 1b trial in patients with heart failure (NCT03534297) and in a phase 2, open label study in Schnitzler's Syndrome (NCT03595371) (44). 3. Studies with MCC950 (also: CP-456,773) were temporarily discontinued due to off-target effects including liver toxicity in patients with rheumatoid arthritis (45–47).

Lastly, CRISPR/Cas9, a third-generation genome editing tool, that can specifically disrupt or repair disease-causing genes by a single gRNA-directed Cas9 nuclease (48) raises hopes for more definitive treatments of inflammasome mediated diseases. In addition to viral delivery systems of the CRISPR/Cas9 system to respective cells that include adeno-associated virus (AAV)-mediated delivery systems which have been tested in murine models of hypercholesterolemia and Duchenne muscular dystrophy (49–51), non-viral delivery systems using modified lipid nanoparticles have been explored. One study used an optimized cationic lipid-assisted nanoparticle (CLAN) system that can encapsulate mCas9 and guide NLRP3 ($CLAN_{mCas9/gNLRP3}$), to disrupt *NLRP3* gene expression in bone marrow derived macrophages (BMDM) *in vitro*, and *in vivo* when administered by injections in murine models of LPS-induced septic shock, MSU-induced peritonitis and high-fat-diet-induced diabetes (52). The uptake of CLAN particles in predominantly phagocytosing macrophages and neutrophils suggest that the system, if safe, may provide a novel treatment strategy in the future.

Recent Developments on the Genetic Diagnosis and Management of NLRP3 Inflammasome Mediated Diseases, Caps (FCAS, MWS, and NOMID/CINCA)

Autosomal-dominant, heterozygous GOF mutations in *NLRP3* cause the disease-severity spectrum of the predominantly familial cold induced autoinflammatory syndrome (FCAS), Muckle Wells syndrome (MWS), and the mostly sporadic severe phenotype Neonatal-onset Multisystem Inflammatory Disease (NOMID) (**Figure 2A**). While the familial mutations are germline mutations, rare sporadic cases of FCAS and MWS and up to 50% of NOMID patients acquire somatic *NLRP3* mutations in pluripotent cells during early embryogenesis (gonosomal inheritance) that are not detected by Sanger sequencing (53). Somatic mutations in more than 5% of transcripts in blood are detected by next generation sequencing (NGS); however, deep sequencing and subcloning may be required to identify lower frequency mutations (54). Somatic *NLRP3* mutations can be acquired in bone marrow myeloid progenitor cells; in rare cases they can cause adult-onset CAPS, that presents with neutrophilic urticaria, fever, conjunctivitis, and arthralgia (55, 56).

IL-1 blockade with anakinra, canakinumab, and rilonacept is standard of care in CAPS with a well-established safety profile. Adverse events include injection site reactions (mostly anakinra) and non-serious skin, urinary tract or GI infections that do not require treatment discontinuation (57–60). A study assessing the safety and efficacy of canakinumab in 33 young children between 1-month and 5-years-of age with mostly FCAS and MWS ($n = 29$); 4 children had NOMID, reported that the treatment was well-tolerated in the young children treated (61). Disease flares post-vaccination are well-documented in MKD/HIDS

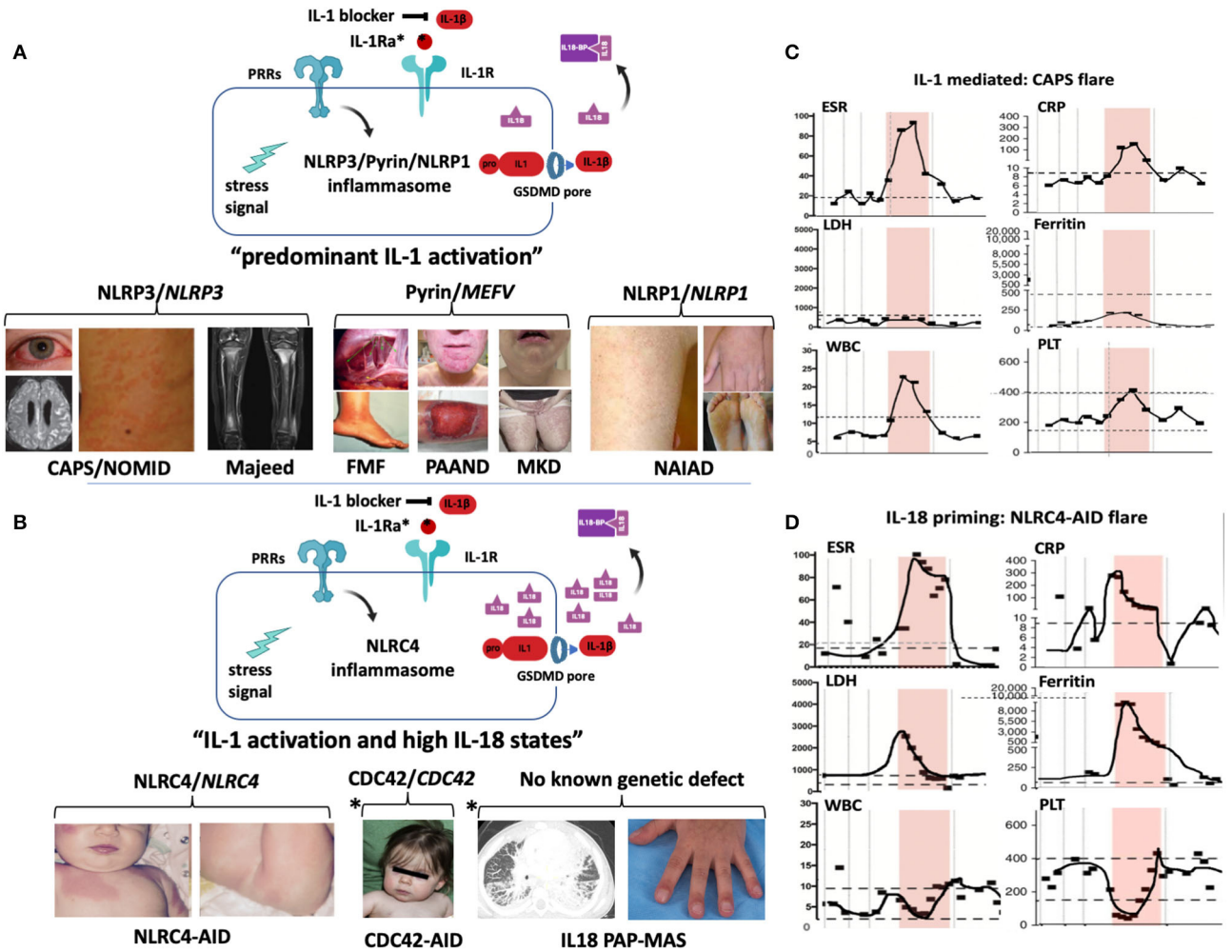

FIGURE 2 | Inflammasomes and inflammasomopathies. GOF mutations in Inflammasome sensors (NLRP3, Pyrin, NLRC4, NLRP1) cause systemic autoinflammatory diseases. While GOF mutations in NLRP3, pyrin, and NLRP1 lead to predominantly IL-1b activation **(A)**, GOF mutations in NLRC4 lead to IL-1b activation and very high IL-18 levels **(B)**. **(A)** GOF mutations in *NLRP3* cause the cryopyrin-associated autoinflammatory diseases (CAPS) (images depict conjunctivitis, MRI with leptomeningeal enhancement in NOMID patient with aseptic neutrophilic meningitis and neutrophilic urticarial rash) and secondary NLRP3 inflammasome activation by recessive LOF mutations in *LPIN2* has been linked to the generation of a metabolic triggers/stress (⚡) that cause Majeed syndrome (depicted is sterile osteomyelitis of the growth plates, the disease is not discussed in the text). Additive GOF mutations in pyrin cause familial Mediterranean fever (FMF) (depicted are abdominal adhesions that can develop in chronic sterile peritonitis and erysipelas-like erythema of the ankle in FMF) or Pyrin-Associated Autoinflammatory with Neutrophilic Dermatosis (PAAND) (depicted is cystic acne and pyoderma gangrenosum in a patient with neutrophilic dermatitis), and secondary pyrin inflammasome activation is caused by LOF mutations in *MVK* that cause Mevalonate kinase deficiency (MKD) (depicted is lymphadenitis and a papular rash). GOF mutations in *NLRP1* cause NLRP1-Associated Autoinflammation with Arthritis and Dyskeratosis (NAIAD) (depicted is follicular dyskeratosis, hyperkeratosis of the soles and lesions on left hand). **(B)** GOF mutations in *NLRC4* cause the NLRC4-Associated Autoinflammatory Disorders (NLRC4-AID) associated with often ultra-high serum IL-18 expression which predisposes to the development of MAS [images depict the rash from a patient with NLRC4-associated macrophage activation syndrome (MAS)]. Other high IL-18 states include *CDC42* mediated autoinflammatory disease (depicted are mild facial dysmorphisms including frontal bossing and nasal bridge depression); IL-18 PAP-MAS (a subset of SOJIA-ILD), an autoinflammatory disease without known genetic defect (depicted are a chest CT scan with interstitial lung disease and clubbing of the fingernails). For the 2 latter diseases, increased NLRC4 inflammasome activation as cause of the high IL-18 has not been demonstrated. **(A,B)** Inflammatory markers during MAS and CAPS flares differ. Flare episodes in patients with CAPS **(C)** differ from flare episodes in patients with high IL-18 levels (shown in a patient with NLRC4-AID in **D**). ESR and CRP are similarly elevated in both diseases, in MAS other features include elevated ferritin and LDH, and cytopenias (leukopenia, thrombocytopenia); while in CAPS, ferritin and LDH increase little if at all and patients develop leukocytosis and thrombocytosis. (The shaded area shows longitudinal laboratory markers collected during a 2-week period during a disease flare). CAPS, Cryopyrin-Associated Periodic Syndrome; CDC42, Cell Division Control protein 42 homolog; FMF, Familial Mediterranean Fever; IL18-BP, IL-18 binding protein; IL-1Ra, IL-1 receptor antagonist; MKD or HIDS, Mevalonate Kinase Deficiency or Hyper-IgD Syndrome; NAIAD, NLRP1 Associated Autoinflammation with Arthritis and Dyskeratosis; NLRC4-AID, NLRC4-Associated Autoinflammatory Disorders; NOMID, Neonatal-Onset Multisystem Inflammatory Disease; PAAND, Pyrin-Associated Autoinflammatory with Neutrophilic Dermatosis; PAP-MAS, Pulmonary Alveolar Proteinosis-Macrophage Activation Syndrome; PRR, Pattern recognition receptor.

(62) and together with concerns regarding vaccination efficacy and safety in other autoinflammatory disease patients receiving biologics led to recent studies in the field. The administration of canakinumab to healthy subjects 2 weeks prior to influenza and meningococcal vaccinations demonstrated that similar protective antibody titers developed in subjects receiving canakinumab and those who did not (63). Vaccination of 17 CAPS patients with polysaccharide or conjugate pneumococcal vaccines, led to disease flares in 12 patients, who all received polysaccharide vaccines (64) and a retrospective survey in 17 patients (5 CAPS, 4 MKD, 1 FMF, 7 sJIA) on IL-1 or IL-6 blockade who received live vaccines (varicella, MMR, oral polio, and yellow fever), recorded disease flares when IL-1 blocking treatment was held for vaccination and possible vaccine-induced infections (one of 5 developed varicella zoster, one of 8 post-MMR pneumonia and 1 of 1 diarrhea post-oral polio vaccination) (65). In the absence of larger studies providers need to balance the risks and benefits.

PYRIN AND NLRP1 INFLAMMASOMES AND ASSOCIATED DISEASES

The Pyrin Inflammasome

Pyrin is encoded by *MEFV* and contains PYD, bZIP (transcription domain), B-Box (zinc finger), CC (coiled-coil), and B30.2/SPRY domains (**Figure 1A**). Pyrin requires the adaptor ASC to recruit and activate caspase-1 (66–68). PTMs modulate the pyrin inflammasome through geranylgeranylation and phosphorylation and microbes evolved to exploit these mechanisms to manipulate virulence and host defense (69). This pivotal paper laid the ground for further explorations of the role of PTMs in activating the pyrin inflammasome in FMF and MKD/HIDS, which are discussed below.

Pyrin Inflammasome Mediated Diseases

Familial Mediterranean Fever (FMF), the most prevalent autoinflammatory disease, is characterized by recurrent episodes of fever, serositis, arthralgia, and monoarticular arthritis (70, 71). Recessive GOF mutations in *MEFV* confer additive pyrin inflammasome activation. Most patients are homozygous or compound heterozygous, but milder disease in heterozygosity occurs predominantly with more severe mutations (70). FMF-causing pathogenic *MEFV* mutations favor an active pyrin state. Missense mutations of serine p.S208 or p.S242 in *MEFV* cause a different phenotype that presents with recurrent episodes of neutrophilic dermatosis, fever, elevated acute-phase reactants, arthralgia, myalgia/myositis, an autosomal dominant disease that was termed pyrin-associated auto-inflammation with neutrophilic dermatosis (PAAND) (72). PAAND and studies that explored the disease pathogenesis of mevalonate kinase deficiency (MKD) or hyper-IgD syndrome (HIDS) uncovered the intriguing PTMs that regulate pyrin activation and linked MKD/HIDS to pyrin dysfunction.

Mevalonate kinase deficiency (MKD) or HIDS is a periodic fever syndrome that is caused by LOF mutations in *MVK,* the gene that encodes mevalonate kinase (MVK), which is an enzyme in the cholesterol pathway. MVK deficiency causes shortage of geranylgeranyl-pyrophosphate, which is not only an intermediate in the cholesterol pathway but is also essential for the biosynthesis of terpenes and terpenoids that are components of anti-microbials, hormones, and molecules that regulate cell differentiation and growth (73). Geranylgeranyl-pyrophosphate is a precursor to geranylated proteins, that include the GTPases, Kras, and RhoA. PTM through geranylation tethers them to the plasma membrane where they regulate TLR-mediated PI3K-Akt1 activation that downregulates MEFV expression (71). Geranylgeranylated RhoA activates phosphokinases, PKN1 and PKN2, that phosphorylate p.S208 or p.S242 on pyrin that enables the binding of a 14-3-3 protein, a member of a family of conserved regulatory proteins, that when bound to pyrin, keeps it inactive (72, 74). Thus, the absence or severe reduction of geranylgeranyl phosphate seen in patients with MKD results in lack of RhoA geranylation which keeps it "inactive/paralyzed" and prevents the protective pyrin phosphorylation that would allow the protective binding of 14-3-3 (74). The pathomechanism of MKD illustrates the link between "metabolic disturbances/stress" and the pyrin inflammasome activation and provides a plausible explanation for the phenotypic similarities between FMF and MKD (60) (**Figure 2B**).

The NLRP1 Inflammasome and Autoinflammatory Diseases

The NLRP1 inflammasome was last to be associated with human disease. In contrast to NLRP3 and pyrin, NLRP1 has a FIIND domain, a PYD and a CARD domain and can activate caspase-1 independent of the adapter ASC (**Figure 1A**). A Cryo-EM model is currently not available, but in contrast to the other disease-causing inflammasomes, NLRP1-inflammasome activation is uniquely dependent on proteasomal degradation. Pathogen-derived proteolytic toxins (75, 76) including lethal factor secreted by *Bacillus anthracis* (75) or IpaH7.8, an E3 ubiquitin ligase secreted by *Shigella flexneri* (76), or host endogenous proteinases (77, 78) modify NLRP1. This process exposes a ubiquitylation site, that initiates proteaseome-dependent degradation of NLRP1. Recent insights have been summarized in "functional degradation model" of NLRP1 that suggests that cleavage of the FIIND domain releases an N-terminal, NBD–LRR–FIIND(ZU5) fragment and a small C-terminal fragment that includes the C-terminal component of the FIIND domain and a CARD domain, or FIIND(UPA)-CARD peptide. Multiple liberated C-terminal fragments self-assemble into an oligomer that serves as a platform for inflammasome assembly and caspase-1 maturation (75). The authors suggest that NLRP1 is a sensor for proteases and pathogen effectors, that can directly induce proteasomal degradation of NLRP1 (76).

Mutations in the PYD domain in *NLRP1* cause the pre-cancerous conditions, palmoplantar carcinoma, familial keratosis lichenoides chronica, and inherited corneal intraepithelial dyskeratosis without systemic inflammation (79–81) and point to a role of NLRP1 in keratinocyte differentiation (82). In contrast, GOF mutations between the NACHT and the LRR domain and at the autolytic cleavage domain, FIIND, cause NLRP1-associated

auto-inflammation with systemic inflammation, arthritis, and dyskeratosis. The systemic features respond to IL-1 blockade; the skin manifestations respond to retinoic acid and vitamin A (83). These conditions suggest activation differences of the NLRP1 inflammasome in keratinocytes and monocytes (**Figure 2C**).

THE NLRC4 INFLAMMASOME AND THE BIOLOGY AND TREATMENT OF "HIGH IL-18 STATES"

The NLRC4 Inflammasome

NLRC4, a tripartite protein with a CARD, a NACHT and an LRR domain (**Figure 1A**) binds to the NLR, NAIP. Mice have five Naips, humans have one NAIP, thus challenging the translation of murine findings and may explain a lack of data on human endogenous triggers of the NLRC4 inflammasome. Interestingly, actin polymerization defects seem to play a role in NLRC4 activation (84). NLRC4 can assemble as filamentous (85) or as disc-like structures (86).

IL-18 Biology and the Association of High IL-18 States With the Development of Macrophage Activation Syndrome (MAS)

The discovery of *de novo* GOF mutations in *NLRC4* presenting with systemic inflammation, high serum IL-18 levels and recurrent macrophage activation syndrome (MAS) linked the NLRC4 inflammasome to IL-18 activation and MAS (87, 88) and triggered exploration of the role of IL-18 in regulating monocyte and macrophage function. *In vivo*, IL-18 is bound to IL-18 binding protein (IL-18 BP) and only extremely high levels of total IL-18 result in measurable free, unbound serum IL-18 (88). Murine studies overexpressing IL-18 (88) or knocking-out of *Il18bp* (87) liked IL-18 to the development of MAS following TLR9 activation. Chimeric bone marrow transplant in mice transgenic for the human disease-causing GOF *NLRC4* mutation, p.T337S, suggest that tissues (including the gut epithelium) and not hematopoietic cells are the major source of the high serum IL-18 levels (88). Phenotypic overlap and similar cytokine profiles link MAS and primary hemophagocytic lymphohistiocytosis (HLH) caused by genetic defects in cytotoxicity (89, 90) and infection-induced MAS (91). Chimeric antigen receptor (CAR) T-cell therapy can cause a "cytokine storm syndrome" that can progress to HLH/MAS-like disease. Recent studies identified recipient macrophages as a source for IL-6, and IL-1 and nitric oxide (NO) (92) that are thought to fuel the "cytokine activation syndrome." Patients are managed with corticosteroids and IL-6 receptor blockade (93), but treatment with IL-1 blocking agents was superior to IL-6 blockade in reducing neurotoxicity in a CAR T cell leukemia mouse models (94) suggesting a role of inflammasome activation. Murine models of free IL-18 (87, 88), HLH (95), and mice transgenic for human IL-6 (IL-6TG mice) that are challenged with LPS all develop MAS/HLH-like disease and respond to neutralization of IFNγ, which reversed HLH/MAS (91). Together these data suggest that increased IFNγ production which is downstream of IL-18, and other "cytokine storm syndromes," may present a common end-pathway that can lead to hemophagocytosis, cytopenias and hypercoagulability, and to the progressive organ failure and high mortality seen in HLH/MAS-like disease.

Novel Diseases Associated With High Serum IL-18 Levels

Disease-causing GOF mutations in *NLRC4* cause a clinical disease spectrum. Although systematic geno-phenotype correlations are lacking, somatic mutations in the NBD domain, p.T177A cause a NOMID-like phenotype (96); germline mutations in the WHD1 domain, p.H443P (97), and p.S445P (98), cause an FCAS-like phenotype, that may be IL-1 mediated; whereas a germline GOF mutation in the NBD domain, p.S171F, can cause MAS and thrombotic vasculopathy (99). Mutations in the HD1 domain, p.T337S (100), p.V341A (101), or in the LRR domain, p.W665C (102), can all cause MAS and early-onset enterocolitis (NLRC4-MAS), which are IL-1 and IL-18 mediated (103) (**Figure 2D**).

The predisposition to MAS is not seen in patients with *NLRP3* mutations, furthermore, laboratory flare characteristics differ in CAPS and NLRC4-MAS. While all patients present with ESR and CRP elevation, different from CAPS flare, in patients with NLRC4-MAS and high IL-18, lactate dehydrogenase (LDH), ferritin, and more variably transaminase (AST and ALT) levels can rise astronomically and are accompanied by cytopenia (granulo-and thrombocytopenia) and splenomegaly (**Figures 2C,D**). Between flares, NLRC4-MAS patients normalize ferritin, but IL-18 levels stay elevated (100). In various hyperferritinemic diseases associated with HLH/MAS that include genetically complex conditions such as systemic juvenile idiopathic arthritis (sJIA), adult-onset Still's disease, infection-induced MAS/HLH (88) or familial hemophagocytic lymphohistiocytosis (fHLH) (104), serum IL-18 levels can be constitutively elevated and/or rise only with flares.

Several newly described autoinflammatory diseases present with constitutively elevated IL-18 levels that predispose to MAS in the context of infections. GOF mutations in the C-terminal region of cell division control protein 42 homolog (*CDC42),* p.C188Y, p.R186C, and c.576A>C p.*192C*24 (105–107) cause an autoinflammatory syndrome with predisposition to MAS (**Figure 2D**). The mutations are hypothesized to affect the diarginine motif, p.R186, and p.R187, which binds to liposomes containing phosphatidylinositol 4,5-bisphosphonate (PIP2) (108) that is critical in mediating actin assembly. As actin polymerization activates the NLRC4 inflammasome in a salmonella infection model (84), a role of the NLRC4 inflammasome in CDC42 mediated AID is possible but requires further studies. In another report, a large homozygous LOF mutation in *IL18BP* causing IL-18 BP deficiency led to fulminant viral hepatitis in an 11-year-old girl. Tissue biopsy identified liver macrophages and hepatocytes as IL-18 source. Although clinical features were consistent with MAS triggered by acute hepatitis A, ferritin, free IL-18 and IFNγ levels were not reported (109). Lastly, rare patients with MAS, constitutively elevated IL-18 and interstitial lung disease and/or pulmonary alveolar proteinosis (**Figure 2D**), who do not have monogenic disease, present with

IL-18 and IFNγ driven cytokine elevation (i.e., CXCL9 and CXCL10) in blood and in bronchoalveolar lavage, suggesting that organ-specific IL-18 dysregulation (i.e., in the liver or pulmonary system) may explain the phenotypic variability in some high IL-18 states (110–113).

Novel Therapies for Patients With High Serum IL-18 Levels

The prominent roles of IL-18 and IFNg in the immune dysregulation of MAS is being validated in treatment studies. Recombinant human IL-18BP, tadekinig alfa, showed efficacy in a patient with NLRC4 MAS (103) and had a good safety profile in an open label phase II trial of adult-onset Still's disease. Patients were able to rapidly taper systemic steroids and achieve clinical remission (114). As suggested by murine models of HLH, the humanized anti-IFNγ monoclonal antibody, emapalumab, was efficacious and is approved in the US for treatment of primary intractable HLH (115, 116). Clinical trials evaluating safety and efficacy of emapalumab and anti-human IL-18 monoclonal antibodies in sJIA-MAS are ongoing (117, 118).

THE ROLE OF INFLAMMASOME ACTIVATION IN DISEASES OTHER THAN SYSTEMIC AUTOINFLAMMATORY DISEASES

Blocking NLRP3 inflammasome improves metabolic diseases in mouse models of insulin-resistance (119), non-alcoholic steatohepatitis (120), and atherosclerosis (121), and is linked to halting progression of neurodegeneration (122), and cellular senescence (123, 124). The role of inflammasomes in modulating innate immune functions in the tumor microenvironments are well-established (125, 126) and are emerging in CAR T-cell biology (94). Blocking IL-1β reduced lung cancer incidence and mortality (127) and prevented disease progression in smoldering myeloma (128, 129). High IL-18 levels were detected in patients with gastrointestinal disease (130), breast cancers (131), and in multiple myeloma (132); NLRC4 overexpression or high IL-18 levels contributed to a poor prognosis in gliomas (133) and acute myeloid leukemia (134). Despite these data, the efficacy of inflammasome modulators as adjuvant therapy in the comprehensive treatment of human malignancies, metabolic and degenerative diseases is yet to be established in clinical settings.

CONCLUSION

Inflammasomes are critical in defense against pathogens and in sensing of endogenous DAMPs signals. Tissue-specific expression and differences in their activating triggers are likely responsible for some phenotypic differences in various inflammasome-mediated autoinflammatory diseases. The role of free IL-18 levels in triggering MAS, combined with improvements in genetic testing and a growing number of targeted anti-cytokine therapies have revolutionized the diagnosis and management of autoinflammatory diseases in recent years and spearhead precision medicine in diagnosis and treatment in inflammatory and a wider spectrum of non-inflammatory diseases.

AUTHOR CONTRIBUTIONS

RG-M developed the outline, wrote, revised the manuscript, and the figures. SA conducted a systematic literature review, wrote, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Management of Monogenic IL-1 Mediated Autoinflammatory Diseases in Childhood

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Monogenic Interleukin 1 (IL-1) mediated autoinflammatory diseases (AID) are rare, often severe illnesses of the innate immune system associated with constitutively increased secretion of pro-inflammatory cytokines. Clinical characteristics include recurrent fevers, inflammation of joints, skin, and serous membranes. CNS and eye inflammation can be seen. Characteristically, clinical symptoms are coupled with elevated inflammatory markers, such as C-reactive protein (CRP) and serum amyloid A (SAA). Typically, AID affect infants and children, but late-onset and atypical phenotypes are described. An in-depth understanding of autoinflammatory pathways and progress in molecular genetics has expanded the spectrum of AID. Increasing numbers of genetic variants with undetermined pathogenicity, somatic mosaicisms and phenotype variability make the diagnosis of AID challenging. AID should be diagnosed as early as possible to prevent organ damage. The diagnostic approach includes patient/family history, ethnicity, physical examination, specific functional testing and inflammatory markers (SAA, CRP) during, and in between flares. Genetic testing should be performed, when an AID is suspected. The selection of genetic tests is guided by clinical findings. Targeted and rapid treatment is crucial to reduce morbidity, mortality and psychosocial burden after an AID diagnosis. Management includes effective treat-to-target therapy and standardized, partnered monitoring of disease activity (e.g., AIDAI), organ damage (e.g., ADDI), patient/physician global assessment and health related quality of life. Optimal AID care in childhood mandates an interdisciplinary team approach. This review will summarize the current evidence of diagnosing and managing children with common monogenic IL-1 mediated AID.

Keywords: treat-to-target, AID management, multidisciplinary team, disease activity, monitoring, autoinflammation

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INTRODUCTION

Monogenic IL-1 mediated autoinflammatory diseases (AID) are rare, often severe disorders caused by variants in innate immunity genes resulting in a constitutive overproduction of pro-inflammatory cytokines (1, 2). Clinical characteristics can include recurrent fevers, inflammation of joints, eyes, skin, and serous membranes (3). Severe phenotypes can include inflammation of CNS, bones, inner ears with hearing loss, and kidneys (4, 5). Patients with AID frequently report fatigue,

irritability, headache, abdominal pain, and musculoskeletal complaints (6–9). Characteristically, clinical symptoms are coupled with increased inflammatory markers, such as C-reactive protein (CRP) and serum amyloid A (SAA) (3). AA amyloidosis is a serious complication with a prevalence of up to 50% in untreated familiar Mediterranean fever (FMF) (10).

The genetic origin of IL-1 mediated AID was first determined for FMF in 1997 (11, 12). In 1999, mutations in the *TNFRSF1A* gene were shown to be associated with the Tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS) previously called Familial Hibernian fever (13–15). In the following disease-causing genes were identified for several AID including the Cryopyrin-Associated Periodic Syndromes (CAPS) and the Hyperimmunoglobulinemia D Syndrome (HIDS)/Mevalonate Kinase Deficiency (MKD). The AID spectrum is continuously expanding due to advancements in genomic technologies such as next generation-sequencing (NGS) (16, 17). Translational research has further advanced the pathophysiological understanding of AID.

The management of AID patients include early diagnosis, effective therapy, treat-to-target (T2T) strategies and standardized monitoring of disease activity and damage. Therefore, a multidisciplinary team approach and attention to disease-related psychosocial burden are important. This review will summarize the available evidence focusing on common monogenic IL-1 mediated AID including CAPS, HIDS/MKD, FMF, and TRAPS.

Introducing the Inflammasome

Inflammasomes are intracellular complexes controlling inflammation and immune cell activation triggered by a variety of exogenous and endogenous triggers (2, 18) (**Figure 1**). The nucleotide-binding domain-like receptor (NLR) family forms a group of proteins involved in the formation of inflammasome sensors (22). These contain a pyrin domain or a caspase activation and recruitment domain (22). One of the most prominent members of NLR families in monogenic AID is NLRP3 (22). Pyrin is another important inflammasome-forming protein (23). Inflammasome assembly (**Figure 1**) leads to the activation of caspase-1, which is able to process the inactive pre-cursor form of IL-1β to its mature bioactive form and induce its release (22, 24). IL-1β is one of the most prominent products of inflammasome activation and a key regulator of systemic inflammation (22). Genetic variants can alter proteins involved in the inflammasome pathways (25).

Pathogenesis in Brief of Common IL-1 Mediated Aid

CAPS pathogenic gain-of-function variants in the *NLRP3* gene result in activation of the NLRP3 inflammasome with increased IL-1β secretion (26, 27). FMF is caused by autosomal recessive variants in the *MEFV* gene encoding for pyrin, a protein involved in the pyrin inflammasome (11, 12). HIDS/MKD results from loss-of-function mutations in the *MVK* gene, encoding for an enzyme of the isoprenoid biosynthesis (28, 29). The impaired isoprenoid biosynthesis leads to accumulation of mevalonate, shortage of end-products and reduced isoprenylated proteins (30, 31). Particularly, the shortage of geranylgeranyl-pyrophosphate affects small GTPases, resulting in IL-1β hypersecretion, activation of the pyrin inflammasome and the nuclear factor κB (NF-κB) pathway (32–34). TRAPS results from variants in the *TNFRSF1A* gene (14). The intracellular retention of the mutated receptor causes several pathological responses including autophagy, increased endoplasmatic reticulum stress, excessive mitochondrial reactive oxygen species and enhanced NF-κB activation with production of pro-inflammatory cytokines including IL-1 (35–37). Four distinctly different pathogenic variants in heterogeneous inflammatory pathways result in increased IL-1β release. It remains unclear, why the common feature of increased IL-1 β is associated with a heterogeneous clinical phenotype across diseases and even within each IL-1 mediated AID.

DIAGNOSIS

In 2012, Toplak et al. reported a medium diagnostic delay for AID of more than 7 years (range 0.3–76). There was a rapid increase in recognition after the first AID gene discovery in 1997 (38). Diagnosis of AID should be made as early as possible to prevent organ damage (39). In patients with suspected AID, a stepwise diagnostic approach should be performed, including patient and family history, ethnicity, physical examination, and inflammatory markers during febrile attacks and symptom free-intervals and genetic testing (40–42) (**Figure 2**). Other differential diagnosis such as immunodeficiencies, infections, autoimmune diseases and malignancies need to be excluded. Red flags are a family history of early hearing loss or renal transplants, Mediterranean background, fever periodicity and specific flare triggers, such as cold exposure.

Clinical Aid Symptoms

Recurrent fever is a leading symptom varying in duration for different AID associated with specific clinical symptoms. The Autoinflammatory Disease Activity Index (AIDAI), a standardized symptom diary (43), captures AID characteristic symptoms and helps identify phenotypic patterns.

The spectrum of **CAPS** includes three phenotypes and their overlaps: familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and the neonatal-onset multisystem inflammatory disease (NOMID)/chronic infantile neurologic cutaneous and articular syndrome (CINCA) (44, 45). The FCAS phenotype is characterized by cold-induced neutrophilic dermatitis, fever, and chills (4). Patients with the moderate MWS may suffer from sensorineural hearing loss, urticaria-like rash and arthritis, while the severe NOMID phenotype is associated with aseptic meningitis, skeletal deformities and papilledema (4). MWS onset is often in early childhood, while NOMID is frequently recognized in the neonatal period (4).

Characteristic symptoms of **FMF** are recurrent fevers ranging 1–3 days, serositis, abdominal pain and/or pericarditis, arthritis, myalgia, and erysipelas-like erythema (46).

HIDS/MKD related features include malaise, fatigue, weight loss, lymphadenopathy, mucocutaneous involvement,

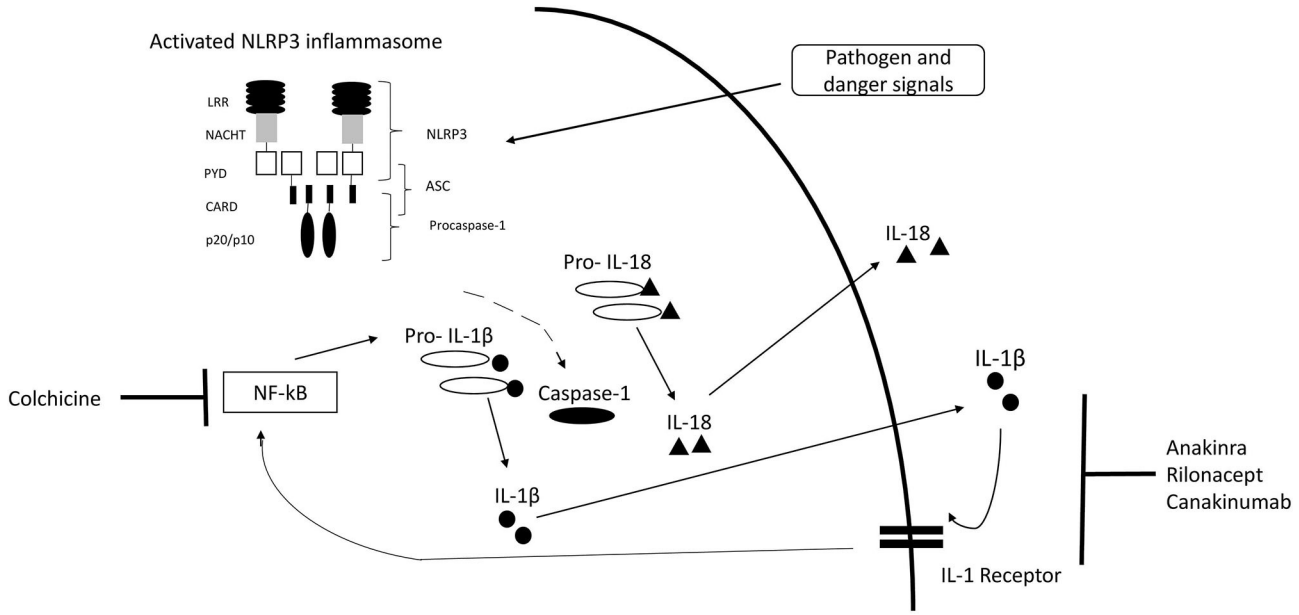

FIGURE 1 | IL-1 inflammasome structure, cytokine release, and treatment target. Inflammasome formation is induced by a variety of triggers. Activated NLRP3 subsequently nucleates ASC forming filaments *via* PYD-PYD interactions and drives procaspase-1 filament formation through CARD-CARD interactions. Caspase-1 mediates cleavage of pro-IL-1β and pro-IL-18 to IL-1β and IL-18. [Modified from McCoy et al. (19), Lachmann et al. (20), Broderick (21)]. CARD, caspase activation and recruitment domain; LRR, leucine-rich-repeats; PYD, Pyrin domain; ASC, Apoptosis related speck-like protein containing CARD.

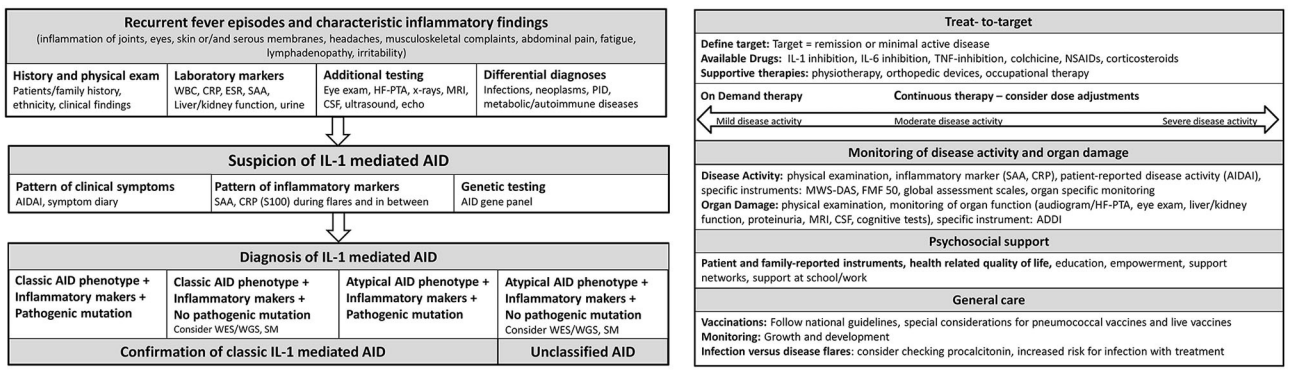

FIGURE 2 | Flow sheet diagnostic steps and management of AID. AID, autoinflammatory disease; WBC, whole blood count; CRP, c reactive protein; ESR, erythrocyte sedimentation rate; SAA, Serum Amyloid A; HF-PTA, high frequency pure tone audiogram; MRI, magnet resonance imaging; CSF, cerebrospinal fluid; PID, primary immune deficiency; IL, Interleukin; AIDAI, Autoinflammatory Disease Activity Index; S100, S 100 proteins; WES, whole exome sequencing; WGS, whole genome sequencing; SM, somatic mosaicism; TNF, Tumor necrosis factor; NSAIDs, Nonsteroidal anti-inflammatory drugs; MWS-DAS, Muckle Wells Syndrome Disease activity score; FMF 50, Familial Mediterranean Fever 50 Score; ADDI, Autoinflammatory Disease Damage Index.

musculoskeletal complaints, and gastrointestinal symptoms (47). Neurological involvement and life-threatening macrophage activation syndrome are observed.

TRAPS patients typically present with musculoskeletal complaints, abdominal pain, maculopapular and migratory rash, and periorbital edema (6). Serositis, pericarditis, arthritis and myalgia may be more prominent in adulthood, while abdominal pain is found more typically in childhood (6, 48). Furthermore, Gaggiano et al. highlight that high penetrance variants are associated with abdominal pain, amyloidosis and subclinical inflammation, whereas oral aphthosis is frequently observed in low-penetrance variants (48). TRAPS may start in adulthood, family history may then be non-contributory.

Laboratory Investigations

CRP, SAA and complete blood count are considered first line laboratory parameters and should be tested during febrile attacks and in symptom-free intervals (42). Liver and muscle enzymes, renal function tests, and urine analysis including 24 h evaluation for proteinuria should be performed serially

(**Figure 2**). Immunoglobulin D (IgD) and mevalonic acid levels may be useful in suspected HIDS/MKD (47).

Functional Testing

AID can affect multiple organ systems and may result in organ damage. Functional testing is required at diagnosis and during monitoring (**Figure 2**). In suspected AID, a thorough physical examination including growth, development, musculoskeletal, neurological, and ophthalmologic examination should be performed (39). In suspected CAPS, high frequency audiograms (HF-PTA) including 0.5–10 kHz, formal cognitive testing, magnetic resonance imaging (MRI) brain, spinal tap with opening pressure, cerebrospinal fluid (CSF) cell count and protein, and a lesional skin biopsy should be considered (39). In patients with severe musculoskeletal involvement, x-rays and bone MRI are recommended (39). In HIDS/MKD, additional cognitive testing and muscle and liver enzyme monitoring are recommended (39). For FMF patients, particularly during colchicine treatment, renal and liver function tests should be monitored serially (49).

Genetic Testing

Molecular testing should be performed, when the clinical phenotype, laboratory and functional tests are suggestive of AID (40). Sanger sequencing had been primarily utilized to identify AID-causing variants. In recent years, NGS-AID panels have become the gold standard (50). Complete coding sequences of AID genes are enriched in these panels. The Genetic Testing Registry database (https://www.ncbi.nlm.nih.gov/gtr/) provides an overview of available panels. The reported diagnostic yield of comprehensive gene testing panels seems to range between 21 and 32% (51, 52). Whole exome sequencing (WES) should be considered in patients with negative panel testing to improve the diagnostic yield preferably using a family-based trio approach (53). In addition, WES enables the discovery of novel variants in known AID genes and in those not yet associated with diseases. Whole genome sequencing (WGS) can identify deep intronic variants and mutations in non-coding regulatory regions and therefore increases the diagnostic yield (53). Additionally, it allows a much more reliable identification of copy number variations compared with WES (53).

Somatic mosaicism results from *de novo* post-zygotic mutations. AID-panel testing in these patients may be negative. AID onset and phenotype may be atypical. Saito et al. first identified somatic mutations causing NOMID (54). Subsequently, Tanaka et al. reported somatic mutations in 70% of previously mutation-negative NOMID patients (55).

The identified pathogenic variants may allow prediction of disease severity; for example FMF patients with homozygous M694V were found to be at high risk for a severe phenotype including early disease-onset (56). Similarly, in HIDS/MKD combined heterozygosity for p.V377I/p.I268T was shown to be a risk factor for AA amyloidosis (47). Genetic variants are typically described as "pathogenic," "likely pathogenic," "uncertain significance," "likely benign," and "benign" (57). Some platforms can assist clinicians and geneticists in determining the pathogenicity of variants including the MOLGENIS platform (58). The Infevers database is an exhaustive registry of sequence variants identified in AID related genes (59).

Diagnostic and Classification Criteria

Diagnostic criteria are used to guide the care of individual patients, establish the correct diagnosis, and start targeted treatment (60). The diagnostic criteria for CAPS mandate raised inflammatory markers (CRP/SAA) *plus* at least two of the following symptoms: neutrophilic dermatitis, cold-triggered episodes, sensorineural hearing loss, musculoskeletal symptoms, chronic aseptic meningitis, and skeletal abnormalities (61). These criteria enable physicians to make a CAPS diagnosis without mandating evidence of a disease causing variant (e.g., in case of low-penetrance variants). For FMF, the first Tel Hashomer criteria were proposed for adult patients in 1967 (62). In 1997, Livneh et al. proposed a set of diagnostic FMF criteria based on the presence of one major or two minor criteria, or one minor plus five supportive criteria (63). The major criteria are typical attacks (lasting 12–72 h, >3 attacks, fever > 38°C) with any one of peritonitis, pleuritis/pericarditis, monoarthritis or unilateral "orchitis", erysipelas-like eruption in the calf, and/or symmetric myalgia with extreme tenderness in the lower extremities. The minor criteria were defined as incomplete attacks, exertional leg pain and favorable response to colchicine. In 2009, Yalçinkaya, Ozen et al. proposed the pediatric FMF criteria requiring ≥2 of the following characteristics: fever, abdominal pain, one-sided chest pain and arthritis, each lasting 6–72 h, ≥3 attacks, and family history of FMF (64), allowing to make a clinical FMF diagnosis in case of inconclusive/negative genetics and are useful in selection which patients should be genetically tested. These criteria were developed in a Turkish population. Subsequently, Demirkaya et al. compared the performance of these existing criteria in the pediatric AID cohort of the Eurofever registry (65). The Yalchinkaya-Ozen criteria yielded a higher sensitivity (87.4%) than 1967 and 1997 criteria. The authors suggest that they can be used for FMF diagnosis in pediatric patients from European and eastern Mediterranean region (65). Classification criteria are primarily used to define patients cohorts for research (60). In 2019, Gattorno et al. published validated evidence-based classification criteria for hereditary AID including CAPS, HIDS/MKD, FMF, and TRAPS with pathogenic/likely pathogenic variants, low-penetrance variants and without genetic testing/findings (66).

Diagnostic Uncertainty

While advanced genetic testing may establish a diagnosis in some patients, testing may still be negative, inconclusive or even misleading (1, 52, 67, 68). Therefore, the correlation of clinical phenotype and genetic result is critical (67). Low-penetrance variants in AID genes can be present in the general population. As some of these low-penetrance variant carriers nevertheless express AID symptoms unlike the known classical phenotype in confirmed pathogenic variants (69), it might be possible that these are mediated by different pathways parallel to the caspase-1 activation (70). Moreover, low-penetrance variants may confer an increased susceptibility to inflammation (71).

EFFECTIVE AID THERAPY: TREAT-TO-TARGET (T2T)

Therapy is comprehensive including medication, psychosocial support, physiotherapy and supportive care such as hearing aids (**Figure 2**). Traditional symptomatic therapy consisted of non-steroidal anti-inflammatory drugs (NSAIDS) and glucocorticoids, which often shortened disease flares but can increase their frequency (39, 49). Today, IL-1 inhibitors play a pivotal role and evidence-based AID treatment plans are available (39, 49).

Targeting of inflammatory pathways enables T2T strategies (19, 72) (**Figure 1**). Key component of T2T is the definition of a target such as remission or minimal disease activity. Standardized serial assessments are required to determine, if the target is achieved (72) (**Figure 2**). Different levels of disease activity require different treatment approaches (39, 72, 73). Frequently, dose adjustments are required, particularly in children and in severe disease subtypes (74, 75).

Colchicine

Colchicine is the established first-line FMF therapy with favorable response and risk reduction of AA amyloidosis (49, 76). Colchicine should be started as early as possible (49). Colchicine is metabolized by CYP3A4 enzymes and the P-glycoprotein (P-gp) efflux transporter, therefore, concomitant treatment with CYP3A4 and/or P-pg inhibitors should be avoided (77, 78).

IL-1 Targeting Drugs

IL-1 inhibition has been shown to be safe and effective in controlling inflammation in CAPS, TRAPS, and MKD/HIDS (75, 79–82). It is a valid therapeutic strategy in FMF patients with colchicine-intolerance/resistance (83–86). Currently, three IL-1 inhibitors are approved by the US Food and Drug Administration for AID.

Long-term efficacy and safety of the short-acting recombinant IL-1 receptor antagonist **anakinra** has been confirmed in several studies (79, 86–90). Anakinra is administered daily subcutaneously and blocks the binding of IL-1α and IL-1β to the IL-1 receptor. In a study of 43 CAPS patients treated with anakinra up to 5 years, serious adverse events reported most frequently were pneumonia and gastroenteritis (79). There is evidence that pediatric patients with undifferentiated AID may also benefit from anakinra (91).

The recombinant soluble IL-1 receptor **rilonacept** binds to IL-1α and IL-1β. Weekly subcutaneous administration has shown a good safety and efficacy profile (92).

Canakinumab is a fully humanized anti-IL-1β monoclonal antibody selectively binding soluble IL-1β. It has to be administered every 4–8 weeks subcutaneously. Several studies confirmed long-term efficacy and safety (93–98). Brogan et al., reported complete response to canakinumab in 17 CAPS patients <5 years of age (71% MWS, 24% NOMID, 6% FCAS) (96). Open label observations suggest that children require higher doses up to 8 mg/kg/4 weekly to achieve remission, particularly in severe CAPS phenotype (94). Recently, efficacy and safety of canakinumab was demonstrated for FMF, TRAPS and HIDS/MKD (75, 99). In patients with HIDS/MKD dose adjustment is frequently needed (75). The rate of serious infections was 7.4/100 patients-years in 181 patients with TRAPS, HIDS and CAPS (75).

Alternatives

In a prospective open-label dose escalation study, etanercept reduce symptoms and inflammatory markers in a dose-dependent manner in TRAPS (100). FMF patients with chronic arthritis and sacroiliitis can benefit from TNF-inhibition (101). IL-6 inhibition may be promising in TRAPS patients (102) and HIDS/MKD, particularly when refractory to anakinra/etanercept (103–105). Hematopoietic stem cell transplantation has been performed in refractory HIDS/MKD patients (39).

Psychosocial Needs in Aid

The care of patients with AID should include psycho-social support, as AID affect all areas of life (106) (**Figure 2**). AID are associated with depression, lower health related quality of life, anxiety and social isolation (107–110). Patients/parents have to deal with work/school-related challenges because of frequent sick-leaves (107). Long-term management should take psychological factors such as illness beliefs, coping strategies and the distribution of dependency into account (108). Patient support networks can provide important support (111).

MONITORING OF AID ACTIVITY AND DAMAGE

Regular monitoring of disease activity is crucial (39). This includes physical examination, measurement of height and weight, neurological and musculoskeletal examination, and determination of SAA and CRP levels to detect ongoing inflammation (**Figure 2**). Repeatedly increased SAA levels between AID flares may indicate a significant risk for AA amyloidosis. Monitoring of SAA and S100 proteins may detect subclinical diseases activity, particularly in FMF (112, 113).

The validated patient-reported AIDAI is a simple tool for assessing disease activity (43). The AIDAI contains 13 items addressing fever (>38°C), overall symptoms, specific AID symptoms, and use of NSAIDs (43). The clinical symptoms are dichotomous and scored as 0 (absent) or 1 (present) (43). The maximum score per day is 12 with a cumulative monthly score ranging from 0 to 372 (43). The clinical meaningful threshold indicating active AID is a score of at least 9 (43). The Autoinflammatory Disease Damage Index (ADDI) is a reliable instrument to assess disease-related organ damage in FMF, CAPS, TRAPS and HIDS/MKD (114). ADDI consists of 18 items grouped in eight categories of reproductive, renal/amyloidosis, developmental, serosal, neurological, auditory, ocular, and musculoskeletal damage (114, 115). Damage is defined as persistent or irreversible change in structure or function present for at least 6 months (114, 115). The ADDI can be used to monitor structural damage in individual patients, and allows

outcome analysis and comparison of damage accrual in clinical trials (114).

INFECTIONS AND VACCINES

Patients can experience febrile inflammatory episodes not primarily related to their AID. Particularly in atypical AID-flares, infections have to be excluded (39). While CRP does not discriminate between infection and flare, procalcitonin (PCT) may be a promising marker (116). Some infectious diseases are preventable by vaccination. However, both vaccination and infection may trigger flares, particularly in HIDS/MKD (117). Patients with CAPS may develop severe local and systemic inflammatory reactions after pneumococcal vaccination (118, 119). The 13-valent pneumococcal conjugate vaccine appears to be more favorable compared to the polysaccharide vaccine (119). In general, vaccination recommendations for patients with immunosuppressive therapy and inflammatory rheumatic diseases can be used for AID, where inactive vaccines are considered as safe and are recommended to national vaccination guidelines (120–123) (**Figure 2**). In accordance with the recommendations of the European League Against Rheumatism, annually influenza vaccinations are recommended for AID patients and immunosuppressive therapy (120, 124). Live -vaccines should be avoided/has to be considered individual for the patient (121, 122, 124).

SUMMARY

AID are rare diseases associated with the risk of severe morbidity, mortality and reduced health-related quality of life. The increasing number of somatic mosaicisms and low-penetrance variants make the diagnosis of these potential live-threatening diseases challenging. A standardized diagnostic approach for suspected AID should include the clinical phenotype, inflammatory markers, functional, and genetic testing (**Figure 2**). AID panels should be performed and may need to be supplemented with WES/WGS. The management of AID mandates a multidisciplinary team and psychosocial support. Medication should be tailored individually using T2T strategies. In IL-1 mediated AID, colchicine, and IL-1 inhibition are effective. Alternative therapies including IL-6 inhibition and TNF-blockade can be beneficial. Regular target evaluation and standardized monitoring of disease activity and organ damage is important. Vaccines should be administered according to national vaccination guidelines, respecting general vaccination recommendations for patients with rheumatic diseases.

AUTHOR CONTRIBUTIONS

TW, SB, and JK-D conceived the concept of the manuscript, wrote the manuscript, drafted the work, and reviewed the article critically. All authors have provided approval for publication.

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