Current understanding of the role of inflammasome activation in the pathophysiology of systemic Juvenile Idiopathic Arthritis

Writing assignment: literature review

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Submission date: 03-11-2022
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Systemic Juvenile Idiopathic Arthritis (sJIA) is a severe disease with autoinflammatory characteristics that mainly affects young children. It is characterised by systemic inflammation and fever. sJIA patients have elevated levels of the proinflammatory cytokines interleukin (IL)-1β, IL-6, and IL-18 with elevated levels of S100A8/A9, S100A12, and the acute-phase protein ferritin. Approximately 10% of the children with sJIA also develop the life-threatening complication macrophage activation syndrome (MAS), which comes with fever and very high levels of ferritin. sJIA patients are treated with nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and biologicals that block the IL-1 and IL-6 pathway. The exact cause of the disease remains unknown but the inflammasome likely plays an important role in the pathophysiology. IL-1β and IL-18 both are products of an activated inflammasome and S100A8/A9, S100A12, and ferritin are associated with inflammasome activation. Furthermore, gain-of-function mutations in the nucleotide-binding domain of nucleotide-binding oligomerization domain leucine-rich repeat receptor (NLR) caspase activation and recruitment domain (CARD) containing 4 (NLRC4) inflammasome are associated with MAS. This review focuses on the current understanding of the possible role of the five best understood inflammasomes (NLR pyrin domain containing 1 (NLRP1), NLRP3, NLRC4, absent in melanoma 2 (AIM2), and pyrin) in the pathophysiology of sJIA. This knowledge is important for future research into the treatment and possible prevention of sJIA. Direct research into the role of the different inflammasomes in sJIA is scarce and therefore the symptoms, cytokine expression and treatments of monogenic autoinflammatory disorders as a comparison to sJIA will also be used to determine the likelihood of the involvement of the respective inflammasome in sJIA pathophysiology. Data of studies focussing on inflammasomes in sJIA and the comparisons with inflammasomopathies, indicate that the NLRC4 and AIM2 inflammasome are most likely involved in the pathophysiology of sJIA.

Layman's summary

Systemische Juveniele Idiopathische Artritis (sJIA) is een ernstige aandoening die kinderen kunnen krijgen. Kinderen met sJIA hebben onder andere last van koorts, gewrichtsontstekingen en huiduitslag. Daarnaast ontwikkelt 10% van de patiënten een levensbedreigende complicatie, genaamd macrofaag activatie syndroom (MAS). Dit zorgt ervoor dat de kinderen nog zieker worden. Gelukkig bestaan er medicijnen waarmee een groot deel van de symptomen tegengegaan kan worden. De oorzaak van sJIA en de bijbehorende symptomen is echter onbekend. We weten dat sJIA patiënten hogere waarden van de cytokinen interleukine (IL)-1β, IL-6 en IL-18 hebben. Deze cytokinen zorgen ervoor dat het immuunsysteem wordt geactiveerd en infecties bestreden kunnen worden. IL-1β en IL-18 worden geactiveerd door inflammasomen. Dit zijn complexen die uit meerdere eiwitten bestaan die er onder andere voor zorgen dat IL-1β en IL-18 geactiveerd worden. Dit is erg belangrijk wanneer je een infectie oploopt en je lichaam de infectie moet bestrijden, maar als er te veel van deze cytokinen worden gemaakt terwijl je geen infectie hebt, word je hier ziek van. Dit gebeurt dan ook in sJIA, want door een onbekende reden worden er meer cytokinen gemaakt dan normaal, waardoor je immuunsysteem geactiveerd wordt en je bijvoorbeeld koorts krijgt. Naast deze hoge waarden van cytokinen, hebben patiënten ook hoge waarden van andere eiwitten, zoals S100A8/A9, S100A12 en ferritine. Deze eiwitten kunnen ervoor zorgen dat inflammasomen geactiveerd worden.

Het lijkt er dus op dat inflammasomen overactief zijn in sJIA patiënten waardoor er te veel IL-1β en IL-18 wordt gemaakt, waardoor de kinderen ziek worden. Er zijn verschillende soorten inflammasomen waaronder NLRP1, NLRP3, NLRC4, AIM2 en pyrin, maar ze kunnen allemaal IL-1β en IL-18 activeren. We weten dat bepaalde mutaties in genen – hiervan worden eiwitten gemaakt – van deze inflammasomen ervoor kunnen zorgen dat de inflammasomen overactief raken. Hierdoor kunnen mensen ziek worden en symptomen krijgen die voor een deel lijken op die van sJIA. De symptomen

verschillen voor een deel per aangedaan inflammasoom, maar bij alle inflammasomen ontstaat er inflammatie door overactiviteit van het inflammasoom.

Aangezien de symptomen lijken op die van sJIA en het er op lijkt dat de inflammasomen overactief zijn in sJIA patiënten, hebben we onderzocht wat er bekend is over de rol van inflammasomen in sJIA patiënten. Tot nu toe zijn er in sJIA patiënten geen mutaties in genen van deze inflammasomen gevonden. Wel is bekend dat in sJIA patiënten de genen die belangrijk zijn voor de NLRC4 en AIM2 inflammasomen meer aanwezig zijn. Dit zorgt er waarschijnlijk voor dat sJIA patiënten meer NLRC4 en AIM2 inflammasomen hebben. Hierdoor kan er meer IL-1β en IL-18 gemaakt worden, waardoor patiënten ziek worden. Wat we nog niet weten, is hoe en waarom deze genen meer aanwezig zijn in sJIA patiënten. Om deze vragen te beantwoorden is extra onderzoek nodig.

Introduction

Systemic juvenile idiopathic arthritis (sJIA) is a severe autoinflammatory disease that mainly affects young children[1]. It is currently classified under the umbrella of juvenile idiopathic arthritis (JIA) of which oligoarthritis and polyarthritis are the most common[2]. All types of JIA are characterised by joint inflammation, but in contrast to the other subtypes of JIA, sJIA is characterised by systemic inflammation and autoinflammatory characteristics[3]. Patients with sJIA experience symptoms related to systemic inflammation, such as a quotidian relapsing-remitting fever, rash, hepatomegaly, splenomegaly, lymphadenopathy, and serositis[3,4]. Approximately 10% of the children with sJIA also develop macrophage activation syndrome (MAS)[5], which is a secondary form of hemophagocytic lymphohistiocytosis (HLH)[6]. Furthermore, it is suggested that MAS occurs subclinically in 30% of the sJIA cases[7]. Patients with MAS experience unremitting fever, pancytopenia, elevated triglycerides, elevated levels of ferritin (>1000 ng/mL), decreased natural killer (NK) cell counts and impaired NK cell activity[8,9]. MAS episodes, which can be recurrent, can be triggered by viral infections, e.g., Epstein-Barr virus, or new medications, but often no trigger is identified[8].

Even though the pathophysiology of sJIA is incompletely understood, it has distinct autoinflammatory characteristics, such as activation of the innate immune system resulting in neutrophilic leukcytosis[3]. sJIA patients have elevated levels of the proinflammatory cytokines interleukin (IL)-1β, IL-6 and IL-18[10–12]. IL-18 is especially elevated in sJIA patients with MAS[13]. The importance of these cytokines in the pathophysiology of sJIA is underlined by the therapeutic effect of anti-IL-1β and anti-IL-6 treatment[14–16]. sJIA patients without severe symptoms or MAS usually are treated with nonsteroidal anti-inflammatory drugs (NSAIDs)[3]. However, not all patients have a full response to this treatment and corticosteroids or biologic agents are required[3]. There are three types of biologic agents sJIA patients are usually treated with: anakinra (a human recombinant IL-1 receptor antagonist (rIL-1Ra))[14], canakinumab (a monoclonal anti-IL-1β)[15] or tocilizumab (a monoclonal anti-IL-6 receptor)[16]. sJIA patients with MAS are usually treated with high dose corticosteroids, cyclosporine, and biologicals[8]. In addition to the elevated levels of cytokines IL-1β, IL-6, and IL-18, patients also have elevated levels of S100A8/A9 (calprotectin) and S100A12 (calgranulin C) proteins and acute-phase protein ferritin[17–19].

Interestingly, these biomarkers are all associated with inflammasome activation[20]. Inflammasomes are multiprotein complexes that play a crucial role in the innate host defence against pathogens[21]. Functional inflammasome complexes have been found in a variety of human cells, such as T cells, monocytes, macrophages, neutrophils, gastric cells, and airway epithelial cells[22]. Formation and activation of inflammasomes results in two major effector functions. One of them is cytokine maturation and release. Pro-IL-1β and pro-IL-18 are cleaved by inflammasome complexes into their bioactive forms, which results in local and systemic responses to infections[23]. IL-1β mediates transmigration of leukocytes, it induces fever, and promotes B cell proliferation, antibody production, and T cell survival[23]. Furthermore, IL-1β and IL-18 promote polarisation of T helper 1 (T_H1), T_H2, and T_H17 responses[23]. In contrast to IL-1β, IL-18 does not induce fever[23]. The other major effector function is pyroptosis, which is a type of inflammatory cell death in which the cell membrane is ruptured and cytoplasmic molecules activating the immune system, such as danger-associated molecular patterns (DAMPs), are released[23].

Assembly of inflammasomes is initiated by pattern recognition receptors (PRRs) that recognise pathogen-associated molecular patterns (PAMPs) or DAMPs[21]. Multiple receptor proteins that activate inflammasome formation are known, such as nucleotide-binding oligomerization domain leucine-rich repeat receptors (NLRs), absent in melanoma 2 (AIM2)-like receptors (ALRs) and pyrin[21,24]. Once the receptor is activated, it oligomerises into the inflammasome complex, which consists of different domains varying between inflammasomes, such as the nucleotide-binding and oligomerisation domain (NACHT), leucine-rich repeat (LRR) domain, function to find domain (FIND), HIN domain, and B30.2 domain (Figure 1A)[21,25].

Next, the inflammasome complex can bind pro-caspase-1. Pro-caspase-1 can bind to the caspase activation and recruitment domain (CARD) which is either present in the receptor itself or in the recruited adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) (Figure 1A)[26]. ASC consists of a pyrin domain (PYD), which binds to the inflammasome, and a CARD, which binds pro-caspase-1[26]. After binding, pro-caspase-1 is activated and cleaves pro-IL-1β and pro-IL-18 into their bioactive forms (Figure 1B)[26]. Furthermore, caspase-1 cleaves gasdermin D into gasdermin D N-terminal, which forms pores in the cell membrane (Figure 1B)[27]. This pore formation allows IL-1β and IL-18 to leave the cell and it can induce pyroptosis[23].

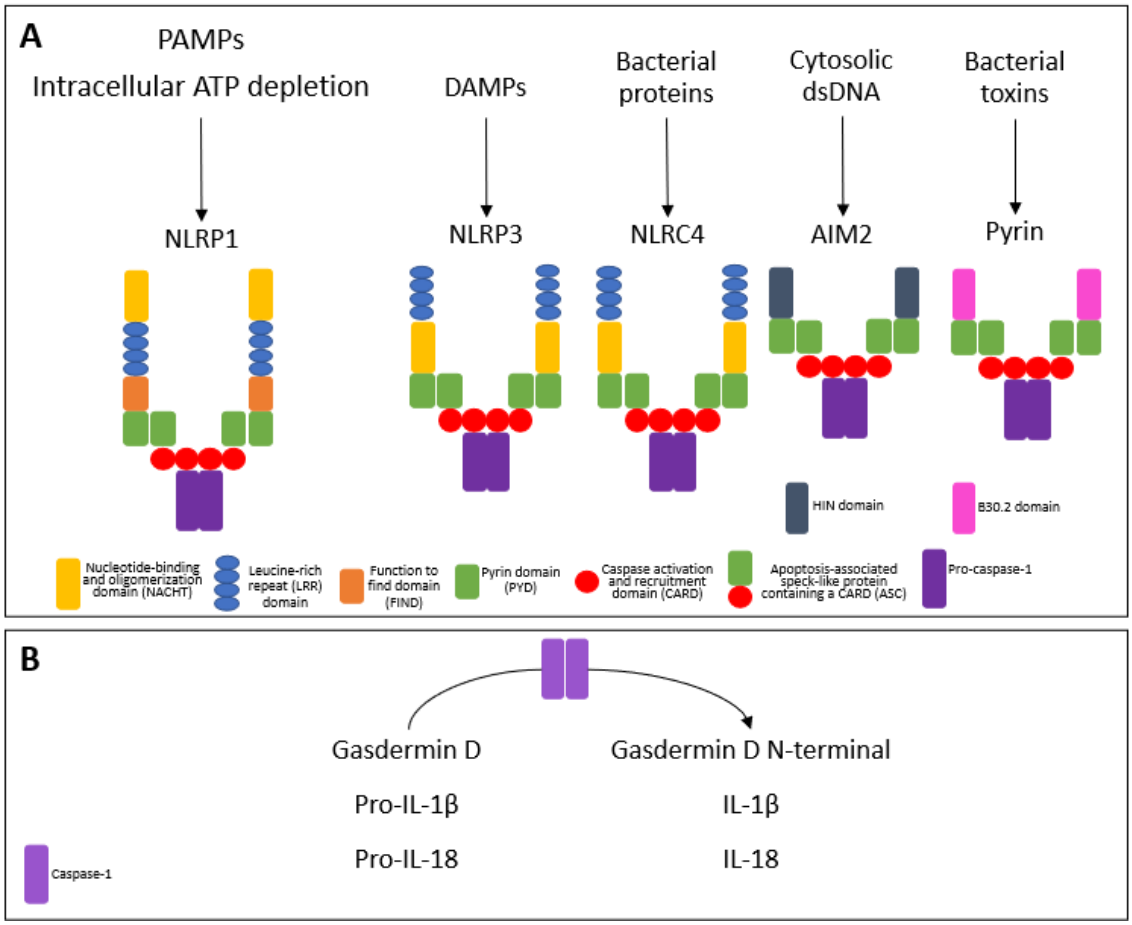

Figure 1: Inflammasome activation. A) The nucleotide-binding oligomerization domain leucine-rich repeat (NLR) pyrin domain containing 1 (NLRP1) is activated by pathogen-associated molecular patterns (PAMPs) and intracellular ATP depletion. The NLRP3 inflammasome is activated by danger-associated molecular patterns (DAMPs). The NLR caspase activation and recruitment domain (CARD) containing 4 (NLRC4) inflammasome is activated by bacterial proteins. The absent in melanoma 2 (AIM2) inflammasome is activated by double stranded DNA. The pyrin inflammasome is activated by bacterial toxins. B) Caspase-1, activated by inflammasomes, cleaves gasdermin D into gasdermin D N-terminal and pro-interleukin (IL)-1β and pro-IL-18 into their bioactive forms.

Inflammasomes are classified into different families based on their receptor. The best understood inflammasomes are NLR pyrin domain containing 1 (NLRP1), NLRP3 (cryopyrin), NLR CARD domain containing 4 (NLRC4), AIM2, and pyrin. The NLRP inflammasomes, AIM2, and pyrin require ASCs to bind pro-caspase-1, while NLRC4 can bind pro-caspase-1 directly via CARD and indirectly via ASC[28]. As mentioned above, the inflammasomes are activated by PAMPs or DAMPs, which vary for the different inflammasomes (Figure 1A). The NLRP1 inflammasome can be activated by PAMPs and intracellular ATP depletion[29]. NLRP3 can be activated by multiple DAMPs, such as the production of reactive oxygen species (ROS), elevated intracellular calcium levels, and potassium efflux out of the cell[26]. The NLRC4 inflammasome forms a complex with NAIP proteins that bind bacterial proteins[24]. The AIM2 inflammasome is activated once ALRs bind cytosolic double stranded DNA[30] and the pyrin inflammasome is activated by bacterial toxins[31].

Interestingly, dysregulations of the NLRP1, NLRP3, NLRC4, AIM2 and pyrin inflammasome have been linked to a variety of autoinflammatory and autoimmune diseases, and they are all linked to childhood rheumatic diseases[20]. The association of these inflammasomes with autoinflammatory diseases and childhood rheumatic diseases makes their involvement in sJIA expected. As mentioned above, multiple biomarkers of sJIA are associated with inflammasomes. IL-1β and IL-18 both are products of inflammasome activation[26], S100A8/A9 and S100A12 proteins can prime NLRP3 inflammasome activation[32], and ferritin is associated with inflammasome activation[33]. These biomarkers thus suggest inflammasomes might be involved in the pathophysiology of sJIA. Furthermore, gain-of-function mutations in the nucleotide-binding domain of NLRC4 are associated with MAS [8,34]. However, the presence and possible role of this mutation in sJIA is unknown. Even though these associations suggest involvement of inflammasomes in sJIA, the question remains if and what inflammasomes are involved in the pathophysiology of sJIA. This knowledge is important for future research into the treatment and possible prevention of sJIA. This review will therefore focus on the current understanding of the possible role of the five best understood inflammasomes in the pathophysiology of sJIA.

Overactivation of inflammasomes in sJIA

As described in the introduction, sJIA patients have biomarkers associated with inflammasome activation. Several of the biomarkers can prime inflammasome activation, which encompasses elevated expression of inflammasome proteins[20]. This priming ensures enough proteins are available for activation of inflammasomes by PAMPs and DAMPs[20]. S100A8/A9 and S100A12 are known to prime NLRP3 inflammasome activation, which will be discussed later[20]. Ferritin is also associated with the priming of inflammasome activation[33]. Two recent studies reported significantly elevated serum ferritin levels in sJIA patients compared to healthy controls[18,19]. Serum ferritin is primarily derived from macrophages and IL-1β is a trigger of ferritin secretion[35]. The elevated ferritin levels could contribute to inflammasome activation since it was found ferritin primes inflammasome activation in macrophages *in vitro*[33]. However, the role of ferritin in inflammasome activation in sJIA patients is unclear.

In addition to biomarkers associated with the priming of inflammasome activation, sJIA patients also have biomarkers associated with the activation of inflammasomes. IL-1β and IL-18 are both products of inflammasome activation and increased levels of these cytokines suggests overactivation of inflammasomes[11,12]. Furthermore, another product of inflammasome activation is gasdermin D N-terminal. Once an inflammasome is activated, gasdermin D is cleaved into the N-terminal p30 fragment, which forms the pore in the cell membrane[18]. Nagai *et al.* measured gasdermin D N-terminal levels in serum of active sJIA patients and found that the levels were significantly increased compared to healthy controls and patients in remission[18]. This suggests inflammasome activation is increased in active sJIA patients. However, it remains unclear which cells are responsible for the increased levels of gasdermin D N-terminal. Furthermore, it is unknown which inflammasomes are responsible for the extra cleavage of gasdermin D. Future research is required to determine which inflammasomes and which cells are responsible for the elevated gasdermin D N-terminal levels in sJIA patients.

Thus, the significantly higher levels of IL-1β, IL-18 and gasdermin D N-terminal indicate overactivation of inflammasomes. Furthermore, elevated S100A8/A9, S100A12 and ferritin levels could result in overactivation of inflammasomes since these proteins are known to prime inflammasome activation. However, none of these biomarkers directly proves the involvement of specific inflammasomes in the pathophysiology of sJIA.

Involvement of different inflammasomes in sJIA

It thus remains unclear which inflammasomes are affected in sJIA and which inflammasomes are responsible for the elevated levels of IL-1β, IL-18, and gasdermin D N-terminal. Here, we will discuss the evidence of the involvement of the five best understood inflammasomes, NLRP1, NLRP3, NLRC4, AIM2, and pyrin, in the pathophysiology of sJIA. Since direct research into the role of the different inflammasomes in sJIA unfortunately remains scarce, we will use the symptoms, cytokine expression and treatments of monogenic autoinflammatory disorders, also called inflammasomopathies, as a comparison to sJIA to determine the likelihood of the involvement of the respective inflammasome in the pathophysiology of sJIA.

Inflammasomopathies can arise from mutations in genes encoding NLRP1, NLRP3, NLRC4, AIM2, and pyrin inflammasomes or genes encoding direct or indirect inflammasome regulators[36]. Since the different inflammasomes are expressed at distinct locations in the body, the inflammasomopathies come with different symptoms of various severity[36]. E.g., NLRP3 and pyrin are expressed in innate immune lineages and mutations in genes encoding these inflammasomes can lead to a widespread immunopathology[36]. On the other hand, the NLRC4 inflammasome is mainly expressed in the gut and the NLRP1 and AIM2 inflammasomes in the skin[36]. Since sJIA is characterised by systemic inflammation[3], the involvement of NLRP3 and pyrin is likely. Below, a selection of inflammasomopathies is described and compared to sJIA. Furthermore, single nucleotide polymorphisms (SNPs) in genes encoding inflammasome proteins and altered gene expression of inflammasome encoding genes in sJIA patients are discussed.

The NLRP1 inflammasome

Gain-of-function mutations in the PYD domain of *NLRP1* can cause multiple self-healing palmoplantar carcinoma (MSPC), familial keratosis lichenoides chronica (FKLC), and inherited corneal intraepithelial dyskeratosis without systemic inflammation, which are all pre-cancerous conditions[37]. These pre-cancerous conditions are characterised by hyperkeratotic ulcerative skin lesions[37]. Furthermore, gain-of-function mutations in other domains of *NLRP1* are associated with systemic inflammation, arthritis, and dyskeratosis[37]. All disorders caused by a gain-of-function mutation in *NLRP1* are characterised by elevated IL-1β levels, which can be treated with IL-1 inhibitors[37,38].

Even though both sJIA and NLRP1 inflammasomopathies are characterised by elevated IL-1β levels, this does not indicate involvement of the NLRP1 inflammasome in sJIA. The symptoms of NLRP1 inflammasomopathies caused by gain-of-function mutations in the PYD domain of *NLRP1* are distinct from sJIA, which suggest this part of the NLRP1 inflammasome is not affected in sJIA (Table 1). Interestingly, gain-of-function mutations in other domains of *NLRP1* are associated with symptoms that are also seen in sJIA patients, such as systemic inflammation and arthritis. However, these symptoms are quite general for inflammasomopathies and thus do not necessary suggest an association between the NLRP1 inflammasome and sJIA.

Studies regarding SNPs in *NLRP1* contribute to this statement since no association between SNPs in *NLRP1* and sJIA has been found. In one study, two SNPs in *NLRP1* (rs6502867 and rs12150220) were assessed, but no association with JIA was found[39]. In another study, sixteen SNPs were studied across the *NLRP1* gene locus and none were associated with JIA susceptibility[40]. In both studies the cohorts consisted of JIA patients with different subtypes, such as sJIA and oligoarthritis.

Together, these data indicate the NLRP1 inflammasome is less likely to be the main inflammasome affected in the pathophysiology of sJIA. So far, no SNPs across the *NLRP1* gene locus are associated with sJIA. Furthermore, symptoms of NLRP1 inflammasomopathies caused by mutations in the PYD domain of *NLRP1* are distinct from sJIA. Gain-of-function mutations in other domains of *NLRP1* are associated with symptoms that are also seen in sJIA patients, but these symptoms are common for inflammasomopathies and thus do not necessary suggest an association between the NLRP1 inflammasome and sJIA.

The NLRP3 inflammasome

Cryopyrin associated periodic syndrome (CAPS) is a monogenic autoinflammatory disorder caused by gain-of-function mutations in *NLRP3* which encodes cryopyrin, also called the NLRP3 inflammasome[41]. Different syndromes of various severity are considered to be a CAPS: familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS) and neonatal onset multisystem inflammatory disease (NOMID)[36]. Symptoms typically occur within the first year of life and are systemic, cutaneous, musculoskeletal, and central nervous system inflammation[36,41]. Furthermore, CAPS patients have increased IL-1β levels, which can be normalised by IL-1 targeted therapy, such as anakinra[41].

CAPS and sJIA are both characterised by systemic and cutaneous inflammation and increased IL-1β levels (Table 1). Furthermore, patients of both diseases respond well to IL-1 targeted therapy. In both diseases the IL-1β levels normalise after treatment. These similarities suggest that overactivation of the NLRP3 inflammasome is involved in sJIA. However, in contrast to sJIA, no elevated levels of IL-18 and gasdermin D have been reported in CAPS patients. Furthermore, CAPS patients also experience musculoskeletal and central nervous system inflammation, which is not characterizing for sJIA. Another difference is the age of onset, patients with CAPS typically experience symptoms within the first year of life, while sJIA patients are older. Overactivation of the NLRP3 inflammasome by a gain-of-function mutation thus induces symptoms that are partially similar to the symptoms of sJIA, but sJIA is characterised by systemic inflammation and more elevated IL-18. This comparison between CAPS and sJIA thus does not clearly indicate or exclude the involvement of the NLRP3 inflammasome in sJIA.

Fortunately, multiple studies have analysed genetic data for a possible association between sJIA and genes encoding inflammasome proteins, such as *NLRP3*. Yang *et al.* (2014) included DNA extracted from whole blood of 22 sJIA patients, 96 non-systemic JIA patients, and 103 healthy controls[42]. The DNA was genotyped for two SNPs, one located in *NLRP3* (rs4353135) and one in *CARD8* (rs2043211), which is an inflammasome sensor involved in the NLRP3 inflammasome[42,43]. The group of non-systemic JIA patients consisted for a significantly higher percentage of rs4353135 variant G allele carriers compared to healthy controls[42]. This variant rs4353135 G allele is associated with increased levels of inflammatory markers and an enhanced lymphocytes IL-17 response, which mediates an IL-6 response[44]. However, in the group of sJIA patients, no difference in the percentage of rs4353135 and rs2043211 SNP carriers was found compared to healthy controls[42].

A second study investigated the Q705K (glutamine is changed to lysine) polymorphism in *NLRP3,* which is a gain-of-function alteration resulting in overactivation of the NLRP3 inflammasome[45]. Furthermore, this polymorphism is associated with rheumatoid arthritis[46]. However, no significant correlation between this polymorphism and JIA patients, among which several sJIA patients, was found[45]. Perica *et al.* also studied a polymorphism in Toll-like receptor (TLR) 2 (R753Q, arginine to glutamine) and two polymorphisms in TLR4 (D299G, aspartic acid to glycine; and T399I, threonine to isoleucine). Both TLRs can activate the NLRP3 inflammasome and the polymorphisms are associated with inflammation[45]. None of these polymorphisms were associated with JIA[45]. However, this study was only performed with 11 JIA blood samples, and it was suggested it should be repeated in a larger study[45]. In another study, DNA samples of JIA patients, of which 175 belonged to sJIA patients, were genotyped for 45 SNPs across the *NLRP3, NOD2*, and *MEFV* loci[47]. These genes are all involved in the activation of inflammasomes[47]. No association between the tested SNPs and sJIA patients was found[47]. Thus, so far, no polymorphisms across the *NLRP3* gene locus or polymorphisms in genes encoding proteins involved in NLRP3 inflammasome assembly or activation are associated with sJIA.

In addition to polymorphisms, altered gene expression of genes encoding inflammasomes could indicate overactivation of inflammasomes. In two studies, the gene expression levels in neutrophils of sJIA patients were studied. In the first study, neutrophils were isolated and purified from whole blood, total RNA was extracted from the neutrophils, and cDNA made of RNA was sequenced. 214 differentially expressed genes were found in neutrophils of sJIA patients compared to neutrophils of

healthy controls[48]. One of the significantly differentially expressed genes in active sJIA patients compared to healthy controls was *TLR2* (log-fold change 1.31), which can activate the NLRP3 inflammasome[48]. No other genes involved in activation or assembly of the NLRP3 inflammasome were found to be significantly differentially expressed in neutrophils of sJIA patients[48].

In the second study, 1693 differentially expressed genes were found in neutrophils of sJIA patients compared to neutrophils of healthy controls[49]. Two of the upregulated genes in neutrophils of sJIA patients were *S100A8* (log-fold change 2.90) and *S100A12* (log-fold change 3.67), which can prime NLRP3 inflammasome activation[49]. No other genes involved in activation or assembly of the NLRP3 inflammasome were found to be significantly differentially expressed in neutrophils of sJIA patients[49].

Thus, no altered gene expression of *NLRP3* was identified in the neutrophils of sJIA patients. Upregulation of *TLR2, S100A8*, and *S100A12* in the neutrophils of sJIA patients could indicate overactivation of the NLRP3 inflammasome since these genes are involved in the activation of the NLRP3 inflammasome. However, since the *NLRP3* gene was not differentially expressed, overactivation of the NLRP3 inflammasome is less likely.

Besides the upregulation of *S100A8* and *S100A12* in neutrophils of sJIA patients, the serum levels of S100A8/A9 and S100A12 are also elevated in sJIA patients compared to healthy controls. S100A8/A9 and S100A12 function as alarmins and amplify innate immune signalling[48]. They are excreted predominantly by phagocytes[17]. S100A8/A9 and S100A12 are sensed by TLRs, mainly TLR4, which leads to activation of the NF-κB pathway, which in turn enhances the transcription of *NLRP3* and of the precursors of IL-1β and IL-18[20]. In three studies, serum levels of S100A8/A9 and S100A12 in sJIA patients were measured. The first study showed significantly higher serum levels in sJIA patients compared to non-systemic JIA patients (S100A8/A9: 31,465 ng/mL versus 1,471 ng/mL; S100A12: 2,075 ng/mL versus 100 ng/mL)[17]. Furthermore, it was found children with active sJIA had significantly higher S100A8/A9 and S100A12 levels than children with inactive sJIA (S100A8/A9: 31,465 ng/mL versus 1,685 ng/mL; S100A12: 2,075 ng/mL versus 130 ng/mL)[17].

The two other studies have shown that S100A8/A9 and S100A12 levels are elevated in sJIA patients and that these levels correlate with the neutrophil counts. In the first study, neutrophil counts and S100A8/A9 and S100A12 serum levels were compared between active and inactive sJIA patients[48]. Significantly higher levels of S100A8/A9 and S100A12 were found in active sJIA patients compared to inactive sJIA patients (S100A8/A9: 6,869 ng/mL versus 956 ng/mL; S100A12: 220 ng/mL versus 76 ng/mL)[48]. Furthermore, the neutrophil counts were strongly correlated with S100A8/A9 serum levels[48]. This study also quantified S100A8/A9 and S100A12 release by neutrophils isolated from sJIA patients. No significant difference in S100A8/A9 and S100A12 levels released by unstimulated neutrophils was found between cells isolated from active and inactive sJIA patients and healthy controls[48]. Furthermore, no significant difference in S100A8/A9 and S100A12 levels excreted by neutrophils stimulated with phorbol myristate acetate (PMA) were found between active and inactive sJIA patients[48]. Stimulation did lead to significantly increased S100A8/A9 levels excreted by neutrophils of sJIA patients compared to neutrophils of healthy controls[48].

In the second study, neutrophil counts and S100A8/A9 and S100A12 serum levels in sJIA patients during disease and three months after initiation of anakinra (rIL-1Ra therapy) were measured[49]. Neutrophil counts were found to be significantly elevated during active disease and the count dropped to normal after three months of anakinra[49]. Furthermore, the levels of S100A8/A9 and S100A12 were significantly increased in active sJIA patients compared to patients that were treated with rIL-1Ra therapy[49]. Both studies thus showed patients with sJIA have significantly elevated S100A8/A9 and S100A12 levels and elevated neutrophil counts.

Thus, S100A8/A9 and S100A12 proteins are elevated in active sJIA patients. This suggests increased activation of the NLRP3 inflammasome in active sJIA patients, since S100A8/A9 and S100A12 can prime activation of the NLRP3 inflammasome. However, as mentioned above, the NLRP3 inflammasome encoding genes are not upregulated. This suggests there is no increased activation of the NLRP3 inflammasome. Therefore, the role of the elevated S100A8/A9 and S100A12 levels on NLRP3 inflammasome activation in sJIA seems to be unclear. It could be possible that in sJIA patients overactivation of the NLRP3 inflammasome is prevented by active downregulation of the NF-κB pathway or *NLRP3* gene to counteract the effect of S100A8/A9 and S100A12. However, further research is required to determine the role of elevated S100A8/A9 and S100A12 levels on the NLRP3 inflammasome in sJIA. Interestingly, S100A8/A9 and S100A12 can also prime inflammasome activation by inducing transcription of pro-IL-1β and pro-IL-18, which can be cleaved into their bioactive forms by caspase-1 in inflammasomes[20]. The elevated levels of these proteins thus could explain the elevated levels of IL-1β and IL-18 found in sJIA patients.

Together, these data do not elucidate the possible role of the NLRP3 inflammasome in the pathophysiology of sJIA. Overactivation of the NLRP3 inflammasome by a gain-of-function mutation induces CAPS, a disease with symptoms that are partially similar to the symptoms of sJIA. This could indicate involvement of the NLRP3 inflammasome in sJIA. So far, no SNPs across the *NLRP3* gene locus are associated with sJIA. Furthermore, no altered gene expression of *NLRP3* has been reported in sJIA patients. Interestingly, genes encoding proteins, S100A8/A9 and S100A12, that can activate the NLRP3 inflammasome, have been reported to be upregulated in sJIA patients. However, the NLRP3 inflammasome encoding genes are not upregulated in neutrophils, which suggests no increased activation of the NLRP3 inflammasome in neutrophils. Of course, this does not exclude the NLRP3 inflammasome since the expression in other cells is unknown. However, the role of the elevated S100A8/A9 and S100A12 levels on NLRP3 inflammasome activation in sJIA seems to be unclear. These proteins could be involved in the elevated levels of IL-1β and IL-18 found in sJIA patients, since they induce transcription of pro-IL-1β and pro-IL-18. In conclusion, there is no prove yet of gain-of-function mutations in *NLRP3* or overexpression of genes encoding the NLRP3 inflammasome in neutrophils of sJIA patients. Although this does not completely exclude NLRP3 as the inflammasome involved in active sJIA, it does make it a less likely culprit.

Other members of the NLRP family

Another study identified two *de novo* microduplications in several genes involved in inflammatory pathways in a sJIA patient[50]. The microduplications encompass *IL11*, which has been reported to correlate with the severity of arthritis in sJIA patients, *HSPBP1*, which is involved in the regulation of immune responses, and *NLRP2, NLRP9*, and *NLRP11*, which are all members of the NLRP family involved in inflammasome activation[50]. According to Tadaki *et al.* duplication of the *NLRP* gene cluster may significantly contribute to the pathophysiology of sJIA since this cluster is correlated with inflammatory pathways[50]. However, this study does not show the effect of the microduplications on gene expression of these genes. So, it remains unclear what the precise effect of the microduplications on this gene cluster is. Furthermore, the microduplications have only been found in one of the fifty tested sJIA patients. Future research is required to determine the effect of the microduplications on the *NLRP* gene cluster and the assembly and activation of inflammasomes. In addition, it would be valuable to know if these microduplications are seen more often in sJIA patients compared to healthy controls.

The NLRC4 inflammasome

In addition to the NLRP family, the NLRC4 inflammasome is also associated with a variety of inflammatory disease. The best known NLRC4 inflammasomopathy is caused by gain-of-function mutations that can lead to systemic inflammation with multiple episodes of MAS, which is characterised by flares with fever, elevated IL-18 levels, and elevated ferritin serum levels[51].

Another NLRC4 inflammasomopathy is autoinflammation with infantile enterocolitis (AIFEC)[36]. These patients experience hyperinflammation from an early age and it is characterised by rash, joint inflammation, severe intestinal disease, hepatosplenomegaly, and elevated IL-1β levels[36,52]. Furthermore, AIFEC patients experience symptoms and biomarkers typical for MAS[36]. Patients are usually treated with IL-1 inhibitors, but the elevated IL-18 levels remain[36].

The symptoms of these diseases suggest the NLRC4 inflammasome is involved in the pathophysiology of sJIA. sJIA and AIFEC are characterised by similar symptoms, such as rash and joint inflammation, and by elevated IL-1β, IL-18 and ferritin serum levels (Table 1). However, sJIA is not characterised by severe intestinal disease, which is the case for AIFEC patients. Since the NLRC4 inflammasome is mainly expressed in the gut, local symptoms in NLRC4 inflammasomopathies are expected to be common. Interestingly, MAS patients do not experience symptoms in the gut even though the NLRC4 inflammasome is affected. Since the symptoms and biomarkers of MAS are partially similar to sJIA, involvement of the NLRC4 inflammasome in sJIA is likely. Furthermore, since 10-30% of the patients with sJIA also develop MAS, NLRC4 might play a key role in sJIA.

However, no studies have reported on polymorphisms across the *NLRC4* gene locus associated with sJIA patients or sJIA patients with MAS, which could have been a direct indicator of the involvement of the NLRC4 inflammasome in sJIA. Fortunately, two studies analysed the gene expression levels in neutrophils of sJIA patients. In the first study, 214 differentially expressed genes were found in neutrophils of sJIA patients compared to neutrophils of healthy controls[48]. One of the significantly differentially expressed genes in active sJIA patients compared to healthy controls, was *NLRC4* (log-fold change 1.42)[48]. No other genes involved in activation or assembly of the NLRC4 inflammasome were found to be significantly differentially expressed in neutrophils of sJIA patients[48]. In the second study, 1693 differentially expressed genes were found in neutrophils of sJIA patients compared to neutrophils of healthy controls[49]. Again, *NLRC4* was found to be upregulated in neutrophils sJIA patients compared to healthy controls (log-fold change 2.99)[49]. A third study investigated the gene expression levels in the blood of sJIA patients and found highly upregulated levels of *CARD12/IPAF* (log-fold change 2.09) compared to healthy controls[14]. *CARD12/IPAF* is involved in the assembly of the NLRC4 inflammasome[14]. No other genes involved in activation or assembly of the NLRC4 inflammasome were found to be significantly differentially expressed in the blood of sJIA patients[14].

These studies thus show upregulation of *NLRC4* and *CARD12/IPAF*, which involved in the assembly of the NLRC4 inflammasome, in sJIA patients. However, none of the studies differentiates between sJIA patients without MAS and sJIA patients with MAS. It thus remains unknown if the upregulation of these genes is caused by, e.g., gain-of-function mutations in *NLRC4* as has been reported for MAS and thus if these upregulation of *NLRC4* is associated with MAS and not sJIA. Therefore, it would be insightful to repeat these studies with sJIA patients with and without MAS to observe the role of *NLRC4* overexpression in sJIA patients with and without MAS. Furthermore, studying SNPs across the *NLRC4* gene locus that are associated with MAS, in the same study, could indicate whether the upregulation of *NLRC4* is linked to MAS or not.

Together, these data indicate involvement of the NLRC4 inflammasome in the pathophysiology of sJIA. The symptoms and biomarkers of NLRC4 inflammasomopathies and sJIA are similar. Furthermore, *NLRC4* is upregulated in neutrophils of sJIA patients, which could indicate overactivation of the NLRC4 inflammasome. However, since 10-30% of the sJIA patients also experience MAS, it could be possible this upregulation is linked to gain-of-function mutations in *NLRC4* known to cause MAS. Therefore, further research is required to elucidate the role of the NLRC4 inflammasome in sJIA patients with and without MAS.

The AIM2 inflammasome

At the time of writing, no AIM2-driven inflammasomopathy has been reported[36]. However, in several autoimmune diseases, such as psoriasis, colitis, and systemic lupus erythematosus (SLE), differential expression of *AIM2* has been observed[53]. Overactivation of the AIM2 inflammasome in these patients is most likely caused by self-DNA present in the cytosol[53]. One of the sources of self-DNA, are neutrophil extracellular traps (NETs), which are made by activated neutrophils of their own DNA as a defence mechanism[54]. In SLE, NETs are known to bind the AIM-like receptor leading to activation of the AIM2 inflammasome[55]. Since the neutrophil count in sJIA patients is significantly elevated[48,49], it is likely more NETs are produced in sJIA patients than in healthy controls, which could induce overactivation of the AIM2 inflammasome.

Interestingly, *AIM2* has been identified as an upregulated gene in neutrophils of sJIA patients. In one study, 214 differentially expressed genes were found in neutrophils of sJIA patients compared to neutrophils of healthy controls[48]. One of the significantly differentially expressed genes in active sJIA patients compared to healthy controls, was *AIM2* (log-fold change 2.08)[48]. In another study, 1693 differentially expressed genes were found in neutrophils of sJIA patients compared to neutrophils of healthy controls[49]. Again, *AIM2* was found to be upregulated in neutrophils of sJIA patients compared to healthy controls (log-fold change 3.64)[49].

These data suggest that the AIM2 inflammasome might be involved in the pathophysiology of sJIA. Overexpression of *AIM2* in neutrophils of sJIA patients has been reported in two studies, but it is unclear whether more AIM2 inflammasomes are assembled. Furthermore, elevated neutrophils counts in sJIA patients suggest higher levels of NETs which can induce activation of the AIM2 inflammasome.

The pyrin inflammasome

Familial Mediterranean fever (FMF) is an inflammasomopathy caused by gain-of-function mutations in *MEFV*, which encodes pyrin[36]. The symptoms of FMF typically start in childhood and consist of fever episodes with serosal inflammation which manifests with abdominal pain, chest pain, arthralgia, monoarticular arthritis, and skin rash[56]. The episodes usually last 48-72 hours and inflammation is controlled by colchicine and IL-1 inhibitors[56]. Furthermore, IL-18 and IL-6 levels are elevated in FMF patients[57].

Another monogenic autoinflammatory disorder caused by gain-of-function mutations in *MEFV*, is pyrin associated autoinflammation with neutrophilic dermatosis (PAAND)[36]. Symptoms of this disease also come in recurrent episodes lasting weeks and include fever, myalgia, myositis, arthralgia, and neutrophilic dermatosis[36,37]. This disorder is treated similarly as FMF, namely with colchicine and IL-1 inhibitors[36,57]. Monocytes of PAAND patients have a significantly increased spontaneous production of IL-1β and IL-18[56].

FMF and sJIA are both characterised by elevated IL-18 and IL-6 levels (Table 1). In contrast to sJIA patients, FMF patients do not have increased IL-1β levels[57]. PAAND on the other hand, is characterised by elevated IL-1β and IL-18 levels, similar as sJIA (Table 1). However, increased levels of pro-inflammatory cytokines are not pyrin inflammasome specific, since other inflammasomes can also cleave the precursors of IL-1β and IL-18. Interestingly, both diseases are characterised by fever episodes that last from a couple of days or a few weeks. sJIA patients, experience quotidian fever, which can also occur in episodes of weeks. Furthermore, other symptoms, such as serositis and rash, are also common for FMF, PAAND and sJIA. These similarities suggest the pyrin inflammasome is involved in the pathophysiology of sJIA. However, no studies have reported on polymorphisms across the *MEFV* locus associated with sJIA. Furthermore, no altered gene expression of *MEFV* has been reported in sJIA patients.

Even though the pyrin inflammasomopathies indicate involvement of the pyrin inflammasome in sJIA, the lack of data showing genetic proof of an association, suggests the pyrin inflammasome is less likely involved in the pathophysiology of sJIA.

Table 1: Overview of symptoms, biomarkers, and treatment of NLRP1, NLRP3, NLRC4 and pyrin inflammasomopathies and systemic juvenile idiopathic arthritis (sJIA). FKLC: familial keratosis lichenoides chronica; MSPC: multiple self-healing palmoplantar carcinoma; CAPS: cryopyrin associated periodic syndrome; MAS: macrophage activation syndrome; AIFEC: autoinflammation with infantile enterocolitis; FMF: familial Mediterranean fever; PAAND: pyrin associated autoinflammation with neutrophilic dermatosis.

Inflammasomopathy	Gene	Symptoms	Biomarkers	Treatment
Pre-cancerous conditions, e.g., FKLC and MSPC	*NLRP1*	Hyperkeratotic ulcerative skin lesions	↑ IL-1β	IL-1 inhibitors
CAPS	*NLRP3*	Systemic, cutaneous, musculoskeletal, and central nervous system inflammation	↑ IL-1β	IL-1 inhibitors
MAS	*NLRC4*	Flares with fever and pancytopenia	↑ IL-18 ↑ Ferritin ↓ NK cell count ↓ NK cell activity	Corticosteroids, cyclosporine, and IL-1 inhibitors
AIFEC	*NLRC4*	Hyperinflammation, rash, joint inflammation, severe intestinal disease, and hepatosplenomegaly + MAS symptoms	↑ IL-1β ↑ IL-18 ↑ Ferritin	IL-1 inhibitors
FMF	*MEFV*	Fever episodes with serosal inflammation which manifests with abdominal pain, chest pain, arthralgia, monoarticular arthritis, and skin rash	↑ IL-6 ↑ IL-18	Colchicine and IL-1 inhibitors
PAAND	*MEFV*	Episodes of fever, myalgia, myositis, arthralgia, and neutrophilic dermatosis	↑ IL-1β ↑ IL-18	Colchicine and IL-1 inhibitors
Symptoms sJIA	Systemic inflammation, such as quotidian fever, rash, hepatomegaly, splenomegaly, lymphadenopathy, and serositis			
Biomarkers sJIA	↑ IL-1β, ↑ IL-18, ↑ IL-6, ↑ Ferritin			
Treatment sJIA	NSAIDs, corticosteroids, IL-1 inhibitors (anakinra, canakinumab), and IL-6 inhibitor (tocilizumab)			

Discussion

Taken together, evidence of the involvement of activation of specific inflammasomes in sJIA patients is limited. Even though the significantly higher levels of IL-1β, IL-18, and gasdermin D N-terminal indicate overactivation of inflammasomes[17,18], and elevated S100A8/A9, S100A12, and ferritin levels could result in overactivation of inflammasomes [17,33], none of these biomarkers directly proves the involvement of specific inflammasomes in the pathophysiology of sJIA. However, based on the data discussed in this review, the inflammasomes that are most likely involved in the pathophysiology of sJIA are NLRC4 and AIM2.

The data described in this study, indicate no involvement of the NLRP1 inflammasome in the pathophysiology of sJIA. So far, no SNPs across the *NLRP1* gene locus are associated with sJIA[39,40]. Moreover, symptoms of NLRP1 inflammasomopathies caused by mutations in the PYD domain of *NLRP1* are distinct from sJIA, which suggests no alterations in the PYD domain are involved in the pathophysiology of sJIA. Furthermore, it is not likely the pyrin inflammasome is involved in sJIA. Even though the symptoms of pyrin inflammasomopathies and sJIA are similar[36,56], the lack of data showing genetic proof of an association, suggests the pyrin inflammasome is less likely involved in the pathophysiology of sJIA.

Moreover, the data do not elucidate the possible role of the NLRP3 inflammasome in the pathophysiology of sJIA. The symptoms of the NLRP3 inflammasomopathy CAPS and sJIA are only partially similar. Furthermore, no SNPs across the *NLRP3* gene locus are associated with sJIA and no altered gene expression of *NLRP3* has been reported in sJIA patients[42,45,47]. Interestingly, genes encoding proteins, e.g., S100A8/A9 and S100A12, that can activate the NLRP3 inflammasome have been reported to be upregulated in sJIA patients[17,48,49]. However, the NLRP3 inflammasome encoding genes are not upregulated in neutrophils, which suggests no increased activation of the NLRP3 inflammasome in neutrophils. Of course, this does not exclude the NLRP3 inflammasome since the expression in other cells is unknown and thus should be examined. Another possibility is that in sJIA patients overactivation of the NLRP3 inflammasome is prevented by active downregulation of the NF-κB pathway or *NLRP3* gene to counteract the effect of S100A8/A9 and S100A12. However, further research is required to determine the role of elevated S100A8/A9 and S100A12 levels on the NLRP3 inflammasome.

Another effect of the S100A8/A9 and S100A12 proteins might be the elevated levels of IL-1β and IL-18 found in sJIA patients, since S100A8/A9 and S100A12 can also prime inflammasomes by inducing transcription of pro-IL-1β and pro-IL-18, which can be cleaved by inflammasomes into their bioactive forms[20]. This suggests elevated IL-1β and IL-18 levels might arise in sJIA patients independent of overactivation of inflammasomes. If there is more pro-IL-1β and pro-IL-18, more IL-1β and IL-18 can be cleaved independent of overactivation of inflammasomes. Normal activation of inflammasomes could be enough to cleave more cytokines. However, since the gasdermin D N-terminal levels are also increased in sJIA patients[18], overactivation of the inflammasome seems to occur.

Not only the role of S100A8/A9 and S100A12 proteins in priming inflammasomes is unclear in sJIA, the role of ferritin is also unclear. Secretion of serum ferritin is triggered by IL-1β and often derived from macrophages[35]. Interestingly, so far it is known that ferritin only primes inflammasome activation in macrophages[33]. This suggests that macrophages play a key role in inflammasome activation by ferritin. However, future research is required to determine the role of ferritin in inflammasome activation in sJIA patients. This role might be revealed by measuring inflammasome products, e.g., IL-1β, IL-18, and gasdermin D, in the supernatant of macrophages and other immune cells that have been primed with ferritin. These data could determine if ferritin can only prime inflammasome activation in macrophages or also in other cells. Furthermore, the cells could be isolated from the blood of sJIA patients and healthy controls to determine whether ferritin has the same effect in healthy cells and cells of sJIA

patients. If ferritin indeed activates inflammasomes in sJIA patients, other inflammasomes than the NLRP3 inflammasome are most likely involved since it has been found that *NLRP3* expression is similar in monocytes of sJIA patients with high and low levels of ferritin[58]. This suggests high levels of ferritin do not induce overexpression of the NLRP3 inflammasome, but other inflammasomes can be involved. If it turns out ferritin activates inflammasomes in sJIA patients, a positive feedback loop might cause the chronic autoinflammation; IL-1β induces ferritin secretion, which induces inflammasome activation, which induces the cleavage of pro-IL-1β into IL-1β. Even though the activator of this loop probably remains unknown based on the suggested experiment, it would present multiple targets for treatment of sJIA.

Inflammasomes that are likely involved in the pathophysiology of sJIA, are NLRC4 and AIM2. Overexpression of *AIM2* in neutrophils of sJIA patients has been reported in two studies[48,49]. Furthermore, elevated neutrophil counts in sJIA patients[48,49] suggest higher levels of NETs which can induce activation of the AIM2 inflammasome. Even though this would explain the involvement of the AIM2 inflammasome in sJIA, the cause still remains unknown, since the cause of the elevated neutrophil counts in sJIA patients is unclear. It might be that sJIA patients are more likely to get infections than healthy persons, which causes elevated neutrophil counts, but the cause of this possible increased sensitivity is unknown.

The role of NLRC4 in sJIA remains uncertain, even though the symptoms and biomarkers of NLRC4 inflammasomopathies and sJIA are similar[51]. Moreover, *NLRC4* is upregulated in the neutrophils of sJIA patients, which could indicate overactivation of the NLRC4 inflammasome[48,49]. However, since 10-30% of the sJIA patients also experience MAS, it could be possible this upregulation is linked to gain-of-function mutations in *NLRC4* known to cause MAS[8]. Therefore, further research is required to elucidate the role of the NLRC4 inflammasome in sJIA patients with and without MAS.

Interestingly, overexpression of *NLRC4* and *AIM2* occurs in neutrophils of sJIA patients, while it is known the NLRC4 and AIM2 inflammasomes are respectively mainly expressed in the gut and skin[36]. It would be insightful to assess whether gut and skin cells also have upregulated *NLRC4* and *AIM2* expression in sJIA patients compared to healthy controls. If there only is overexpression in the neutrophils, this would indicate that neutrophils are important players in the pathophysiology of sJIA. On the other hand, if these genes are also overexpressed in other cells, the type of inflammasome might be more determinative in the pathophysiology than the cell type. Since the neutrophil counts are upregulated in sJIA patients, it is likely these cells have a key role in the pathophysiology of sJIA.

If neutrophils would be the only cell type with overexpression of *NLRC4* and *AIM2*, it would be interesting to determine the trigger of overexpression of *NLRC4* and *AIM2* in these cells in sJIA patients. Are these the usual activators of these inflammasomes, respectively bacterial proteins and double stranded DNA, or other molecules? If bacterial proteins indeed activate the NLRC4 inflammasome in neutrophils, the question remains why neutrophils of sJIA patients are more sensitive to bacterial proteins. This might be because of a polymorphism in *NLRC4* that has not been discovered yet. Another option causing the overexpression of *NLRC4* and *AIM2* could be priming of the inflammasomes by S100A8/A9 and S100A12 proteins, which are upregulated in neutrophils. Furthermore, it is also possible polymorphisms in genes involved in the activation pathway of these inflammasomes, e.g., in TLRs, have not been found yet. If it is elucidated what triggers the activation of these inflammasomes in neutrophils of sJIA patients, this could help targeting the correct cells or inflammasomes to treat or prevent sJIA.

Although data suggesting inflammasome overactivation in neutrophils sJIA patients is present, other cells might also be involved. It is likely neutrophils are important cells in sJIA since *NLRC4* and *AIM2* are overexpressed in these cells and NETs are known to activate the AIM2 inflammasome. However, it could be possible macrophages are also involved in the pathophysiology of sJIA by contributing to a

positive feedback loop with ferritin. Experiments as suggested above should elucidate the role of macrophages and possibly also other cells in inflammasome activation by ferritin. Besides neutrophils and macrophages, other cells, such T cells, monocytes, gastric cells, and airway epithelial cells, could also be involved since these cells can have functional inflammasome complexes[22].

Furthermore, it is important to keep in mind that inflammasomes might not be the only factor in the pathophysiology of sJIA. Other proteins or pathways could also be involved in sJIA. Moreover, it remains unknown if no, only one, or multiple inflammasomes are involved in the pathophysiology of sJIA. The data suggest the involvement of multiple inflammasomes, which could possibly explain the variety of symptoms between patients and why some patients get MAS and others not. It might be possible that based on certain symptoms and a possible source, e.g., involvement of the AIM2 inflammasome, some patients can be distinguished from other patients, e.g., patients with sJIA and MAS. However, this should be determined by future research that separates sJIA patients with and without MAS. Another option might be that the NLRC4 and AIM2 inflammasome can interact in some sJIA patients causing different symptoms or complications. Furthermore, even though this review hints towards the involvement of the NLRC4 and AIM2 inflammasome, other inflammasomes that have or have not been discussed in this review could also be involved in the pathophysiology of sJIA.

Even though the knowledge on inflammasome activation in sJIA patients is scarce, the data still hints toward inflammasome involvement. If inflammasomes are indeed involved, new possibilities for treatment become available, such as inhibitors of caspase-1, IL-18, and specific inflammasome inhibitors. However, so far, no drugs specifically targeting the NLRC4 or AIM2 inflammasome are on the market, nor inhibitors of caspase-1 and IL-18. Although, an IL-18 inhibitor, Tadekinig Alfa, is currently in clinical trial for Adult-onset Still's disease and for patients with MAS caused by mutations in *NLRC4*[59]. In combination with an IL-1 inhibitor, an IL-18 inhibitor could possibly further prevent the symptoms of sJIA patients since both cytokines will be blocked. S100A8/A9, S100A12, and ferritin are less likely to be a good target, since their exact role in sJIA remains unknown. However, if these proteins indeed prime inflammasome activation in sJIA patients, it might be useful to inhibit these proteins, if possible.

Another possible target might be the inflammasome product gasdermin D N-terminal. Since sJIA patients already react well to IL-1 inhibitors, which target the products of inflammasome overactivation, targeting other products might be effective. Recently, it has been found that disulfiram, an FDA-approved drug for alcohol addiction, can inhibit pore formation by gasdermin D N-terminal[60]. This drug thereby prevents cytokine release and would thus target more cytokines than IL-1 inhibitors, which would especially be useful in sJIA patients with MAS, since the IL-18 levels are also extremely high in these patients. However, before possibly adding this drug to the treatment options, it is important to study the possibilities of gasdermin D N-terminal as a biomarker in sJIA patients, e.g., are the levels elevated in every patient and can it be measured easily. If this is the case, it might be useful to test disulfiram for sJIA patients with and without MAS.

The elevated levels of IL-1β, IL-18, and gasdermin D N-terminal in sJIA patients thus suggest overactivation of inflammasomes and they (might) serve as biomarkers and targets. Furthermore, the elevated levels of S100A8/A9, S100A12, and ferritin suggest elevated priming of inflammasomes. However, no gain-of-function mutations in sJIA have been reported in genes encoding NLRP1, NLRP3, NLRC4, AIM2, and pyrin. Overexpression of *AIM2* and *NLRC4* has been reported in sJIA patients, but the role of MAS and NETs in this altered gene expression profile remains unknown. Future studies are required to elucidate the role of specific inflammasomes and cell types in the pathophysiology of sJIA.

In conclusion, the NLRC4 and AIM2 inflammasomes most likely are involved in the pathophysiology of sJIA. However, the mechanism behind overexpression of *NLRC4* and *AIM2* and the effect of MAS and NETs on inflammasome activation in sJIA patients remains unknown.

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