

# Inflammasomes and Their Activation

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**ABSTRACT:** The innate immune system relies on the recognition of pathogens by pattern recognition receptors as a first line of defense and to initiate the adaptive immune response. Substantial progress has been made in defining the role of Nod (nucleotide-binding oligomerization domain)-like receptors and AIM2 (absent in melanoma 2) as pattern recognition receptors that activate inflammasomes in macrophages. Inflammasomes are protein platforms essential for the activation of inflammatory caspases and subsequent maturation of their pro-inflammatory cytokine substrates and induction of pyroptosis. This paper summarizes recent developments regarding the function of Nod-like receptors in immunity and disease.

**KEY WORDS:** inflammation, caspase-1, Nod-like receptor, pattern recognition, autoinflammatory disease

## ABBREVIATIONS

**AIM2**, absent in melanoma-2; **ASC**, apoptosis-associated speck-like protein containing a caspase recruitment domain; **bZIP**, basic leucine zipper; **BWS**, Beckwith-Wiedemann syndrome; **CAPS**, cold-induced autoinflammatory syndrome-1 (CIAS1) gene-associated periodic syndrome; **CARD**, caspase recruitment domain; **CC**, coiled coil; **CD2BP1**, cluster of differentiation-2 (CD2) binding protein-1; **CDKN1C**, cyclin-dependent kinase inhibitor 1C; **CIAS1**, cold-induced autoinflammatory syndrome-1; **CINCA**, chronic infantile neurological cutaneous and articular syndrome; **DAMP**, danger-associated molecular pattern; **dsDNA**, double-stranded DNA; **HEK293**, human embryonic kidney 293 cell line; **HSP90**, heat-shock protein 90; **IκBα**, inhibitor of nuclear factor of kappa light chain enhancer of activated B-cells (NF-κB) alpha; **IGF2**, insulin-like growth factor-2; **IKK**, IκB kinase; **IL**, interleukin; **IL-18BP**, interleukin-18 binding protein; **IL-1R**, interleukin-1 receptor; **IL-1Ra**, interleukin-1 receptor antagonist; **ERK**, extracellular signal-regulated kinase; **FCAS**, familial cold autoinflammatory syndrome; **FCAS2**, Guadeloupe variant fever syndrome; **FCU**, familial cold urticaria; **FIIND**, function-to-find domain; **FMF**, familial Mediterranean fever; **FRHM**, familial recurrent hydatidiform mole; **KCNQ1oT1**, potassium voltage-gated channel KQT-like subfamily member-1 (KCNQ1) overlapping transcript-1; **LeTx**, lethal toxin; **LRR**, leucine-rich repeat; **MEFV**, Mediterranean fever gene; **MAPK**, mitogen-activated protein kinase; **MARTX**, multifunctional repeat-in toxins; **MDP**, muramyl dipeptide; **MWS**, Muckle-Wells syndrome; **NAD**, NACHT-associated domain; **NF-κB**, nuclear factor kappa light-chain enhancer of activated B cells; **NLR**, Nod-like receptor; **NLRB**, NLR containing a baculovirus inhibitor of apoptosis domain; **NLRC**, NLR containing a CARD; **NLRP**, NLR containing a PYD; **Nod**, nucleotide-binding oligomerization domain; **NOMID**, neonatal-onset multisystem inflammatory disease; **P2X<sub>7</sub>R**, ATP-activated purinergic (P2X) receptor-7; **PAI-2**, plasminogen activator inhibitor-2; **PAMP**, pathogen-associated molecular pattern; **PAPA**, pyogenic arthritis, pyoderma gangrenosum and acne; **PI3K**, phosphoinositide 3-kinase; **PRR**, pattern recognition receptor; **PSTPIP1**, proline serine threonine phosphatase-interacting protein-1; **PYD**, pyrin N-terminal homology domain; **RIG1**, retinoic acid-inducible gene-1; **RIP2**, receptor-interacting protein-2; **RLR**, retinoic acid inducible gene-1 like receptor; **ROS**, reactive oxygen species; **SH3**, Src homology-3; **SGT1**, suppressor of G2 allele of SKP1; **SMA**, spinal muscular atrophy; **SREBP**, sterol

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regulatory element-binding protein; **Syk**, spleen tyrosine kinase; **TIR**, Toll/interleukin-1 receptor; **TLR**, Toll-like receptor; **TNFR1**, tumor necrosis factor receptor-1; **TRIM**, tripartite motif-containing protein; **TRX**, thioredoxin; **TXNIP**, thioredoxin interacting protein

## I. INTRODUCTION

In addition to the mechanical barrier of the skin, mammals are protected by two arms of the immune system, the innate and the adaptive immune systems, which cooperatively function to eradicate invading pathogens. The innate immune system is responsible for initial defense and also activates the adaptive immune system for long-lasting and highly specific immunity by antigen-specific, clonally expanded B and T lymphocytes. A key requirement in this process is the discrimination between self and non-self to specifically eliminate pathogens. Therefore, germ line-encoded pattern recognition receptors (PRRs) of innate immune cells sense pathogens directly at the infection site through recognition of pathogen-associated molecular patterns (PAMPs), which are conserved, and essential structures that are present on microorganisms but absent on host cells.<sup>1</sup> PRRs can be classified into phagocytic PRRs, which directly bind pathogens and mediate their internalization, and sensor PRRs, which respond to recognition of PAMPs by initiating pro-inflammatory signaling pathways.<sup>2</sup> Sensor PRRs, such as the membrane-bound Toll-like receptors (TLRs) recognize PAMPs on the cell surface and lumen of endosomes and lysosomes.<sup>3</sup> More recently, PRRs that sense the cytosolic presence of PAMPs have been discovered, and these include the RIG-1 (retinoic acid-inducible gene-1)-like receptors (RLRs),<sup>4</sup> the nucleotide-binding and oligomerization domain (Nod)-like receptors (NLRs),<sup>5-8</sup> and AIM2 (absent in melanoma-2).<sup>9</sup>

Macrophages also express a number of phagocytic PRRs, including scavenger receptors, C-type lectins, collectins, and pentraxins.<sup>2</sup> The broad activity of the innate immune system is provided by phagocytes such as monocytes, macrophages, neutrophils, dendritic cells, and epithelial cells, which engulf infectious agents and initiate an inflammatory host response. This response is mediated by the release of

cytokines and chemokines that act on the surrounding tissue cells, as well as by up-regulation of adhesion and co-stimulatory molecules to promote recruitment of immune cells into the infected tissue. Two of these pro-inflammatory cytokines, the related interleukin (IL)-1 $\beta$  and IL-18, are produced early during host defense and are responsible for perpetuating this inflammatory response and exerting multiple effects during infection, tissue damage, inflammation, and autoimmune and autoinflammatory disease.<sup>10</sup>

## II. IL-1 $\beta$ AND IL-18: TWO CASPASE-1-DEPENDENT PRO-INFLAMMATORY CYTOKINES

The processes that generate the biologically active cytokines IL-1 $\beta$  and IL-18 have a similar mechanism. In contrast to most cytokines, which are only transcriptionally induced and immediately secreted in response to inflammatory and infectious stimuli, IL-1 $\beta$  and IL-18 require two signals. First, activation of TLRs, RLRs, and other PRRs that induce the inflammatory response by activation of primarily NF- $\kappa$ B (nuclear factor kappa light-chain enhancer of activated B cells) and MAPK (mitogen-activated protein kinase) signaling pathways that collectively promote a transcription factor-mediated response, is required for the up-regulation of *IL1B* transcripts. In contrast, the *IL18* transcript is constitutively produced in most cell types. Transcription/translation, however, only produces the intracellular, inactive precursors, pro-IL-1 $\beta$  (31 kDa) and pro-IL-18 (24 kDa). The second signal required for cytokine release causes the activation of caspase-1. Active caspase-1 proteolytically cleaves the prodomain to liberate the 17- and 18-kDa mature cytokines, which are then released by an atypical, leader peptide-independent mechanism, which is still controversial.<sup>11-13</sup> This caspase-1-dependency of IL-1 $\beta$  and IL-18 maturation appears to be restricted to monocytes and macrophages.

While monocytes express constitutively active caspase-1, and therefore only require the priming step for IL-1 $\beta$  release,<sup>14</sup> other cells such as neutrophils and mast cells release serine proteases that are frequently present in inflammatory fluids. These proteases, including proteinase-3, elastase, chymase, matrix metalloproteinases, and others, can mature pro-IL-1 $\beta$  and pro-IL-18, which can be released

from short-lived and damaged cells.<sup>15</sup> Mature IL-1 $\beta$  and IL-18 are recognized by their receptors, IL-1RI and IL-18R $\alpha$ , respectively, and cause a conformational change that allows high-affinity binding in the complex with the IL-1R accessory protein (IL-1RAc or IL-1RIII) or the IL-18R $\beta$ , respectively.<sup>10</sup> Signal transduction is then mediated by the TIR (Toll/IL-1 receptor) domain, which is also present in TLRs, further emphasizing their link to innate immunity. Furthermore, both cytokines have a naturally occurring inhibitor, the IL-1R antagonist (IL-1Ra) and the IL-18 binding protein (IL-18BP), respectively.

In addition to its well-established role in maturing pro-IL-1 $\beta$  and pro-IL-18, caspase-1 has been implicated in unconventional protein secretion extending beyond cytokine release.<sup>16</sup> Caspase-1 is able to also promote cell survival in response to microbial pore-forming toxins, which activate NLRP3 (NLR containing a PYD-3) and NLRC4 (NLR containing a CARD-4) inflammasomes, through activating lipid metabolic pathways to mediate membrane biogenesis and repair.<sup>17,18</sup> In this capacity, caspase-1 activates the sterol regulatory element-binding proteins (SREBPs) 1 and 2, which in turn facilitate fatty acid metabolism and cholesterol and lipid biosynthesis.<sup>19</sup> This might appear to contradict the better known role of caspase-1 in promoting an inflammatory form of cell death called pyroptosis, which causes loss of plasma membrane integrity and local inflammation.<sup>20-24</sup> However, morphologically, pyroptosis is reminiscent of pyronecrosis, yet another form of inflammatory cell death, which is NLRP3, ASC (apoptosis-associated speck-like protein containing a CARD), and cathepsin B dependent, but independent of caspase-1.<sup>25,26</sup> Thus, the consequence of caspase-1 activation might depend on the state of the infection. While maturation of proinflammatory cytokines is a first step in limiting the spread of a pathogen, the activation of membrane biogenesis could repair damage resulting from the microbial insult. There is now evidence that active caspase-1 is rapidly released from macrophages, which might prevent it from causing any further cell damage.<sup>27</sup> However, if the infection persists and cannot be cleared, caspase-1 is capable of inducing pyroptosis to eliminate the pathogen through cell death.

Caspase-1, the first member of the caspase family of cysteine proteases to be identified, belongs to the

inflammatory caspase subfamily, which also includes caspase-4, caspase-5 (and their mouse paralogue, caspase-11), and caspase-12.<sup>28</sup> The inflammatory caspases belong to the initiator caspases and contain a CARD as their pro-domain. Caspases are initially synthesized as inactive precursor proteins (zymogens) and require activation to achieve catalytic activity. CARD is essential for the clustering of two or more pro-caspases required for their autocatalytic transactivation by the induced proximity mechanism.<sup>29</sup> The clustering of pro-caspases occurs in large protein platforms specific for each caspase, which is the inflammasome for the inflammatory caspases.<sup>27</sup>

### III. INFLAMMASOMES: PROTEIN PLATFORMS TO ACTIVATE INFLAMMATORY CASPASES

While the activation of TLRs, RLRs, and some NLRs initiates signaling cascades that ultimately promote a transcriptional response to up-regulate pro-inflammatory mediators, the activation of several NLRs and AIM2 causes the formation and activation of inflammasomes, multi-protein platforms that mediate the activation of inflammatory caspases, including caspase-1 and caspase-5, by the induced proximity mechanism.<sup>27</sup> For most characterized inflammasomes, ASC is the required adaptor for pro-caspase-1 activation.<sup>30,31</sup> It binds to the CARD-containing pro-domain of caspase-1 to mediate its oligomerization. Accordingly, enforced oligomerization of ASC is sufficient to activate caspase-1.<sup>27,32-34</sup> Therefore, ASC and caspase-1 are shifted into a high-molecular-weight protein complex following *in vitro* activation. ASC is the adaptor to bridge activated NLRs with pro-caspase-1, and consequently NLRP1 and caspase-5 are shifted into these large-molecular-weight complexes in response to *in vitro* activation.<sup>27</sup> However, in response to MDP (muramyl dipeptide) activation of macrophages *in vivo*, only NLRP1, Nod2, and caspase-1 are shifted into high-molecular-weight complexes.<sup>35</sup>

Consistently, *in vitro* reconstituted NLRP1 inflammasomes are able to assemble in the absence of ASC, although ASC is able to enhance inflammasome activity.<sup>36</sup> In contrast to this ASC-independent response of NLRP1 inflammasomes, immunodepletion of ASC is able to abrogate NLRP1

inflammasome activation in the human monocytic cell line THP-1 in response to lipopolysaccharide, which is reminiscent of the best-studied NLRP3 inflammasome.<sup>27</sup> ASC directly interacts with NLRP3 by a PYD-PYD interaction, and recruits pro-caspase-1 by a CARD-CARD interaction.<sup>34,37</sup> For NLRC4 inflammasomes, genetic evidence suggests that ASC is required for caspase-1 activation in spite of the direct interaction of NLRC4 with pro-caspase-1 by a CARD-CARD interaction.<sup>30,38,39</sup> Thus, although ASC is required for caspase-1 activation by some inflammasomes, and ASC-deficient macrophages fail to respond to most tested stimuli with caspase-1 activation and release of IL-1 $\beta$  and IL-18,<sup>30,31,40</sup> caspase-1 activation in response to MDP and perhaps some other stimuli is mostly uncoupled from ASC.<sup>35,36</sup> These data suggest that while in some inflammasomes ASC is essential for assembly, ASC might play a less prominent role in other inflammasomes. Indeed, recent studies demonstrated that knockdown of ASC in THP-1 cells impaired the NF- $\kappa$ B-mediated up-regulation of several pro-inflammatory cytokines, including pro-IL-1 $\alpha$ , in response to several TLR agonists and infection with *Escherichia coli* and *Porphyromonas gingivalis*, which could explain the partial requirement for ASC<sup>41</sup>: NLR oligomerization is required for their interaction with ASC.<sup>42</sup>

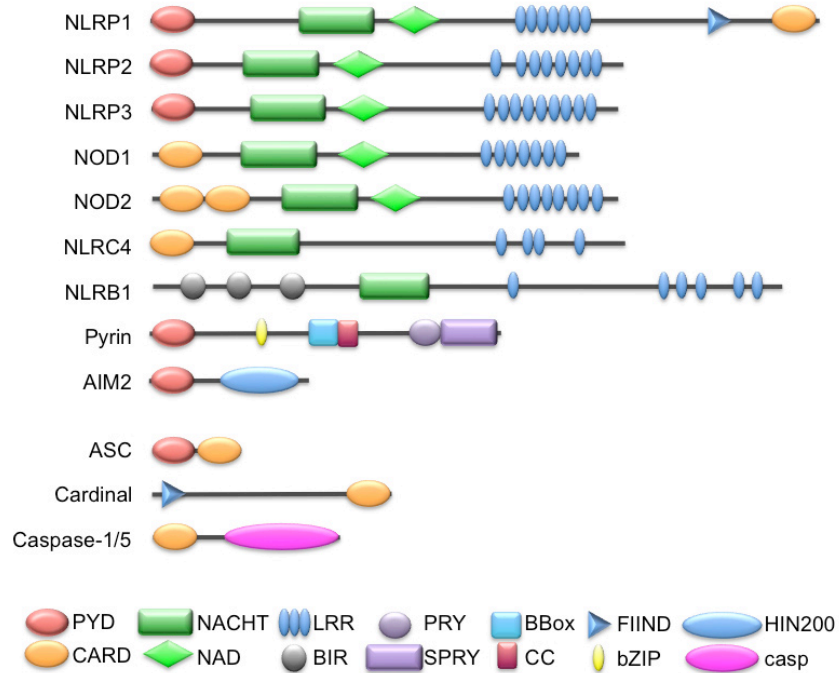
The NACHT domain of at least some NLRs has ATPase activity, and ATP binding is a prerequisite for NLR oligomerization.<sup>36,43,44</sup> Several NLRs are complexed via their LRRs (leucine-rich repeats) with the ubiquitin ligase-associated protein SGT1 (suppressor of G2 allele of SKP1) and HSP90 (heat-shock protein 90) in unstimulated cells, which might keep the receptors in an inactive, but signaling ready state. In the absence of SGT1 or upon treatment of cells with geldanamycin to impair HSP90 chaperon activity, NLRs are degraded.<sup>45,46</sup> However, upon inflammatory activation of cells, SGT1 and HSP90 dissociate from NLRs, followed by nucleotide binding and NLR oligomerization.<sup>45,46</sup> Although 22 human NLRs and 33 mouse Nlr genes exist,<sup>47</sup> the function of most NLRs has yet to be elucidated, and so far only a few NLRs have been linked to inflammasome activation.

## IV. NOD-LIKE RECEPTORS AND AIM2: SENSORS OF PATHOGEN INFECTION AND CELLULAR STRESS

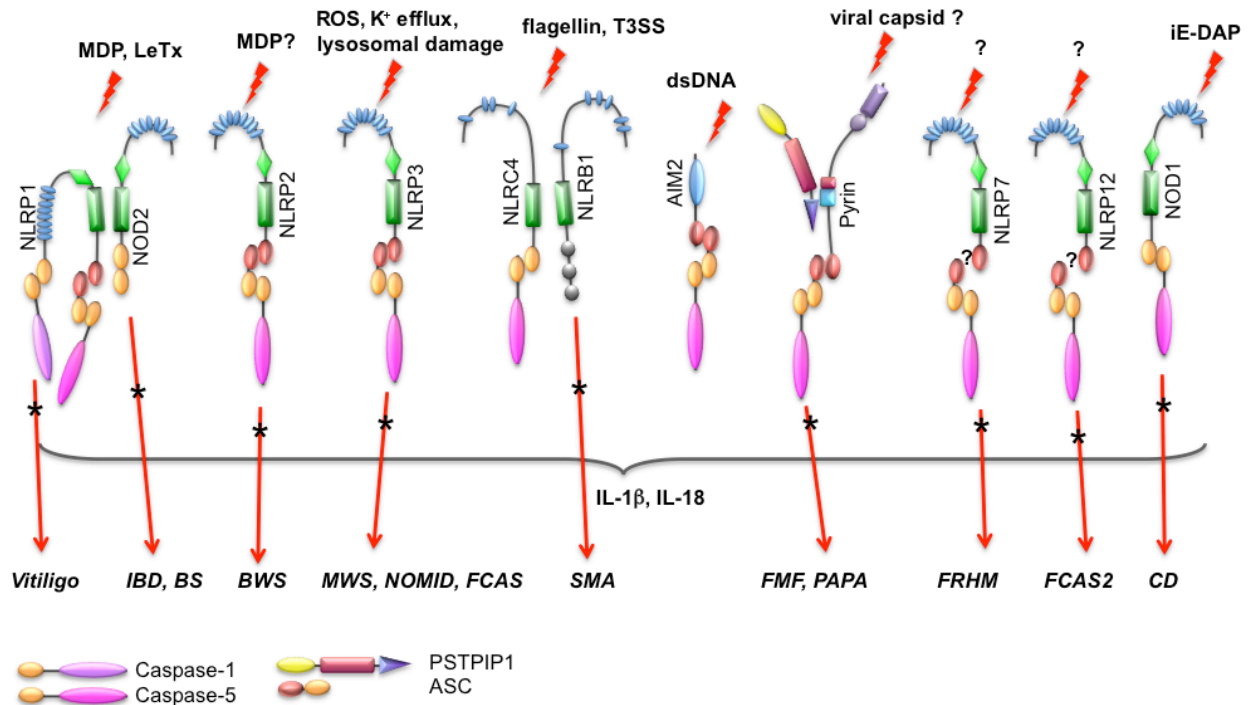
### A. NLRP1 and Nod2

The first identified inflammasome was that of NLRP1.<sup>27</sup> NLRP1 (also known as NALP1, DEFCAP, NAC, CARD7, and CLR17.1) is unique among NLRPs because, in addition to its PYD, NACHT, NACHT-associated domain (NAD), and LRRs, it contains a C-terminal function-to-find (FIIND) domain and CARD (Figs. 1 & 2).<sup>48,49</sup> Although there is only one *NLRP1*, there are three paralogous *Nlrp1* genes encoded in mice, all of which lack the PYD. The NLRP1 inflammasome contains caspase-1, which is recruited by ASC, and caspase-5, which directly interacts with the CARD of NLRP1.<sup>27</sup> However, the roles of ASC and caspase-5 in the NLRP1 inflammasome are still uncertain. ASC interacts with NLRP1, immunodepletion of ASC impairs NLRP1 inflammasomes, and the presence of ASC enhances in vitro reconstituted NLRP1 inflammasomes that assemble in response to MDP.<sup>36,27</sup> However, ASC was also found to be not essential for NLRP1 inflammasome function, and mouse *Nlrp1* lacks the PYD and therefore cannot interact with ASC.<sup>35,36</sup> Caspase-5, although part of the NLRP1 inflammasome, is lacking from other inflammasomes, including NLRP2 and NLRP3-containing inflammasomes, and thus might not have an essential role in all inflammasomes.<sup>37</sup>

MDP is also sensed by Nod2 (also known as NLRC2, CARD15, CD, BLAU, IBD1, PSORAS1, and CLR16.3), an NLRC that can directly interact with the CARD of pro-caspase-1. However, Nod2-mediated pro-caspase-1 activation and IL-1 $\beta$  maturation also require the presence of NLRP1 or NLRP3 (see below) (Figs. 1 & 2).<sup>35,50</sup> This interaction does not require the adaptor protein RIP2 (receptor-interacting protein-2; also known as Cardiak, RICK, CCK, and Ripk2), which bridges Nod2 to the NF- $\kappa$ B activating IKK (I $\kappa$ B kinase) complex, although RIP2 itself can bind to caspase-1.<sup>51-54</sup> Both NLRP1 and Nod2 form an MDP-sensing inflammasome, and in reconstitution assays, both proteins enhanced each other's interaction with caspase-1 and inflam-



**FIGURE 1.** Domain structure of NLR and adaptor proteins involved in inflammasome activation. Proteins were analyzed using the simple modular architecture research tool (SMART) (<http://smart.embl.de/>)<sup>48,49</sup> and conserved domains are indicated.



**FIGURE 2.** Composition and activators of known inflammasomes. Known and putative inflammasomes are shown. Inflammatory diseases caused by mutations or polymorphisms of inflammasome proteins are indicated by an asterisk. A question mark indicates that the inflammasome agonist or precise inflammasome composition is not known.

masome activation, which is most likely explained by their ability to heterodimerize.<sup>35,55</sup> However, this MDP-induced inflammasome does not contain NLRP3, although NLRP3 has also been linked to MDP-mediated inflammasome activation, which also required RIP2 and ASC.<sup>35,50,56</sup>

In addition to MDP, Nlrp1 is involved in sensing *Bacillus anthracis* infections. The lethal toxin (LeTx) is responsible for mortality in systemic anthrax infections; inbred mice show a variable degree of sensitivity to LeTx, and this trait has been mapped to *Nlrp1b*.<sup>57</sup> LeTx activation of Nlrp1b depends on lysosomal membrane destabilization.<sup>58</sup> Similar to MDP, recognition of LeTx also requires NLRP1 and Nod2, indicating that NLRP1/Nod2 heterodimers might be more prevalent.<sup>35</sup> It is still not known if MDP or LeTx are directly bound by NLRP1 or Nod2, but in a minimal in vitro reconstitution system NLRP1 is still able to respond to MDP, which suggests that MDP could be directly recognized.<sup>36</sup> LeTx, on the other hand, causes macrophage death through inactivation of p38 $\alpha$ , which is required for macrophage survival, through up-regulation of the caspase-1 inhibitor PAI-2 (plasminogen activator inhibitor-2).<sup>59-61</sup> Thus, it is more likely that LeTx is rather indirectly recognized by NLRP1/Nod2. As proposed for NLRP1, the Nod2 LRRs also engage with the CARD-NACHT region by intramolecular interaction, and this autoinhibitory interaction is reversed by MDP.<sup>35,36</sup>

## B. NLRP2

NLRP2 (also known as NALP2, PYPAF2, NBS1, PAN1, and CLR19.9) interacts with ASC to form an inflammasome that also contains Cardinal (also known as TUCAN and CARD8) (Figs. 1 & 2).<sup>37,62</sup> Cardinal is a FIIND and CARD-containing protein that can interact with caspase-1.<sup>63</sup> The interaction of Cardinal with NLRP2 occurs via the NACHT and FIIND domains and requires an activated NLRP2 or deletion of the LRR (which can activate NLRs), but does not interact with full-length naive protein, due to its inactive conformation.<sup>37</sup> Cardinal might provide the FIIND-CARD that is also present in NLRP1, but lacks in other NLRs, including NLRP2. However, unlike NLRP1, this interaction is insuff-

icient to recruit caspase-5 to NLRP2.<sup>37</sup> While cell rupture can activate NLRP2 inflammasomes, similar to NLRP1 and NLRP3, the physiological NLRP2 agonist is still elusive.

## C. NLRP3

NLRP3 (also known as cryopyrin, cold-induced autoinflammatory syndrome-1 [CIAS1], NALP3, PYPAF1, and CLR1.1) is perhaps the best-studied NLRP and requires the adaptor ASC to bridge to caspase-1 (Figs. 1 & 2).<sup>30</sup> Like NLRP2, NLRP3 inflammasomes also contain Cardinal, although the precise role of Cardinal in inflammasomes is elusive, given the fact that it is absent from the mouse genome.<sup>37</sup> The interaction with ASC is induced by enforced NLRP3 oligomerization, which occurs upon ligand binding or upon expression of disease-associated mutations in the NLRP3 protein, indicating that inflammatory disease-linked mutants are constitutively active and uncoupled from ligand sensing.<sup>42</sup> Accordingly, disease-associated NLRP3 mutants cause enhanced oligomerization of ASC.<sup>64</sup> Because the LRRs are the ligand-sensing/-binding domain and keep the NLR inactive, deletion of the LRR renders the protein constitutively active.<sup>42</sup>

A first insight into the physiological role of NLRP3 came from macrophages isolated from NLRP3-deficient mice, which demonstrated that NLRP3 is activated by a large number of diverse agonists, including PAMPs, DAMPs (danger-associated molecular patterns), pathogens, and synthetic substances that cause potassium efflux, lysosomal damage, and/or reactive oxygen species (ROS) generation.<sup>18,40,65,66</sup> PAMPs and other exogenous ligands that are recognized by NLRP3 include MDP, viral and bacterial dsRNA, poly(I:C), or the imidazoquinolines R848/R837, mainly in conjunction with ATP.<sup>56,65,67,68</sup> The precise mechanism of how diverse PAMPs activate NLRP3 is not clear, but potentially promotes macrophage priming to up-regulate pro-IL-1 $\beta$ , while ATP potentially provides the second signal for inflammasome activation and IL-1 $\beta$  maturation. Activation and opening of the pannexin-1 channel may allow entry of PAMPs into the cytosol. In fact, pannexin-1 channel opening occurs as a consequence of binding of exogenous ATP, which is a

danger signal released from stressed and dying cells following tissue injury to the purinergic P2X<sub>7</sub> receptor (P2X<sub>7</sub>R) and subsequent potassium efflux.<sup>69-72</sup> Indeed, exogenous ATP is a potent activator of the NLRP3 inflammasome, which causes production of ROS and activation of PI3K (phosphoinositide 3-kinase).<sup>18,72,73</sup> Exogenous triggers appear to require ATP/P2X<sub>7</sub>R/K<sup>+</sup> efflux for inflammasome activation, while intracellular pathogens, such as *Salmonella* or *Listeria* and intracellular flagellin, do not require this step.<sup>69</sup> Furthermore, while mouse macrophages require exogenous ATP for IL-1 $\beta$  release in response to certain stimuli, human macrophages release ATP upon treatment with inflammatory or infectious stimuli, which then acts through an autocrine mechanism on P2X<sub>7</sub>R to promote IL-1 $\beta$  release.<sup>74</sup>

In support of these findings, the action of ATP can be mimicked with microbial pore-forming toxins such as streptolysin O or pneumolysin.<sup>72</sup> Similarly, maitotoxin and nigericin, two K<sup>+</sup>/H<sup>+</sup> antiport ionophores, cause intracellular K<sup>+</sup> ion depletion and promote NLRP3 activation.<sup>18</sup> Preventing K<sup>+</sup> depletion, for example by culturing of macrophages in medium containing 130 mM KCl, also reduces inflammasome activation and emphasizes the significance of K<sup>+</sup> efflux for NLRP3 activation.<sup>22,75,76</sup> In the case of *Staphylococcus aureus* MDP, phagocytosis and lysozyme-based cell wall degradation are essential to make MDP accessible for inflammasome activation, while preventing degradation also diminishes inflammasome activation.<sup>77</sup> However, caspase-1 activation can also occur in the absence of infectious stimulation during a sterile inflammatory response. Caspase-1 can be activated in macrophages and dendritic cells by incubation with inflammatory cytokines such as TNF $\alpha$  and IL-1 $\beta$ , but not with CD40L, Fas, IFN $\gamma$ , or RANKL in combination with ATP or particulates (see below).<sup>78</sup>

Crystalline and polymeric structures, including environmental pollutants and danger signals, are also potent activators of NLRP3. Asbestos, silica, calcium pyrophosphate dehydrate crystals, dextran sulfate sodium polymers, aluminum salt, monosodium urate crystals, cholesterol crystals, and amyloid- $\beta$  fibrillar peptides all activate NLRP3.<sup>66,79-87</sup> These NLRP3 agonists undergo similar phagocytosis that leads to subsequent inflicted phagolysosomal damage, caus-

ing the release of lysosomal proteases, most prominently cathepsin B and L, suggesting that NLRP3 might be activated downstream of a proteolytic pathway. Cathepsin B- and L-deficient mice display impaired IL-1 $\beta$  processing, and short-interfering RNA (siRNA)-mediated knockdown of cathepsin B abrogates caspase-1 activation in response to nigericin or dextran sulfate sodium.<sup>87-89</sup> However, siRNA-mediated knockdown of cathepsin B did not affect inflammasome activation in response to LeTx, which, however, is an Nlrp1b agonist.<sup>58</sup> The same study also pointed out that CA-074Me, a previously considered cathepsin B-specific inhibitor, blocks LeTx-induced Nlrp1b activation independently from acting on cathepsin B through inhibiting alternative protease activity induced or released by lysosomal membrane permeabilization.<sup>58</sup> The NLRP3-agonist role of such crystals can be mimicked by direct lysosomal rupture using pharmacological or mechanical mechanisms, which cause NLRP3 activation, supporting the essential role of lysosomal destabilization for NLRP3 activation.<sup>81,83,90</sup>

NLRP3 is also responsible for sensing diverse pathogens, including the gram-positive bacteria *S. aureus* and *Listeria monocytogenes*<sup>18,91</sup> and the gram-negative *V. vulnificus* and *V. cholerae*.<sup>90</sup> *S. aureus* is recognized by NLRP3 through  $\alpha$ -hemolysin, a pore-forming virulence factor responsible for pneumonia.<sup>92</sup> Similarly, *V. vulnificus* and *V. cholerae*-mediated NLRP3 activation occurs by hemolysins and MARTX (multifunctional repeat-in toxins).<sup>90</sup> NLRP3 also senses infections by *C. pneumoniae*, which is dependent on potassium efflux, lysosomal damage, and cathepsin B release.<sup>93</sup> *Chlamydia trachomatis* also activates NLRP3, which requires Syk (spleen tyrosine kinase), potassium efflux, and ROS generation.<sup>94</sup> NLRP3 further senses infections by DNA and RNA viruses, including Sendai virus and influenza virus, and recognizes the adenoviral capsid.<sup>67,68,95-97</sup> The influenza proton-selective ion channel M2 is localized to the Golgi, and its acidification is required for influenza M2-induced NLRP3 inflammasome activation, which is caused by imbalances in H<sup>+</sup> and other cations.<sup>97,98</sup> However, caspase-1 activation by viral RNA occurs independently of NLRP3 by a complex of the RNA helicase RIG-I and ASC.<sup>99</sup> Parasites such as *Plasmodium chabaudi adami* DS are

recognized by NLRP3 through its metabolic waste hemozoin, which has a crystalline appearance, and deficiency of NLRP3 augments survival in a mouse model of *P. chabaudi adami* DS infection.<sup>100</sup>

In agreement with its crystalline structure, hemozoin recognition depends on phagocytosis, lysosomal damage, potassium efflux, ROS generation, and cathepsin B release.<sup>100</sup> Moreover, hemozoin causes activation of the tyrosine kinases Syk and Lyn (a Yamaguchi sarcoma viral oncogene homolog) and subsequently activation of PI3K and ERK (extracellular signal-regulated kinase), providing evidence for the link between inflammasome activation and kinase signaling.<sup>100</sup> Fungal infections, such as by *Candida albicans* and *Aspergillus fumigatus*, are sensed by NLRP3 inflammasomes in concert with Syk-coupled C-type lectin receptors with an immunoreceptor tyrosine-based activation motif, such as dectin-1 and dectin-2.<sup>75,101</sup> *C. albicans*-mediated NLRP3 activation is independent of lysosomal damage and cathepsin B release, but dependent on ROS generation, which is in agreement with an essential role of Syk in the generation of ROS in response to fungal infections.<sup>75,102</sup> Similarly, monosodium urate and other particulates that activate NLRP3 have been shown to activate Syk. Therefore, the generation of ROS is a common requirement for most, if not all, of the NLRP3 activators.

Further support for the importance of ROS comes from experiments with ROS scavengers such as N-acetyl cysteine.<sup>70,73,75,76,85,100</sup> Recently, the link between ROS and NLRP3 has been elucidated. The LRRs of NLRP3, but not other NLRs, interact specifically with the thioredoxin (TRX)-interacting protein (TXNIP). While TXNIP is bound to TRX during homeostasis, elevated ROS levels induce the dissociation of TXNIP from TRX, allowing TXNIP to interact and activate NLRP3 in a ROS-dependent manner.<sup>103</sup> However, not all stimuli causing the generation of ROS also cause activation of NLRP3 and maturation of IL-1 $\beta$ , suggesting some form of regulation downstream of ROS. Thus, there might be several distinct mechanisms responsible for NLRP3 activation, including ATP/P2X<sub>7</sub>R/pannexin-1/K<sup>+</sup> depletion, lysosomal destabilization and potentially release of proteases such as cathepsin B and L, and the generation of ROS.

## D. NLRC4 and NLRB1

NLRC4 (also known as CARD12, NOD27, CLAN, IPAF, and CLR2.1) is an NLR containing a CARD, which can directly recruit caspase-1, but also interacts with ASC (Figs. 1 & 2).<sup>104-106</sup> NLRC4 is activated by the intracellular gram-negative *S. typhimurium* (but not by bacteria lacking the salmonella invasion protein B), *Legionella pneumophila*, *Shigella flexneri*, and *Pseudomonas aeruginosa*.<sup>30,38,39,107-109</sup> Although NLRC4 can interact directly with caspase-1, ASC is also required for NLRC4 inflammasome activation in response to *S. typhimurium*, *S. flexneri*, and *P. aeruginosa*.<sup>30,38,108,109</sup> The common PAMP that is recognized by NLRC4 is flagellin.<sup>38,39,107</sup> Accordingly, a *L. pneumophila* mutant lacking flagellin is unable to activate NLRC4 and avoids clearance due to a defective lysosomal fusion of phagosomes, and thus efficiently replicates in NLRC4-deficient macrophages.<sup>107</sup> Also the flagellin-deficient *S. typhimurium* fliC-fljB or fhCD mutants fail to activate NLRC4, whereas cytosolic delivery of flagellin is sufficient.<sup>38,39</sup> NLRC4 recognizes a conserved C-terminal 35-amino-acid peptide in flagellin in addition to another region.<sup>110</sup>

Flagellin is not the only PAMP recognized. *P. aeruginosa* recognition by NLRC4 is independent of flagellin, since the flagellin-deficient *P. aeruginosa* mutant PAK $\Delta$ fliC is still capable of activating caspase-1 by an NLRC4-dependent mechanism.<sup>109</sup> Similarly, the non-flagellated *S. flexneri* is sensed by NLRC4, indicating that other structures are also recognized by NLRC4.<sup>108</sup> NLRC4 recognition of *L. pneumophila* depends on a competent type IV secretion system.<sup>107</sup> Similarly, *S. typhimurium*- and *S. flexneri*-mediated caspase-1 activation requires a functional type III secretion system<sup>39,108,111-113</sup> The *S. typhimurium* type III secretion system rod structure encoded by PrgJ from the pathogenicity island-1 (SPI1), but not the rod SsaI from SPI2, resembles the hollow flagellin tube, which is injected into the host cell, and both share a conserved C-terminal peptide sequence sufficient for NLRC4 detection and inflammasome activation. This demonstrates that in addition to flagellin, the type III secretion system itself is also recognized by NLRC4.<sup>114</sup> Other rod proteins recognized by NLRC4 are BsaK from



*Burkholderia pseudomallei*, EprJ and EscI from *E. coli*, MxiI from *S. flexneri*, and PscI from *P. aeruginosa*, indicating a conserved mechanism of NLRC4 recognition.<sup>114</sup>

Activation of NLRC4 also induces pyroptosis of macrophages. Depending on the pathogen, macrophage death is either beneficial for pathogen spread and selectively induced, or is a mechanism to suppress or subvert macrophage-mediated inflammatory host responses.<sup>115,116</sup> *S. flexneri* and *P. aeruginosa*-induced pyroptosis is blocked in cells lacking caspase-1 or NLRC4, but not in cells lacking ASC, although caspase-1 activation requires both NLRC4 and ASC, suggesting two distinct mechanisms.<sup>108,109</sup>

A PRR role for NLRB1 (NLR containing a baculovirus inhibitor of apoptosis domain-1; also known as NAIP5, BIRC1e, and CLR5.1) has been proposed due to its ability to restrict *L. pneumophila* infection in vitro and in vivo.<sup>117-120</sup> Although NLRB1 does not have a CARD or PYD, its signaling is dependent on caspase-1 (Figs. 1 & 2). While ASC is not required for *L. pneumophila* growth restriction by NLRB1, it does require NLRC4.<sup>118</sup> NLRB1 also recognizes flagellin and detects the same conserved region in flagellin as NLRC4.<sup>110,119</sup> While NLRB1, but not NLRC4, is essential to restrict *L. pneumophila* infections via recognition of flagellin, flagellin-dependent recognition of *P. aeruginosa* and *S. typhimurium* is mediated by both NLRC4 and NLRB1.<sup>110</sup> Similarly, *L. monocytogenes* infection activates several NLRs, including NLRC4, NLRP3, and AIM2.<sup>121-123</sup> While humans encode one NLRB1, mice encode seven paralogous genes (Naip1-7).<sup>124</sup>

## E. Nod1

Like Nod2, Nod1 (also known as NLRC1, CARD4, and CLR7.1) contains a CARD and has a well-characterized role in activation of NF- $\kappa$ B and MAPKs via its adaptor RIP2 in protein platforms called nodosomes (Figs. 1 & 2).<sup>125,126</sup> Also similar to Nod2, Nod1 detects breakdown products from peptidoglycans, specifically meso-diaminopimelic acid from gram-negative bacteria.<sup>127,128</sup> There is very limited evidence for an involvement of Nod1 in inflammasome activation. However, based on an overexpression study, Nod1 interacts with pro-caspase-1 by

CARD interaction, causing its activation and maturation of IL-1 $\beta$ .<sup>129</sup> There is also indirect evidence for this interaction, since meso-diaminopimelic acid-induced caspase-1 activation and IL-1 $\beta$  release correlates with Nod1 expression in uveitis.<sup>130</sup>

## F. NLRP12

Little is known about NLRP12 (also known as Monarch-1, NALP12, PYPAF7, RNO2, PAN6, and CLR19.3), which can inhibit NF- $\kappa$ B.<sup>131-133</sup> NLRP12 also has ATPase activity, which is required for its NF- $\kappa$ B-inhibitory function.<sup>44</sup> However, based on overexpression studies, NLRP12 can also interact with ASC to form a caspase-1 activating inflammasome (Fig. 2).<sup>34</sup>

## G. AIM2

AIM2 belongs to the HIN-200 (hematopoietic interferon-inducible nuclear protein with a 200-amino acid repeat; also known as p200 or PYHIN) family of interferon-response genes, which consists of four human (MNDA, IFI16, IFIX, and AIM2) and six mouse proteins, with AIM2 being the most conserved member.<sup>134</sup> HIN-200 proteins share a conserved domain architecture consisting of an N-terminal PYD (except p202a and p202b) and one or two copies of the HIN-200 domain (Figs. 1 & 2). Although HIN-200 members were initially considered to be transcriptional regulators of cell proliferation and differentiation, it has been recently established that AIM2 functions as a PRR by directly binding to cytosolic double-stranded DNA (dsDNA). The DNA-binding capacity of the HIN-200 proteins was recognized early on, and is thought to be responsible for their multiple functions in transcriptional regulation.<sup>135-139</sup> DNA binding occurs by the HIN-200 domain, which consists of two consecutive oligonucleotide-/oligosaccharide-binding domains.<sup>140</sup>

AIM2 is the only cytosolic HIN-200 protein and the sole HIN-200-binding partner of ASC.<sup>141,142</sup> Based on these requirements, AIM2 has been identified as a cytosolic PRR that activates an inflammasome upon recognition of cytosolic dsDNA but not single-stranded DNA.<sup>141-144</sup> In agreement, reconstitution of AIM2 inflammasomes in human

embryonic kidney (HEK) 293 cells resulted in a fully functional inflammasome upon transfection of dsDNA.<sup>141,145</sup> AIM2 recognizes cytosolic dsDNA of any origin and size, including synthetic DNA (poly dA:dT), DNA isolated from vaccinia virus, plasmid DNA, bacterial DNA, and genomic DNA. AIM2 oligomerizes upon DNA binding and recruits ASC to activate caspase-1.<sup>142</sup> Accordingly, knockdown of AIM2 abrogates caspase-1 activation and IL-1 $\beta$  maturation in response to dsDNA, but not other inflammasome agonists.<sup>141-144</sup>

The essential role of AIM2 in dsDNA-induced inflammasome activation has been validated in macrophages from AIM2-deficient mice, which fail to activate caspase-1 and fail to release IL-1 $\beta$  in response to transfected dsDNA, but not to other inflammasome activators.<sup>122,145</sup> Also, infection of AIM2<sup>-/-</sup> macrophages with the bacterial pathogens *Francisella tularensis* and *L. monocytogenes*, as well as the DNA viruses vaccinia virus, herpes simplex virus-1, and mouse cytomegalovirus, results in impaired caspase-1 activation and IL-1 $\beta$  release.<sup>122,145</sup> As discussed above, *L. monocytogenes* is also sensed by other PRRs such as NLRC4 via flagellin, while a flagellin-deficient mutant is primarily sensed by AIM2 inflammasomes.<sup>123</sup> AIM2-deficient mice are also more susceptible to infection by *F. tularensis*, which is not sensed by other inflammasomes, and lack IL-18 production in response to mouse cytomegalovirus infection resulting in higher virus titers than controls.<sup>122,145</sup> Analogous to other inflammasomes, the AIM2 inflammasome also activates pyroptosis, and AIM2<sup>-/-</sup> macrophages are protected from caspase-1-induced cell death.<sup>122,145</sup> The dsDNA inflammasome response in mice is also regulated by p202, another HIN-200 member, which has no human ortholog. Because p202 binds dsDNA similarly to AIM2 (p210), but lacks the PYD, it cannot interact with ASC to assemble an inflammasome, and thus blocks dsDNA-induced caspase-1 activation. Therefore which causes knockdown of p202, increasing caspase-1 activation in response to dsDNA.<sup>144</sup>

## H. Pyrin

Pyrin is the founding member of the PYD family. Like AIM2, it has a different domain architecture

from NLRs. It consists of a PYD, a central BBox/Coiled Coil (CC), and a C-terminal PRY/SPRY domain (Figs. 1 & 2). Pyrin interacts with several inflammasome proteins, but its role in inflammasome activation is still controversial. Evidence for its inflammasome-activating function came recently from the discovery that pyrin specifically interacts with PSTPIP1 (proline serine threonine phosphatase-interacting protein 1), also known as cluster of differentiation-2 (CD2) binding protein-1 (CD2BP1).<sup>146</sup> This association is mediated by the BBox/CC in pyrin and by the SH3 (Src homology-3)/CC region in PSTPIP1, and is markedly increased in PSTPIP1 harboring pyogenic arthritis, pyoderma gangrenosum, and acne (PAPA) syndrome-associated mutations such as A230T and E250Q.<sup>146</sup> These PSTPIP1 mutations also cause increased IL-1 $\beta$  levels in monocytes. Pyrin can form ASC and caspase-1 containing inflammasomes to promote IL-1 $\beta$  release in reconstituted inflammasomes.<sup>33,64,147</sup> On endogenous level, PSTPIP1 binding to pyrin promotes the unmasking of its PYD and the interaction with ASC, to recruit caspase-1, which is enhanced by the presence of PSTPIP1 proteins harboring the PAPA-linked mutations.<sup>148</sup> Any pathogens or PAMPs that are potentially sensed by pyrin remain elusive.

Pyrin is transcriptionally up-regulated in response to pro-inflammatory stimuli and inhibited by anti-inflammatory cytokines in myeloid cells.<sup>149</sup> Furthermore, type I interferons and retroviral infection induces expression of pyrin, raising the possibility that pyrin is involved in the antiviral host response.<sup>148</sup> Pyrin is also known as TRIM20 (tripartite motif-containing protein 20). Other TRIM family members, most notably TRIM5 $\alpha$ , function to restrict retroviral replication via SPRY domain-mediated viral capsid recognition. Furthermore, TRIM22 mediates its antiviral activity by directly interacting with the HIV core.<sup>150-152</sup> Zebrafish contain several hybrid proteins comprised of NLRs and the SPRY of pyrin, further suggesting a role of pyrin in pattern recognition. A significant number of familial Mediterranean fever (FMF)-linked mutations in pyrin are located inside the SPRY domain, further emphasizing the importance of this domain in promoting inflammasome activation.<sup>153</sup>

In addition to its role in assembling an inflam-

masome, there is also evidence for an inflammasome-antagonistic role of pyrin. Macrophages expressing a truncated pyrin protein retaining only the PYD express increased levels of active caspase-1 and are hyperactive with regard to secreting IL-1 $\beta$ .<sup>154</sup> The PYD of pyrin is still expressed in these cells. Because pyrin interacts with ASC through their PYDs to modulate downstream signaling pathways, expression of the pyrin-PYD could potentially affect IL-1 $\beta$  maturation.<sup>64,155,156</sup> This result was confirmed by the observation that knockdown of pyrin in the human THP-1 monocytic cell line augmented release of IL-1 $\beta$ , providing further evidence that pyrin potentially negatively regulates inflammasomes.<sup>157,158</sup> Mechanistically, pyrin interacts via its SPRY domain with several inflammasome components, including the NACHT domains of NLRP1, NLRP2, and NLRP3; the caspase domain of caspase-1; and the pro-domain of pro-IL-1 $\beta$ , suggesting that pyrin potentially complexes with these proteins to prevent their activation. Furthermore, the commonly FMF-associated pyrin mutations M694V and M680I impair the interaction of pyrin with caspase-1, thus providing a possible explanation for the excessive IL-1 $\beta$  production reported in FMF patients.<sup>158</sup> However, in another study, knockdown of pyrin in the same THP-1 cell line and in primary human monocytes impaired maturation of IL-1 $\beta$ , suggesting that the limited pyrin expression in macrophages is the cause of their reduced ability to release IL-1 $\beta$ .<sup>147</sup> In addition, pyrin itself is cleaved by caspase-1, and the N-terminal fragment comprised of the PYD and the bZIP (basic leucine zipper) domain activates NF- $\kappa$ B.<sup>159</sup> This fragment directly interacts with p65 and potentially acts as a transcription factor, and it also binds to I $\kappa$ B $\alpha$  (inhibitor of NF- $\kappa$ B-alpha), which induces calpain-mediated degradation of I $\kappa$ B $\alpha$  and activation of NF- $\kappa$ B.<sup>159</sup> Certainly, activation of NF- $\kappa$ B is essential for transcription of IL-1 $\beta$ . Consequently, the precise role of pyrin in IL-1 $\beta$  maturation is still controversial and is likely to be dependent on the context of cellular activation.

## V. NOD-LIKE RECEPTORS: LINK TO AUTOINFLAMMATORY AND AUTOIMMUNE DISEASES

Because IL-1 $\beta$  and IL-18 play such essential and

prominent roles in inflammation and innate immunity, it is not surprising that excessive production of both cytokines is closely linked to an expanding number of periodic fever syndromes and autoinflammatory diseases.<sup>160</sup> The term “systemic autoinflammatory disease” was first described for the TNFR1 (tumor necrosis factor receptor-1)-associated periodic syndromes,<sup>161</sup> and has since been expanded to include an increasing number of self-directed tissue-inflammatory conditions that are generally characterized by the absence of high-titer autoantibodies and antigen-specific T cells, thus having limited autoimmune origin.<sup>162</sup> The more details emerge on inflammasomes and their activation, the more evidence also points to a direct contribution of inflammasome defects, such as hereditary mutations in inflammasome activators leading to exaggerated immune responses in, IL-1 $\beta$  inflammasomopathies.

### A. Pyrin

FMF is a recessively inherited, prototypic autoinflammatory disease characterized by repeated and recurrent episodes of fever, which can be asymptomatic or coincide with abdominal pain; signs of peritonitis; joint pain in primarily one large joint; pain in the chest with pleuritis and pericarditis, myalgia, or erysipeloid; and elevated acute-phase reactions that can lead to secondary amyloidosis.<sup>163</sup> Positional cloning identified the FMF-linked gene, which was called *MEFV* (Mediterranean fever gene) and which encodes pyrin.<sup>164,165</sup> There have been 193 hereditary mutations in *MEFV* linked to FMF, with the majority being single-nucleotide substitutions that cluster in exon 2, which contains the bZIP, and exon 10, which contains PRY/SPRY (Fig. 2). Fewer mutations are found in exon 3 (containing the BBox) and exon 5 (see Infefvers, an online database for autoinflammatory mutations, at <http://fmf.igh.cnrs.fr/ISSAID/infefvers>).<sup>166-168</sup> As discussed above, the precise inflammatory role of pyrin is still controversial, and it is feasible that it has a dual physiological role. If pyrin has a predominantly anti-inflammatory role, then the recessively inherited FMF mutations could render the protein defective, causing a loss-of-function phenotype. Alternatively, if a pro-inflammatory role with inflammasome assembly is the main func-

tion of pyrin, then the dominant inherited FMF mutations could manifest as a gain-of-function phenotype.<sup>169</sup>

Notably, some of the most common mutations are located inside the PRY/SPRY domain, including M694V, V726A, M680I, M694I, together with E148Q in exon 2, which account for more than 70% of all cases.<sup>170</sup> This high frequency can only be explained by a positive selection mechanism, which possibly acts by conferring resistance to infection.<sup>169</sup> This hypothesis is supported by the observations that some of the FMF mutations inside the PRY/SPRY domain, including M680I and V726A, are found in wild-type primate alleles, indicating that some FMF mutations recapitulate an ancestral state and that primates might not be exposed to this putative pathogen.<sup>171</sup> Furthermore, the PRY/SPRY domain is missing in rodents, and therefore is a recent evolutionary development, further emphasizing a human-specific function.<sup>172</sup> Several of the severe FMF mutations localize close to a putative binding pocket in the PRY/SPRY domain, suggesting that pyrin could potentially directly recognize a pathogen.<sup>153</sup>

## B. NLRP3

NLRP3, initially known as cryopyrin and encoded by the *CIAS1* gene, was initially cloned based on its frequent mutation in the hereditary fever syndromes familial cold autoinflammatory syndrome (FCAS, also known as familial cold urticaria or FCU) and Muckle-Wells syndrome (MWS) (Fig. 2).<sup>173</sup> Subsequently, mutations in NLRP3 have also been identified in patients with neonatal-onset multisystem inflammatory disease (NOMID, also known as chronic infantile neurological cutaneous and articular syndrome or CINCA).<sup>174,175</sup> NLRP3 inflammasome-linked disorders are now collectively referred to as cryopyrinopathies or *CIAS1*-associated periodic syndromes (CAPS), and are caused by autosomal dominant or de novo mutations in NLRP3, which are generally linked to excessive production and maturation of IL-1 $\beta$ .<sup>153</sup> These disorders usually develop during childhood, and are characterized by unexplained episodes of fever and severe localized inflammation. Monocytes from cryopyrinopathy patients also display a deranged redox homeosta-

sis, characterized by elevated ROS levels and thus a more accelerated IL-1 $\beta$  maturation, which might partially explain the episodic nature of this disorder.<sup>176</sup> NOMID is characterized by recurrent episodes of fever, urticarial rash beginning in the neonatal period, chronic meningitis, uveitis, loss of hearing, and growth retardation.<sup>177</sup> FCAS displays recurrent episodes of rash, cold-induced urticaria, fever, arthralgia, and conjunctivitis frequently triggered by cold exposure.<sup>177</sup> MWS patients have fever, progressive loss of hearing, urticaria, arthralgia, and rare amyloidosis.<sup>177</sup> At least 120 mutations are described in NLRP3, although not all are clearly linked to cryopyrinopathies. Most of these mutations are located in exon 3, which encodes the NACHT domain and the flanking sequences, and represent single-nucleotide substitutions.<sup>166-168</sup> NLRP3 mutations cause spontaneous oligomerization, recruitment of ASC, inflammasome activation, and elevated production of IL-1 $\beta$ .<sup>42,64</sup>

Recently, mouse models of cryopyrinopathies that reflect the human IL-1 $\beta$ -dependent diseases have been generated by introducing FCAS and MWS mutations (A350V and L351P, corresponding to humans A352V and L353P), or by using the shared R258W mutation (corresponding to human R260W) into *Nlrp3*, and also show a skewed Th17 response.<sup>178,179</sup> Most importantly, the mutations do not cause spontaneous IL-1 $\beta$  release, but macrophages isolated from these mice display significantly higher sensitivity to TLR ligands and do not require exogenous ATP for IL-1 $\beta$  release.

NLRP3 polymorphisms are also linked to a number of autoimmune conditions. Gout and pseudogout are common autoinflammatory arthritides, which are caused by deposition of monosodium urate and calcium pyrophosphate dihydrate crystals in articular and periarticular tissues. Monosodium urate is a danger signal and a potent activator of the NLRP3 inflammasome.<sup>66</sup> Cholesterol crystals are also recognized by NLRP3 inflammasomes, and the inflammasome contributes to atherosclerosis in mice.<sup>84</sup> Fibrillar  $\beta$ -amyloid also activates the NLRP3 inflammasome, which is linked to the pathology of Alzheimer's disease, and silicosis and fibrosis are at least partially dependent on the NLRP3 inflammasome.<sup>83,85,86,180</sup> Schnitzler syndrome is a rare disease characterized

by a chronic urticarial eruption and monoclonal gammopathy, and the symptoms include bone pain, fever, arthralgia, lymphadenopathy, leucocytosis, hepatomegaly and/or splenomegaly. NLRP3 (V198M) predisposes to Schnitzler syndrome and dysregulated NLRP3 inflammasomes contribute to the pathogenesis of Schnitzler syndrome.<sup>181,182</sup>

Crohn's disease is a multifactorial, chronic inflammatory bowel disease mostly affecting colon and terminal ileum, which is characterized by excessive production of NF- $\kappa$ B-dependent proinflammatory cytokines, including IL-1 $\beta$ . Mutations and polymorphisms in NLRP3 (Q705K) alone and in combination with Cardinal (C10X), contribute to increased IL-1 $\beta$  production and the susceptibility to Crohn's disease, and polymorphisms in both genes also influence the severity of rheumatoid arthritis.<sup>183-186</sup> Two NLRP3 single-nucleotide polymorphisms (rs4612666 and rs10754558) are significantly associated with the susceptibility to food allergy, food-induced anaphylaxis, and aspirin-induced asthma, and also predispose to type-1 diabetes and celiac disease, indicating that derailed NLRP3 inflammasomes affect the pathogenesis of multiple diseases, further emphasizing the central role of NLRP3.<sup>187,188</sup>

### C. NLRP1

Generalized vitiligo is a polygenic, multifactorial, non-contagious disorder that causes autoimmune loss of melanocytes and results in progressive loss of pigmentation from skin, overlying hair, and oral mucosa. Polymorphisms in NLRP1 predispose to generalized vitiligo and associated autoimmune diseases autoimmune thyroid disease, latent autoimmune diabetes in adults, rheumatoid arthritis, psoriasis, pernicious anemia, systemic lupus erythematosus, and Addison's disease (Fig. 2).<sup>189-192</sup> Polymorphisms are located within the promoter region and could affect transcription of NLRP1 or cause one amino acid substitution (L155H), but whether these mutations affect inflammasome activity warrants further investigation.

### D. NLRP2

As discussed above, little is known about NLRP2-mediated inflammasome activation. Beckwith-Wiedemann syndrome (BWS), a genetically complex congenital overgrowth syndrome, is a genomic imprinting disorder caused by mutation or altered expression of genes located in the imprinted 11p15.5 chromosomal region that are implicated in cell cycle and growth control, such as IGF2 (insulin-like growth factor-2), KCNQ1oT1 (potassium voltage-gated channel KQT-like subfamily member-1 [KCNQ1] overlapping transcript-1), and CDKN1C (cyclin-dependent kinase inhibitor 1C).<sup>193</sup> A positional-candidate gene approach identified a homozygous frameshift mutation in exon 6 of NLRP2 in the mother of a BWS patient (Fig. 2).<sup>194</sup> The frameshift c.1479delAG causes p.Arg493SerfsX32, resulting in a truncated protein lacking 539 amino acids encoding the LRR. The disturbed imprinting of BWS patients suggests a role of NLRP2 in establishing or maintaining genomic imprinting/methylation.<sup>194</sup> This scenario is intriguing, because methylation might have evolved as a transposon defense mechanism. However, BWS patients do not show any inflammation.<sup>194,195</sup>

### E. NLRP7

NLRP7 has been implicated in the regulation of caspase-1 activity based on one overexpression study. Therefore, information on its contribution, to inflammasome activation is also very limited.<sup>196</sup> Familial recurrent hydatidiform mole (FRHM) is an autosomal-recessive condition that results in abnormal human pregnancy with no embryo and hydropic degeneration of chorionic villi.<sup>197</sup> Like BWS, FRHM is a genomic imprinting disorder, thus linking a second NLR to imprinting defects. There have been 145 NLRP7 mutations described, and at least 23 hereditary mutations in NLRP7 have been identified as a causative for FRHM. Women homozygous or compound heterozygous for NLRP7 mutations were shown to be at risk for reproductive wastage (Fig. 2).<sup>166-168</sup> Several NLRP7 mutations linked to FRHM have been identified, and the majority of the mutations affect the LRRs or the

NACHT of NLRP7.<sup>198-205</sup> There is speculation that FRHM might result from an immune-related defect during oogenesis or early embryonic development, but there is no information about whether NLRP7 mutations could cause gain-of-function, similar to cryopyrinopathies, or loss-of-function, as shown for Nod2 mutations in Crohn's disease. NLRP7 mutations might not be linked to inflammasomes after all, because NLRP7 has a role in cell proliferation, is up-regulated in testicular seminoma cells, and gene silencing of NLRP7 suppresses cell growth.<sup>206</sup> Expression levels of NLRP7 are also correlated with the myometrical invasion in endometrial cancer.<sup>207</sup>

#### F. NLRP12

Little is known about any inflammasome-activating role of NLRP12, but using a candidate gene approach, nonsense and splice site mutations were identified in the *NLRP12* gene in two families with a hereditary periodic fever syndromes referred to as Guadeloupe variant fever syndrome (FCAS2) (Fig. 2).<sup>208</sup> FCAS2 causes clinical symptoms similar to those of FCAS and MWS, including cold-induced fever episodes, arthralgia, and myalgia. At least 25 mutations have been identified, and two mutations, c.2072+3insT, which causes a deletion of the LRRs, and R284X, which truncates the protein within the NACHT, are linked to FCAS2.<sup>208</sup> NLRP12 (R284X) is significantly impaired in NF- $\kappa$ B inhibition, but NLRP12 (c.2072+3insT) is also less potent in NF- $\kappa$ B inhibition than the wild-type protein.<sup>208</sup> Whether these mutations also affect inflammasome activity is not known.

#### G. NLRB1

Spinal muscular atrophy (SMA) is an autosomal-recessive neuromuscular disease characterized by the degeneration of motor neurons in the spinal cord, which results in progressive weakness and wasting of voluntary muscles; IL-1 $\beta$  contributes to SMA.<sup>209</sup> Deletions and mutation of NLRB1 have been shown to cause SMA (Fig. 2).<sup>210</sup>

#### H. NOD1 and NOD2

Autosomal dominant mutations in Nod2, mostly within the NACHT domain, are linked to Blau syndrome, which is characterized by granulomatous dermatitis, arthritis, and uveitis (Fig. 2).<sup>211</sup> Blau syndrome-associated Nod2 mutations are gain-of-function mutations characterized by elevated NF- $\kappa$ B activation, and are mostly clustered in the central NACHT domain.<sup>166-168</sup> In addition, one of the inflammatory bowel disease risk alleles is Nod2, and Nod2 mutations are linked to the susceptibility to Crohn's disease, which is characterized by diarrhea and abdominal pain.<sup>212,213</sup> More than 77 mutations are linked to Crohn's disease and 24 to ulcerative colitis, which partially overlap with Crohn's disease; the most common ones are a frameshift mutation (c.L1007fsinsC) and substitutions within the LRR, such as R702W and G908R.<sup>213</sup> In contrast to inflammatory bowel disease, the Crohn's disease-associated mutations reflect a loss-of-function phenotype.<sup>214</sup> The Crohn's disease phenotype could result from reduced epithelial cell barrier function and  $\alpha$ -defensin expression, and thus impaired growth restriction and clearance of invasive bacteria such as *S. typhimurium*.<sup>215-217</sup> Accordingly, Crohn's disease-linked mutations show impaired cytokine release, including IL-1 $\beta$ .<sup>218,219</sup> However, macrophages engineered for Nod2 Crohn's disease mutations show elevated IL-1 $\beta$  secretion, suggesting that mutations could rather reflect a gain-of-function mutation.<sup>220</sup> Nod1 polymorphisms are also linked to susceptibility to Crohn's disease (Fig. 2).<sup>221,222</sup> Seven gain-of-function Nod2 mutations are linked to early-onset sarcoidosis.<sup>223,224</sup> Furthermore, the c.802C>T polymorphism is associated with chronic gastritis and predisposition to cancer in *H. pylori*-infected patients.<sup>225,226</sup>

### VI. INFLAMMASOMES: A TARGET FOR THERAPEUTIC INTERVENTION

Direct IL-1 $\beta$  inhibitors, such as recombinant IL-1Ra (anakinra), IL-1TRAP (rilonacept), or humanized IL-1 $\beta$  antibody (canakinumab), are effective in several IL-1 $\beta$  autoinflammatory conditions, most prominently the cryopyrinopathies, but are also effective

in rheumatic diseases and psoriasis.<sup>227-229</sup> Based on the interaction of IL-1Ra with the IL-1RI, the small peptide antagonist peptide 101.1 was developed, and is effective in vivo in inflammatory bowel disease and phorbol 12-myristate 13-acetate-induced dermatitis mouse models.<sup>230</sup> Caspase-1 has also been directly targeted (pralnacasan and VX765), which reduced several inflammatory conditions in vivo despite some toxicity.<sup>87,231,234</sup> An adverse effect, as for any targeted biological anti-cytokine therapy, is that global cytokine blocking is linked to a slight increase in emerging opportunistic infections.<sup>235,236</sup> Thus, a more targeted approach could be superior, such as specifically inhibiting a particular NLR. Colchicine, a microtubule inhibitor, is a standard treatment for FMF and gout, which not only impairs neutrophil migration and cytokine production, but also blocks phagocytosis.<sup>237</sup> In addition, colchicine and nocodazole, another microtubule inhibitor, reduce the interaction of PSTPIP1 with pyrin to block pyrin-induced activation of caspase-1.<sup>148</sup> The type 2 diabetes drug glyburide (glibenclamide), which inhibits ATP-sensitive K<sup>+</sup> channels, blocks the activation of the NLRP3 inflammasome by a distinct mechanism downstream of the P2X<sub>7</sub>R, and is the first NLRP-specific drug.<sup>238</sup> Potentially, the pannexin peptide could be used to similarly block NLRP3 activation, although no in vivo application has yet been reported.<sup>72</sup> Increasing evidence points to an intrinsic ATPase activity of NLRs, which is required for receptor function, whether this is modulation of NF- $\kappa$ B in the case of NLRP12 or activation of caspase-1 by NLRP1 and NLRP3.<sup>36,43,44</sup> Thus, one potentially could explore exploiting the ATP-binding pocket specific for any given NLR as an NLR-targeted therapy.

## VII. CONCLUSIONS

The study of NLRs and related PRRs and the discovery of the inflammasome have offered tremendous insights into innate immunity and host defense over the past decade. They have also provided molecular mechanisms for the pathogenesis of inflammatory diseases and for adjuvant activity, and thus enabled the availability of novel treatment opportunities, such as anakinra and its derivatives and the

prospect for novel therapies specifically targeting activation of inflammasomes for auto-inflammatory disease patients. However, there is still much room for further investigation, considering that we currently only have limited understanding of the nature of agonists initiating activation and of the physiological and potentially pathological consequences resulting from activation of the majority of NLRs.

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