

## REVIEW ARTICLE OPEN



# Molecular mechanisms and regulation of inflammasome activation and signaling: sensing of pathogens and damage molecular patterns

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The inflammasome is an inflammatory signaling protein complex comprising a sensor protein, the adaptor protein ASC, and the cysteine protease caspase-1. Inflammasome sensor proteins are activated by microbial molecular patterns, endogenous self-derived damage signals, or exogenous environmental danger signals. Multiple inflammasomes that differ in their mechanisms of action and structural composition have been identified. The best characterized are the canonical NLRP1, NLRP3, NAIP-NLRC4, AIM2, and Pyrin inflammasomes and the noncanonical inflammasomes activated by caspase-4, caspase-5 or caspase-11. The lesser known inflammasomes are the NLRP6, NLRP7, NLRP9, NLRP10, NLRP12, CARD8, and MxA inflammasomes. Following inflammasome assembly, caspase-1 promotes the secretion of the proinflammatory cytokines IL-1 $\beta$  and IL-18, and pyroptosis is mediated by the membrane-disrupting proteins gasdermin D and ninjurin-1. These functional activities control innate and adaptive immune responses and the initiation, development, and progression of autoinflammation, cancer, infectious diseases, and neurodegenerative diseases. Understanding how inflammasomes respond to pathogens and sterile signals has refined our view of innate immunity and offered new therapeutic targets. In this review, we present a comprehensive overview of inflammasomes with an emphasis on the mechanistic principles that govern inflammasome formation. We also discuss the contributions of inflammasome activation to health and disease.

**Keywords:** GSDMD; Infection; Interferons; LPS; NINJ1; Pattern-recognition receptors

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## INTRODUCTION

The inflammasome is a cytoplasmic protein complex that contains an inflammasome sensor protein, the adaptor protein called apoptosis-associated speck-like protein containing a CARD (known as ASC or PYCARD), and the cysteine protease caspase-1 [1–3]. Following the formation of this complex, caspase-1 undergoes activation and drives proteolytic cleavage or processing of a range of substrates. Among these substrates are pro-interleukin-1 $\beta$  (IL-1 $\beta$ ) and pro-IL-18, which, via caspase-1-dependent cleavage, are converted to their biologically active forms [4, 5]. Caspase-1 also cleaves the pore-forming protein gasdermin D (GSDMD) [6–12]. Once cleaved, the N-terminal fragment of GSDMD forms pores in the plasma membrane, allowing the bioactive forms of IL-1 $\beta$  and IL-18 to escape from within the cytoplasm to outside of the cell [13, 14]. The build-up of GSDMD pores on the plasma membrane creates osmosis and the influx of water into the cell, causing the typical ballooning morphology and eventual lysis of the cell, called pyroptosis [15–18]. The physical rupture of the plasma cell membrane requires the membrane protein ninjurin-1 (also known as NINJ1) [19], and this tearing process

liberates the remaining cellular content into the extracellular environment [20, 21].

Inflammasome formation is initiated by inflammasome sensor proteins. These sensor proteins are part of a larger family of germline-encoded pattern-recognition receptors (PRRs) that control inflammation, cell death, and the activation and recruitment of immune cells, resulting in hallmarks of inflammation characterized by redness, swelling, heat, and pain [22, 23]. PRRs, including inflammasome sensor proteins, detect all types of pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), and exogenous environmental danger signals [24, 25]. All microbial components, including LPS, bacterial toxins, and viral proteins and nucleic acids, are considered PAMPs. DAMPs are endogenous self-derived molecules, such as nuclear and mitochondrial DNA and ATP, and when they are mislocalized, they are sensed by PRRs. Exogenous environmental danger signals are broadly defined and can include pollutants, silica, asbestos, and venom.

Several families of PRRs can form inflammasomes. The nucleotide-binding domain and leucine-rich repeat (LRR)-containing gene family (also known as NOD-like receptors or NLRs) carry

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the largest number of inflammasome-forming proteins. NLRP1, NLRP3, NLRC4, NLRP6, NLRP7, NLRP9, NLRP10, NLRP11, NLRP12, and NALPs can form inflammasomes. Many NLRs contain a centrally located NACHT (also known as the domain present in NAIP, CIITA, HET-E, and TP-1; SPRY, Spla/Ryanodine receptor domain) and a C-terminal LRR. In general, NLRs carrying an N-terminal caspase activation and recruitment domain (CARD) are called NLRC, whereas those carrying an N-terminal Pyrin domain (PYD) are called NLRP [26–28].

Five other groups of PRRs can form inflammasomes. AIM2 and interferon gamma-inducible protein 16 (IFI16) from AIM2-like receptors (ALRs) are inflammasome sensor proteins. Pyrin is the only inflammasome sensor from the tripartite motif-containing protein receptor (TRIM). CARD8 from a family of loosely classified CARD-containing proteins and MxA from the interferon-inducible GTPase family are both poorly characterized proteins that have been shown to assemble inflammasome complexes. The last group of PRRs that can form inflammasomes is human caspase-4, human caspase-5, and mouse caspase-11. They are collectively referred to as noncanonical inflammasomes because they directly sense cytoplasmic LPS and subsequently drive the activation of the NLRP3 inflammasome [29, 30]. This unique activating step is referred to as the noncanonical inflammasome pathway. Since then, all other inflammasomes that do not require human caspase-4, human caspase-5, or mouse caspase-11 as part of their activation mechanisms have been known as canonical inflammasomes.

The discovery of new PRRs capable of initiating inflammasome activation via conventional or novel mechanisms has substantially advanced our understanding of innate immunity. Given the diverse range of signals that can drive inflammasome activation, aberrant or disrupted inflammasome signaling is linked to inflammatory diseases. As such, inflammasomes have emerged as novel therapeutic targets for human diseases. In this review, we provide a comprehensive overview of the molecular mechanisms governing the activation of inflammasome sensors and the implications of their dysregulated activity in health and disease.

### NLRP1 INFLAMMASOME

Human NLRP1 (also known as CARD7, DEFCAP, KIAA0296, NAC and NALP1) is the first receptor that was found to assemble an inflammasome complex [31]. Human NLRP1 has a C-terminal CARD, a centrally positioned function-to-find (FIIND), an LRR, NACHT, and an N-terminal PYD [32]. The NACHT consists of Walker A and B motifs that facilitate ATP binding and hydrolysis for NLRP1 activation [33, 34]. A single gene encodes NLRP1 in humans, whereas three paralogs of NLRP1, encoding NLRP1a, NLRP1b and NLRP1c, are found in mice [35, 36]. Mouse NLRP1a and NLRP1b paralogs possess a C-terminal CARD, and via this CARD, they can bypass ASC by directly recruiting caspase-1 [33, 34]. In humans, NLRP1 is expressed in the stomach, intestines, lungs, testis, and skin and is enriched in barrier cell types such as bronchial epithelial cells and keratinocytes [37–39]. In mice, NLRP1 paralogs are expressed in the hippocampus [40] and in macrophages [41], with little to no expression in keratinocytes [39].

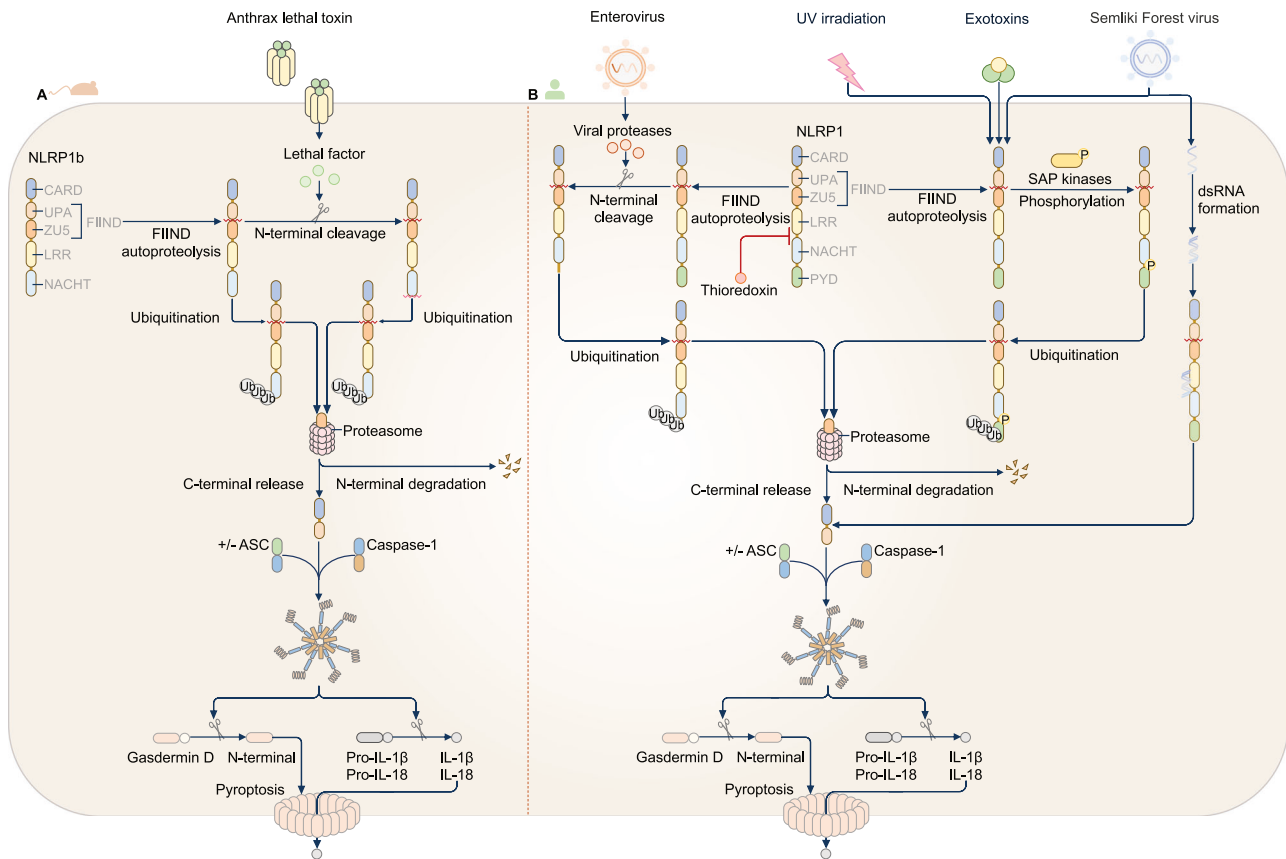
An initial 2006 study demonstrated that mouse NLRP1b induces caspase-1-mediated cell death in response to anthrax lethal toxin [35]. Subsequent structural and mechanistic studies clarified the mechanisms of NLRP1 activation (Fig. 1A, B). Prior to activation, NLRP1 undergoes FIIND-mediated autoproteolysis between the ZU5 subdomain and the UPA subdomain, resulting in the generation of an N-terminal fragment and a C-terminal UPA-CARD, which remain noncovalently attached [33, 34, 42]. A key mechanism driving the activation of NLRP1 is the degradation of its N-terminal domain, which releases the C-terminal UPA-CARD that forms the inflammasome [39, 43–45]. This is achieved by stimulation with certain microbial factors, such as toxins, or the chemical inhibitor Val-BoroPro (also known as VbP, Talabostat, or

PT100), which inhibits the proteolytic enzymes dipeptidyl peptidase (DPP) 8 and DPP9 [46, 47]. Under steady-state conditions, the FIIND of full-length human, mouse or rat NLRP1 interacts with DPP8 or DPP9 to form an inactive ternary complex that traps the UPA-CARD [48, 49]. Val-BoroPro disrupts this interaction between NLRP1 and DPP8 or DPP9, promoting the accelerated proteasomal degradation of the N-terminal fragment and the release of the UPA-CARD of NLRP1 [49].

Other factors or drivers leading to N-terminal NLRP1 degradation include microbial components, protein folding stress, reductive stress, metabolic stress, and tissue damage [50–53]. In human keratinocytes, for example, NLRP1 can be activated by ribosomal stress in response to ultraviolet light [54–57]. Upon exposure to ultraviolet light, stalled ribosomes cause the activation of stress-activated protein (SAP) kinases, leading to the hyperphosphorylation of serine residues between the PYD and NACHT of NLRP1 [57]. This hyperphosphorylation causes N-terminal NLRP1 degradation through an unknown pathway, leading to the release of UPA-CARDs. Other triggers of SAP kinase activation include exotoxin-A from the bacterium *Pseudomonas aeruginosa*, which activates NLRP1 in human keratinocytes and corneal and airway epithelial cells [58, 59]. Proteases from viruses in the Picornaviridae family induce NLRP1 inflammasome activation by cleaving human NLRP1 between the PYD and NACHT, called the tripwire region [60]. Human NLRP1 is also activated in keratinocytes and bronchial epithelial cells in response to viral dsRNA [61, 62]. NLRP1 may serve as an alternative responder to cellular stress in mammalian cells where other known nucleic acid sensors are absent. For example, NLRP1 is activated in response to the dsDNA poly(dA:dT) in human keratinocytes that lack AIM2 [62]. Similarly, in primary human skin and nasal and corneal epithelial cells that lack NLRP3, NLRP1 can be triggered by the ionophore nigericin, leading to potassium (K<sup>+</sup>) efflux and inflammasome activation [63].

Germline mutations in the gene encoding human NLRP1 are found in patients with autoinflammatory disorders, eye disorders, mucosal inflammation, multiple myeloma, and neurodegenerative diseases [64]. Variations in the gene encoding human DPP9, which can lead to aberrant activation of NLRP1, also contribute to inflammasomopathies. These conditions may present as skin abnormalities, immune response defects, anemia, and increased susceptibility to herpes virus infections [65]. Thus, pharmacological modulation of NLRP1, such as the use of the small-molecule dual NLRP1 and NLRP3 inhibitor ADS032 [66] or the modulation of DPP9 activity, holds substantial promise in targeting NLRP1-mediated immune responses in these disorders. In addition, endogenously oxidized thioredoxin can bind to NACHT and LRR and inhibit human NLRP1 [50, 67], which provides another therapeutic target. Additionally, uncovering the mechanisms of NLRP1 activation, particularly posttranslational modifications that trigger NLRP1 N-terminal degradation, may reveal how NLRP1 responds to different triggers and whether this degradation can be accelerated to enhance the killing of virus-infected cells or inhibited to control sepsis. Furthermore, studying the tissue-specific functions of NLRP1 and its potential coactivation with other immune sensors in response to distinct stimuli could shed light on its broader role in orchestrating immune responses.

A major challenge remains in defining the full spectrum of endogenous and pathogen-derived triggers that induce NLRP1 N-terminal degradation in specific tissues. The lack of NLRP1 expression in murine keratinocytes complicates the use of mouse models in the study of NLRP1-mediated inflammation. Humanized mouse models expressing human NLRP1 in epithelial tissues or organoids and primary human keratinocyte cultures can be employed to more accurately recapitulate NLRP1 activation in vivo. Dissecting the regulation of FIIND autoproteolysis under physiological versus stress conditions is also a key priority. Additional approaches using tissue-specific knockout models



**Fig. 1** The NLRP1 inflammasome. **A** Murine NLRP1b undergoes autoproteolytic cleavage in the function-to-find domain (FIIND), generating two noncovalently associated fragments that maintain an autoinhibitory state. The activation of NLRP1b is initiated by extracellular stimuli, including anthrax lethal toxin, which cleaves the N-terminal domain of the nucleotide-binding domain (NBD)-leucine-rich-repeat domain (LRR)-FIIND fragment, marking it for ubiquitination and degradation by the proteasome. This process releases the C-terminal fragment (containing a caspase-activation and recruitment domain; CARD), which initiates inflammasome assembly with or without ASC. The assembled inflammasome leads to caspase-1-dependent cleavage of pro-IL-1 $\beta$  and pro-IL-18 and pyroptosis (left). **B** The activation of human NLRP1 also requires a series of proteolytic cleavage events. Human NLRP1 also undergoes autoproteolytic cleavage in the function-to-find domain (FIIND), generating two noncovalently associated fragments that maintain an autoinhibitory state. In addition, oxidized thioredoxin binds to the NACHT-LRR region of NLRP1 and suppresses its activation. Diverse stimuli, including viral protease cleavage, ultraviolet (UV) irradiation, exposure to exotoxins, double-stranded RNA (dsRNA), or stress-activated protein (SAP) kinase-mediated phosphorylation, promote the ubiquitination and degradation of the N-terminal fragment. Proteasomal degradation of the N-terminus releases the active C-terminal UPA-CARD fragment, which forms the NLRP1 inflammasome, triggering caspase-1-dependent cleavage of pro-IL-1 $\beta$  and pro-IL-18 and pyroptosis (right)

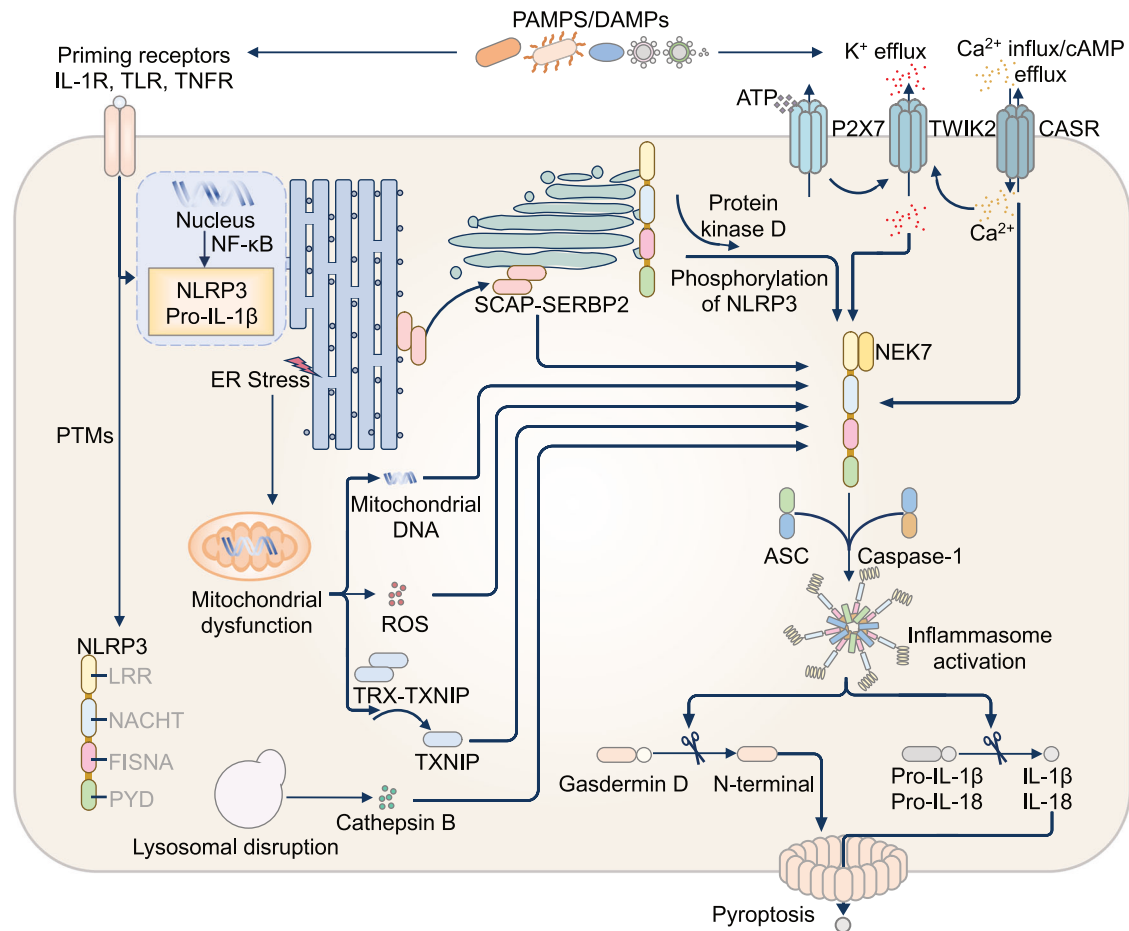
and inducible NLRP1 mutants may help address these pressing questions and guide therapeutic targeting of NLRP1 in autoinflammatory and infectious diseases.

### NLRP3 INFLAMMASOME

NLRP3 (also known as NALP3, Pypaf1, Cryopyrin and CIAS1) is the best characterized NLR and is expressed in the spleen, intestine, liver, kidneys, lungs and brain of humans and mice, with the highest expression in immune cells [37, 68–70]. Like many other NLR family members, it contains an N-terminal PYD, NACHT and C-terminal LRR. NLRP3 was identified through its association with a group of rare autoinflammatory diseases collectively known as cryopyrin-associated periodic syndrome (CAPS) [71–73]. Earlier studies established that NLRP3 interacts with ASC to form an inflammasome complex [31, 74, 75] following the sensing of PAMPs, DAMPs, and exogenous danger signals [76–79]. Since then, the pathways activated by NLRP3 have been broadly defined as the canonical NLRP3 inflammasome or the noncanonical NLRP3 inflammasome. This section focuses on canonical NLRP3 inflammasome activation, whereas noncanonical NLRP3 inflammasome activation will be further discussed in a separate section.

Activation of the canonical NLRP3 inflammasome requires a two-step process involving priming and activation signals (Fig. 2). The priming process is triggered by Toll-like receptors (TLRs) that sense PAMPs and/or DAMPs, leading to the activation of the NF- $\kappa$ B signaling cascade and the transcription of genes encoding NLRP3, pro-IL-1 $\beta$  and other proinflammatory cytokines [80, 81] (Fig. 2). In some cases, cell-surface cytokine receptors such as tumor necrosis factor (TNF) receptors and cytosolic PRRs such as NOD1 and NOD2 can also induce the activation of NF- $\kappa$ B signaling and, therefore, the priming process [80, 81]. Priming also induces posttranslational modifications such as phosphorylation by the kinase PKD [82], palmitoylation by the palmitoyltransferase ZDHHC5 [83], and SUMOylation by the regulatory TRIM protein TRIM28 [84], which collectively promote NLRP3 structure stabilization and inflammasome assembly. NLRP3 activation and inflammasome formation can also be suppressed before or during priming by other inhibitory posttranslational modifications, such as ubiquitination [85–90].

The second step of the activation signal is initiated by a variety of triggers, ranging from PAMPs from bacteria, viruses, parasites, and fungi to endogenous and exogenous signals [91, 92]. These signals alter cellular homeostasis, typically through K<sup>+</sup> efflux



**Fig. 2** The NLRP3 inflammasome. The NLRP3 inflammasome can be activated via canonical or noncanonical pathways. Canonical NLRP3 activation occurs in a two-step process. The first step, known as priming, is triggered by several classes of receptors in response to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). The activation of priming receptors stimulates nuclear factor (NF)- $\kappa$ B signaling, resulting in the transcriptional upregulation of NLRP3 and proinflammatory cytokines, including pro-IL-1 $\beta$ . In addition, the priming step promotes posttranslational modifications (PTMs) of NLRP3 to maintain it in a poised state. The second step, or activation, is driven by diverse stimuli, including microbial products, environmental irritants, and cellular stressors, that induce cellular perturbations such as potassium ( $K^+$ ) efflux, calcium ( $Ca^{2+}$ ) influx, lysosomal disruption, mitochondrial dysfunction, and endoplasmic reticulum (ER) stress. For example, extracellular ATP binds the P2X7 receptor, affecting TWIK2 channels to mediate  $K^+$  efflux.  $Ca^{2+}$  influx is triggered via the calcium-sensing receptor (CASR), which in turn reduces the level of intracellular cyclic AMP (cAMP), relieving the inhibitory effect of cAMP on NLRP3. Mitochondrial damage results in the release of mitochondrial DNA, reactive oxygen species (ROS), and thioredoxin-interacting protein (TXNIP), all of which contribute to NLRP3 activation. ER stress exacerbates mitochondrial dysfunction to facilitate NLRP3 activation. The translocation of the cholesterol transcription factor and its chaperone, the SCAP-SREBP2 complex, from the ER to the Golgi apparatus under stress promotes NLRP3 activation. Moreover, protein kinase D can phosphorylate Golgi-bound NLRP3, facilitating its release and activation. Disruption of lysosomes leads to the release of cathepsin B, which can also activate NLRP3. Upon activation, NLRP3 binds to NIMA-related kinase (NEK) 7, which stabilizes the active conformation of NLRP3 and facilitates its oligomerization. Activated NLRP3 then recruits the adaptor protein ASC, which in turn binds with caspase-1 to form the functional inflammasome complex

[93–95], lysosomal disruption [96, 97], and mitochondrial dysfunction [98, 99]. In general, NLRP3 activation occurs through  $K^+$  efflux-dependent or -independent pathways. The  $K^+$  efflux-dependent pathway is the best characterized pathway and is triggered by the majority of NLRP3 activators [93–95]. In the case of ATP stimulation, for example, ATP binds to the nonselective cation channel P2X purinoceptor 7 (P2X7), which cooperates with the two-pore domain  $K^+$  channel TWIK2, leading to  $K^+$  efflux and NLRP3 activation [76, 93, 100, 101] (Fig. 2). In the case of bacterial pore-forming toxins, such as *Bacillus cereus* hemolysin BL and nonhemolytic enterotoxin, these toxins bind to host cell-surface receptors and oligomerize into a membrane pore [102, 103]. These toxin pores disrupt plasma membrane integrity and release  $K^+$  ions through osmosis, which drive NLRP3 activation [102, 103].

How  $K^+$  efflux activates NLRP3 is unclear, but it likely requires relieving the autoinhibition of NLRP3. Furthermore, the kinase NEK7 is a crucial component of the NLRP3 inflammasome

[104–106], and  $K^+$  efflux promotes the interaction between NLRP3 and NEK7 [104]. However,  $K^+$  efflux-independent and reactive oxygen species (ROS)-dependent NLRP3 activation by the chemical compound imiquimod also requires NEK7 [98], indicating that  $K^+$  efflux must cause other NLRP3-activating cellular changes in addition to promoting the NLRP3–NEK7 interaction. Structural analysis indicated that NEK7 binds to the LRR of the inactive “cage” conformation of NLRP3 and enables opening of the cage into two halves anchored by an NLRP3 PYD filament [104, 106, 107]. These halves then assemble into an NLRP3 wheel-like oligomer through NLRP3–NLRP3 interactions, with the LRR facing outward and the PYD forming a disk at the center [108]. NLRP3 oligomerization is an ATPase-dependent process in which ATP binds the nucleotide-binding site within NACHT and stabilizes NLRP3 in the active state when hydrolyzed by ATPase elements such as the Walker A and B motifs [109, 110]. The NLRP3 oligomer then acts as a scaffold for ASC recruitment [111–113]. Recruited

ASC proteins form a long helical filament where caspase-1 binds to the ASC CARD as the presumed last step of inflammasome formation [111–114]. NEK7 is also phosphorylated at threonine-190 or -191 by the kinase JNK1 following  $K^+$  efflux, NLRP3 activation, and GSDMD pore formation, providing a positive feedback loop that enhances the binding between NLRP3 and NEK7 [115].

Other forms of cation signaling, such as calcium ion ( $Ca^{2+}$ ) flux, function independently or in concert with  $K^+$  efflux to trigger NLRP3 inflammasome activation [116–118]. The calcium-sensing receptor (CASR) activates NLRP3 by increasing the level of intracellular  $Ca^{2+}$  and decreasing the level of cellular cyclic AMP (cAMP), with cAMP binding to and inhibiting NLRP3 [116, 119]. CASR increases intracellular  $Ca^{2+}$  levels by interacting with phospholipase C to increase inositol-1,4,5-trisphosphate production and the efflux of  $Ca^{2+}$  ions from the endoplasmic reticulum (ER) [116]. Moreover, CASR decreases cAMP levels by binding to and inhibiting the adenylate cyclase enzyme needed for the conversion of ATP to cAMP [116]. Additionally, an increase in intracellular  $Ca^{2+}$  during opening of the P2X7 channel increases  $K^+$  efflux by activating the Ras-related protein Rab11a, which is necessary for the translocation of TWIK2 to the plasma membrane [120]. Moreover, efflux of chloride ions ( $Cl^-$ ) through  $Cl^-$  channels either promotes IL-1 $\beta$  transcription, the NEK7–NLRP3 interaction, and ASC speck formation and oligomerization [121, 122] or inhibits NLRP3 [123].

In addition to ion flux, cell organelles contribute to NLRP3 inflammasome activation [124]. When dysregulated, mitochondrial components and products activate the NLRP3 inflammasome [125–129]. These include oxidized mitochondrial DNA, which activates NLRP3 and facilitates inflammasome formation [125–127], and the mitochondrial apoptotic effectors BAX and BAK [128]. BAX and BAK trigger NLRP3 inflammasome formation indirectly by increasing caspase-3 and caspase-7 activation and caspase-3- and -7-dependent  $K^+$  efflux, driving the activation of NLRP3 [128]. Similarly, mitochondrial ROS increase the expression of inflammasome components to mediate inflammasome assembly and additionally dissociate thioredoxin-interacting protein (TXNIP) from thioredoxin to activate NLRP3 [130]. Further work also suggested that certain NLRP3 activators, such as imiquimod, extracellular ATP and the bacterial ionophore nigericin, can inhibit oxidative phosphorylation and, in turn, suppress mitochondrial ATP production and induce damage to the architecture of the mitochondrial cristae [131]. These mitochondrial stressors alone are insufficient to trigger NLRP3 activation but do so in the presence of secondary signals, such as the TLR7/8 agonist resiquimod or Yoda1, an activator of the mechanosensitive ion channel PIEZO1 [131].

The ER contributes to protein synthesis and modifications, such as protein folding, and serves as the assembly line for the active NLRP3 inflammasome. In its resting state, NLRP3 localizes to the *trans*-Golgi network (TGN) as a monomer or in the inactive cage conformation [132]. It is thought that the activation signal prompts a conformational change in NLRP3 and the dispersion of the TGN into vesicles containing NLRP3 [132]. NLRP3 binds to the dispersed TGN through ionic bonding of the polybasic region of NLRP3 and the negatively charged phospholipid phosphatidylinositol 4-phosphate (PtdIns4P) on the dispersed TGN [132]. In response to nigericin,  $K^+$  efflux does not affect TGN dispersion but is required for NLRP3 recruitment to the remodeled TGN [132]. This model is not universal because, in response to Type A cholesterol-dependent cytolytic toxins, exemplified by perfringolysin O from *Clostridium perfringens*,  $K^+$  efflux affects neither TGN dispersion nor NLRP3 recruitment to the remodeled TGN [133]. Instead, a small amount of toxins enter the cytoplasm and peel away the PtdIns4P-negative TGN membrane into multiple vesicles, exposing the remodeled PtdIns4P-positive TGN membrane for NLRP3 recruitment [133].

Dispersed TGN vesicles are thought to traffic to the microtubule organizing center, where NEK7 is recruited and activates NLRP3 [108, 132, 134]. The ER further modulates NLRP3 activation through calcium signaling and organelle crosstalk. Inhibition of ER-to-mitochondria  $Ca^{2+}$  flux has been shown to impair NLRP3 activation in bone marrow-derived macrophages (BMDMs) [116, 117]. In contrast, ER stress promotes mitochondrial dysfunction, ROS generation, and NLRP3 activation [135]. In addition, perturbed trafficking between organelles can facilitate NLRP3 activation. For example, disruption of ER-endosome membrane sites causes impaired endosome-to-TGN trafficking and accumulation of PtdIns4P in endosomes, which in turn increases NLRP3 recruitment and inflammasome formation [136, 137].

The Golgi apparatus sorts proteins from the ER for transport to the cell membrane and works as a hub for NLRP3 activation signals [113]. A complex formed by the cholesterol transcription factor sterol regulatory element binding protein 2 (SREBP2) and its chaperone SREBP cleavage-activating protein (SCAP) binds to NLRP3 in a ternary complex and escorts it to the Golgi to optimize inflammasome assembly [138]. PKD further phosphorylates NLRP3 on the Golgi, enabling the release of NLRP3 from mitochondria-associated ER membranes and the formation of an inflammasome in the cytoplasm [82]. Lysosomes, which breakdown cellular waste and intracellular pathogens, also enable NLRP3 inflammasome activation. Lysosome-related NLRP3 activation is triggered by the phagocytosis of self or foreign particles, including amyloid- $\beta$  [139], uric acid crystals [79], cholesterol or deoxycholic acid crystals [140, 141], silica and aluminum salts [96], or bacterial enzymatic toxins [97]. Furthermore, the stress granule protein DD3X3 interacts with NLRP3 to promote inflammasome activation and pyroptosis or relieves NLRP3 to form stress granules to promote cell survival [142].

In human monocytes, NLRP3 activation can occur through alternative pathways. In primary human monocytes, but not in mouse monocytes, LPS-TLR4 engagement triggers the TRIF–RIPK1–FADD–caspase-8 signaling cascade, which drives NLRP3 inflammasome assembly, recruiting ASC and caspase-1 to process IL-1 $\beta$  [143]. Unlike the canonical pathway, this route bypasses  $K^+$  efflux and induces IL-1 $\beta$  secretion without triggering pyroptotic cell death, representing a nonlytic mode of inflammasome activation [143]. A subsequent study revealed that, in addition to TLR4, other TLRs, including TLR1/2, TLR2/6, and TLR7/8, can also activate NLRP3 via an alternative, nonlytic pathway, in which RIPK1 is dispensable, while  $K^+$  efflux and pyroptosis are similarly bypassed [144]. Heat-killed gram-negative bacteria also induce NLRP3 activation via a single-step alternative pathway in human monocytes. This pathway is negatively regulated by the short isoform of cellular FLICE-like inhibitory protein (cFLIP<sub>S</sub>), which inhibits caspase-8 and reduces IL-1 $\beta$  release [145]. cFLIP<sub>S</sub> expression is controlled by TGF- $\beta$ -activated kinase 1 (TAK1)-dependent NF- $\kappa$ B signaling, and indeed, TAK1 activity is essential for caspase-8 cleavage in response to these bacterial stimuli [145]. Collectively, these studies highlight the breadth of nonlytic alternative mechanisms regulating NLRP3 in human monocytes, underscoring their mechanistic distinction from the canonical two-signal model.

Given the abundance of NLRP3 activators, it is not surprising that the NLRP3 inflammasome has been implicated in many forms of infectious and inflammatory diseases. Indeed, the NLRP3 inflammasome plays an important role in the clearance of bacterial, viral and fungal infections [76, 77, 146–152]. NLRP3 inflammasome activation is stimulated by gram-positive bacteria, such as *Staphylococcus aureus* [76], *Streptococcus* species [153, 154], and *Clostridium* species [97, 155], and gram-negative bacteria, such as *Salmonella* [156–158] and *Yersinia* [159] species, *P. aeruginosa* [160] and *Escherichia coli* [29, 30]. Some bacteria have evolved mechanisms to suppress the NLRP3 inflammasome to increase survival in the host. These bacteria include *Helicobacter*

*pylori*, which reduces NLRP3 activation by mediating mitophagy-mediated degradation of damaged mitochondria via the virulence factor CagA [161], and *Yersinia pestis*, which uses the type III secreted outer effector protein YopK to alter the structure of the type III secretion system and disguise it from NLRP3 sensing [159].

The NLRP3 inflammasome is also involved in antiviral responses to RNA and DNA viruses, namely, influenza A virus (IAV) [151, 162, 163], hepatitis B virus (HBV) [164, 165], Japanese encephalitis virus (JEV) [166], Rift Valley fever [167], encephalomyocarditis virus (EMCV) [168], foot-and-mouth disease virus [169], Mayaro virus (MAYV) [170], varicella-zoster [171], dengue [172] and Zika [173] viruses. NLRP3 is not known to bind directly to viral products. Instead, NLRP3 senses cellular perturbations induced by viral infections, including the activation of the mitochondrial antiviral-signaling protein MAVS, RIPK1-RIPK3-DRP1 signaling, K<sup>+</sup> efflux, ROS accumulation, lysosome and mitochondrial damage, and the release of oxidized DNA and the lysosomal protease cathepsin B [174–179]. Notably, NLRP3 also has inflammasome-independent functions in antiviral immunity by promoting type I interferon responses. In mice, acetate produced by the gut bacterium *Bifidobacterium pseudolongum* NjM1 activates the host G protein-coupled receptor GPR43, which engages NLRP3 to facilitate MAVS oligomerization on mitochondria [180]. This response triggers TANK-binding kinase 1 (TBK1)-mediated phosphorylation of interferon regulatory factor 3 (IRF3) and subsequent transcription of type I interferons, enhancing antiviral defense against IAV independently of caspase-1 or IL-1 $\beta$  activation [180].

Furthermore, NLRP3 inflammasome signaling mediates immunity to fungal species *Candida albicans* [147, 181], *Aspergillus fumigatus* [182, 183], and *Talaromyces marneffeii* [184]. These fungi activate the NLRP3 inflammasome via  $\beta$ -glucan zymosan [185], fungal DNA [182] or the polysaccharide galactosaminogalactan [186]. The production of pro-IL-1 $\beta$  and the release of mature proinflammatory cytokines by the NLRP3 inflammasome during fungal infection can involve crosstalk with other fungal-sensing receptors. For example, Dectin-1 recognizes many fungal species, such that the activation of the NLRP3 inflammasome by certain fungal species is partially mediated by Dectin-1 [187–192]. The NLRP3 inflammasome can also mediate chronic and pathological inflammation during infectious diseases [97, 102, 103, 155, 193–199]. For example, excessive NLRP3 inflammasome signaling is a key contributor to cytokine storms, respiratory distress and organ failure during COVID-19 [193, 194] and exacerbates injury to the lungs and intestinal barrier during bacterial infection [195–197]. Furthermore, it enables persistent human immunodeficiency virus (HIV) infection by contributing to CD4<sup>+</sup> T-cell death [200].

In humans, gain-of-function mutations in the gene encoding NLRP3 drive overactivation of NLRP3 and the development of CAPS [72, 73]. CAPS can be divided into three subtypes on the basis of severity and onset. The mildest form of CAPS is familial cold autoinflammatory syndrome (FCAS), followed by Muckle-Wells syndrome (MWS), with the most severe form of CAPS being neonatal multisystemic inflammatory syndrome (NOMID) [72–74, 201]. NLRP3 mutations enhance NLRP3 inflammasome activity through various mechanisms, including increased ATP binding, oligomerization of the PYD, and reduced binding affinity with NLRP3-inhibiting cAMP molecules [116, 202]. Mapping of CAPS mutations to the NLRP3 structure revealed that most mutations, including the common R260W, L305P, T348M and A439V mutations, are located within the NACHT [107]. These mutations destabilize the inactive conformation of NLRP3, thereby lowering the activation threshold [107]. Certain mutations hypersensitize NLRP3 to cold exposure and nigericin, and most CAPS-associated variants are responsive to the NLRP3 inhibitor MCC950 (also known as CP-456,773 and CRID3) [202]. Although

MCC950 has shown efficacy in preclinical models, its toxicity has limited its clinical utility [203]. Therefore, the development of next-generation safer NLRP3 inhibitors remains a critical therapeutic goal for the treatment of CAPS and other NLRP3-dependent inflammatory diseases.

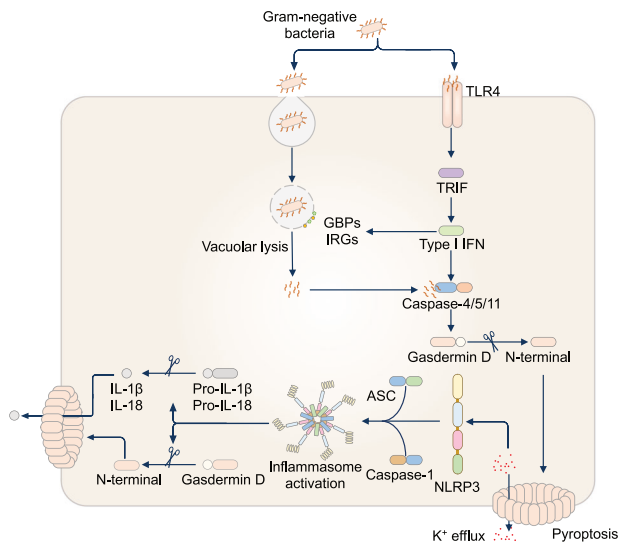
In addition to infectious and genetic conditions, NLRP3 activation contributes to the development of neurodegenerative disorders, Alzheimer's disease [139, 204], Parkinson's disease [205], cancer [206–212], atherosclerosis [140], gout [79], inflammatory bowel disease (IBD) [206, 213–215], liver diseases [216–219], obesity [216], rheumatoid arthritis [220], and type 2 diabetes [221]. In the case of IBD, for example, the NLRP3 inflammasome can elicit both protective and damaging effects. NLRP3 inflammasome activation in the gut causes excessive inflammation during IBD; however, defective NLRP3 inflammasome formation results in a loss of gut epithelial integrity, bacterial overgrowth, and increased susceptibility to dextran sodium sulfate (DSS)-induced colitis [206, 213, 214]. A similar duality in NLRP3 function is observed in certain cancers. The NLRP3 inflammasome has antitumor effects in colon cancer but promotes tumor growth in pancreatic and breast cancers and confers resistance to checkpoint inhibition therapy [206–208, 214, 222]. The mechanisms underlying these context-dependent effects remain unclear, highlighting the need for further research into NLRP3 activation and regulatory pathways and their crosstalk with other immune signaling networks.

A key challenge is to define the context-specific mechanisms that render NLRP3 activation protective or pathological across tissues and disease states. It remains unclear how upstream signals, posttranslational modifications, and organelle crosstalk integrate to fine-tune NLRP3 activation in vivo, particularly under chronic or low-grade inflammatory conditions. Experimental strategies to address these questions may include proximity labeling and proteomics to identify novel NLRP3-interacting factors under distinct activation states, to map posttranslational modifications regulating NLRP3, CRISPR-based screens to reveal upstream regulators or inhibitory pathways and single-cell RNA sequencing to define cell-specific transcriptional programmes associated with canonical or alternative activation. Collectively, these approaches may help unravel the molecular, cellular, and contextual determinants of NLRP3 signaling and could guide the development of safer, context-specific therapeutic interventions for infections, autoinflammatory diseases and cancer.

### NONCANONICAL INFLAMMASOMES AND CASPASE-4/5/11

The outer layer of gram-negative bacteria comprises LPS, a potent endotoxin and a widely studied PAMP that triggers the immune response. The extracellular sensing of LPS by Toll-like receptor 4 (TLR4) initiates NF- $\kappa$ B signaling, whereas the cytosolic sensing of LPS by caspase-11 in mice and the orthologs caspase-4 and caspase-5 in humans initiate the activation of the noncanonical inflammasome [29, 223, 224] (Fig. 3). Activated caspase-11, caspase-4, or caspase-5 induce direct proteolytic cleavage of GSDMD, liberating the N-terminal domain of GSDMD, which forms plasma membrane pores, leading to pyroptotic cell death [8–12]. These GSDMD pores also mediate the efflux of K<sup>+</sup> ions from within the cell, which drives intracellular ionic perturbation and the activation of the NLRP3 inflammasome, leading to caspase-1-dependent proteolytic cleavage of IL-1 $\beta$  and IL-18 [225–227]. The requirement for caspase-11, caspase-4, and caspase-5 in the activation of the NLRP3 inflammasome is referred to as the noncanonical inflammasome pathway (Fig. 3).

Murine caspase-11 shares 60% protein identity with human caspase-4 or caspase-5 [224, 228]. However, a notable difference is that caspase-11 cannot directly induce the proteolytic cleavage of IL-1 $\beta$  and IL-18 into their bioactive forms, whereas caspase-4 and caspase-5 can form a protein complex with pro-IL-18, inducing the



**Fig. 3** Noncanonical inflammasomes and caspase-4/5/11. In the noncanonical inflammasome pathway, lipopolysaccharide (LPS) from gram-negative bacteria activates the Toll-like receptor 4 (TLR4)–TIR-domain-containing adapter-inducing interferon (IFN)- $\beta$  (TRIF) signaling pathway, leading to the upregulation of guanylate-binding proteins (GBPs) and immunity-related GTPases (IRGs) via the type I IFN pathway. GBPs and IRGs target outer membrane vesicles or bacterial and vacuolar membranes to facilitate the release of LPS into the cytoplasm. The binding of LPS to murine caspase-11 or human caspase-4/5 leads to the cleavage of gasdermin D, triggering pyroptosis and potassium ( $K^+$ ) efflux. This efflux of  $K^+$  activates the noncanonical NLRP3 inflammasome

cleavage of pro-IL-18 into its bioactive form with a similar efficiency as that of caspase-1 [229–231]. In some cases, a p37 form of caspase-4 can be generated that has an impaired ability to process IL-18 [232]. The recognition and processing of substrates by these inflammatory caspases are nuanced because these caspases can cleave IL-1 $\beta$ , generating an inactive 27-kDa IL-1 $\beta$  fragment that deactivates IL-1 $\beta$  signaling [229].

Caspase-11 is composed of 373 amino acids with a molecular weight of 43 kDa and has an N-terminal CARD and a C-terminal caspase catalytic domain [224]. Caspase-11 is activated following direct binding between the N-terminal CARD and LPS [9]. LPS is composed of an O-antigen, a core oligosaccharide, and lipid A [233]. Most lipid A species bind and activate caspase-11, but underacylated lipid IVa and LPS from *Rhodobacter sphaeroides* can bind to caspase-11 but cannot induce caspase-11 activation [9]. Furthermore, pentaacylated and hexaacylated lipid A, but not tetraacylated lipid A, can activate caspase-11 [234]. Upon binding to LPS, caspase-11 proteins form oligomers via CARD–CARD interactions [9, 235], but chemical-induced dimerization of caspase-11 is sufficient for activation [236]. Caspase-11 then cleaves itself at D285 within the linker connecting the large and small enzymatic subunits within the C-terminal caspase catalytic domain, which is required for subsequent proteolytic cleavage of GSDMD [236, 237]. Further studies suggest that GSDMD pores that form on the mitochondrial membrane induce the release of mitochondrial DNA into the cytoplasm, which, along with LPS, facilitates the interaction between NLRP3 and another cytosolic sensor, Nur77, triggering inflammasome activation [238]. Additionally, the LPS-binding protein CD14, independent of TLR4, mediates the cytosolic localization of LPS to enable caspase-11 activation [239].

Caspase-4 comprises 377 amino acids with a molecular weight of 42 kDa [240–242], whereas caspase-5 comprises 434 amino acids with a molecular weight of 48 kDa [242, 243]. Like caspase-11, the two human orthologs also possess an N-terminal CARD

and a C-terminal caspase catalytic domain required for cleaving GSDMD [12]. Like caspase-11, caspase-4 and caspase-5 also interact with the lipid A motif of LPS through their respective CARD [9]. However, caspase-4 and caspase-5 seem to have broader detection repertoires than does caspase-11, with these human caspases being able to bind pentaacylated, hexaacylated, and tetraacylated LPS [244]. Furthermore, caspase-11, caspase-4 and caspase-5 can be activated by LPS presented on bacterial outer membrane vesicles (OMVs) [245–247] and by lipooligosaccharide (LOS), which contains a core oligosaccharide and lipid A domain but not the O-antigen chain [233], which is found in bacteria such as *Moraxella catarrhalis* and *Neisseria gonorrhoeae* [245, 248].

Type I interferon signaling plays a fundamental role in licensing the activation of caspase-11 [249–251]. TLR4 recognition of extracellular LPS induces the expression of procaspase-11, whereby endosomal uptake of LPS initiates the TLR4–TRIF signaling pathway, leading to the activation of the transcription factors IRF3 and IRF7 and the upregulation of type I interferons [150, 249–252]. Secreted type I interferons can act in an autocrine and/or paracrine manner by binding to the type I interferon receptors IFNAR1 and IFNAR2, activating the STAT1–STAT2–IRF9 pathway that drives pro-caspase-11 expression [249–253]. Caspase-4 differs from caspase-11 and caspase-5 in that it is constitutively expressed [253]. In addition to the upregulation of the procaspase-11 protein, the type I interferon pathway increases the expression of guanylate-binding proteins (GBPs) and immunity-related GTPases (IRGs) [254, 255]. Following the phagocytosis of bacteria such as *Citrobacter koseri* [256], *E. coli* [257], *Enterobacter cloacae* [256], *Legionella pneumophila* [258], and *Salmonella enterica* serovar Typhimurium (also known as *S. Typhimurium*) [256], GBPs and/or IRGB10 can permeabilize bacteria-containing vacuoles and/or lyse bacterial cells directly, releasing LPS into the host cell cytoplasm. For example, mouse GBP2 is recruited to vacuoles containing *C. koseri*, *Enterobacter cloacae*, *L. pneumophila* and *S. Typhimurium* as early as 20 minutes after infection [258], rupturing the vacuolar and bacterial membranes and leading to LPS-induced caspase-11 activation in macrophages [256, 259, 260]. Similarly, mouse GBP1, GBP2, GBP3 and GBP5 are recruited to *M. catarrhalis*, promoting GBP-dependent bacterial lysis and LOS-induced caspase-11 activation in macrophages [245]. Caspase-11 activation, therefore, requires GBPs to liberate LPS or LOS from pathogen-containing vacuoles and pathogens themselves. In human cells, caspase-4 activation by LPS also requires GBPs [261, 262]. In this case, human GBP1 can directly bind to the LPS of *S. Typhimurium* and *Shigella flexneri* [263, 264]. Up to 30,000 human GBP1 molecules are thought to be recruited to the outer membrane of bacteria [265, 266] and facilitate rupture of the bacterial membrane [267, 268]. The initial coating by human GBP1 mediates the recruitment of human GBP2, GBP3 and GBP4 to the bacterial surface, where caspase-4 can subsequently dock to this GBP complex and interact with LPS [263, 264].

The importance of caspase-11 in mediating host defense against gram-negative bacteria has been demonstrated in mouse models of *Acinetobacter baumannii*, *Burkholderia* species, *E. coli*, and *M. catarrhalis* [29, 245, 269–271]. Caspase-11 reduces bacterial burden and/or lethality following infection with *A. baumannii*, *Burkholderia thailandensis*, *Burkholderia pseudomallei*, or *M. catarrhalis* [245, 269, 271]. In contrast, systemic activation of caspase-11 in response to LPS leads to sepsis and lethality in mice [10, 29, 30, 223, 224, 234, 238, 272–274]. These opposing outcomes highlight the protective role of caspase-11 during localized infections and its detrimental role during systemic inflammation.

Studies have shown a broader role for caspase-4, caspase-5, and caspase-11 in mitigating infectious diseases that are not driven by LPS or gram-negative bacteria [272, 275]. The gram-positive

bacterium *Streptococcus pyogenes* and its lipoteichoic acid (LTA) and extracellular vesicles can activate caspase-4 and caspase-5 in human monocytes via MyD88, RIPK1, and caspase-8 [276]. Both the parasite *Toxoplasma gondii* and the fungal pathogen *Aspergillus fumigatus* lack LPS or LOS, but *Casp11*<sup>-/-</sup> mice have reduced inflammation and attenuated disease severity in response to infection with *T. gondii* [275], and *Casp11*<sup>-/-</sup> mice infected with *A. fumigatus* succumb faster than do wild-type mice [272]. These unexpected observations indicate that these inflammatory caspases may sense additional PAMPs or DAMPs. In addition to LPS or LOS, caspase-11 can bind and sense oxidized phospholipids in murine dendritic cells [277], but caspase-11 also appears to be inhibited by oxidized phospholipids in murine macrophages [278]. Furthermore, caspase-11 and caspase-4 can bind to and be inhibited by mitochondrial cardiolipin [279]. Regardless, these observations support a model in which host phospholipids, often released by damaged mammalian cells, can activate or interfere with noncanonical inflammasome functions.

The importance of caspase-11 has been further demonstrated by studies showing that pathogens evolve strategies to evade immune detection by caspase-11 [234, 280–284]. For example, caspase-11 is unable to bind to the tetraacylated LPS of *Francisella* species and *Chlamydia trachomatis* [234, 281]. The virulence factor NleF from enteropathogenic *Citrobacter rodentium* and *E. coli* can bind to and inhibit the catalytic domains of caspase-11 and caspase-4, respectively, resulting in impaired host defenses against these pathogens [282, 283]. Other evasion strategies against caspase-4 have been reported. The virulence factor OspC3 in *S. flexneri* binds to the p19 subunit of the caspase-4 CARD, hindering the heterodimerization between the p19 subunit and the p10 subunit required for caspase-4 activation. This inhibition ultimately prevents pyroptosis and promotes bacterial replication in epithelial cells [284]. Therapeutic blockade of these virulence proteins may enhance inflammasome-mediated defense against immune-evading pathogens.

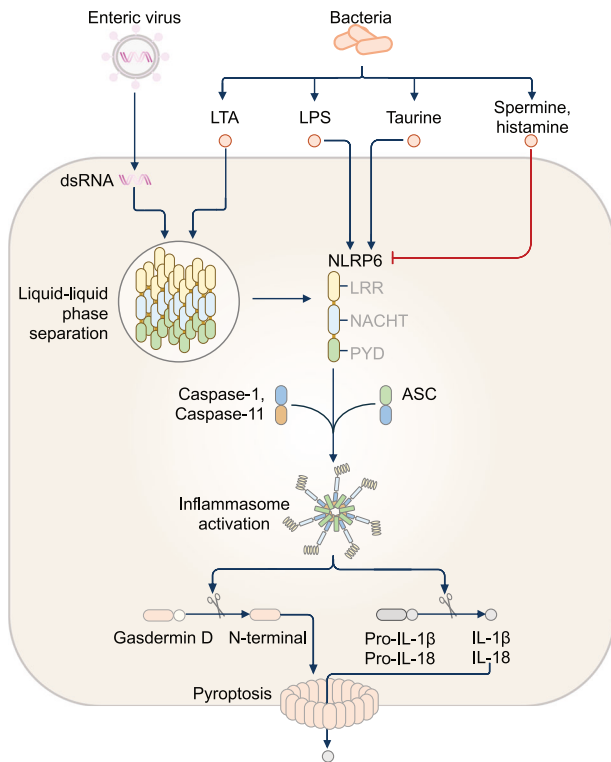
Caspase-11 also protects against intestinal inflammation and colorectal cancer [285–287]. During acute intestinal inflammation, *Casp11*<sup>-/-</sup> mice treated with DSS are more susceptible and have impaired IL-18 production and epithelial proliferation [285, 286]. Following treatment with the carcinogen azoxymethane (AOM) in combination with DSS, *Casp11*<sup>-/-</sup> mice develop more intestinal tumors and have impaired IL-1 $\beta$  secretion and STAT1 activation [287]. The possible antitumor role of caspase-11 may inspire further studies into the role of caspase-4 or caspase-5 in IBD and colorectal cancer in humans. Importantly, differences between mice and humans have hindered the development of therapeutics. In sepsis research, for example, experimental mice are highly resilient to LPS, with the doses of LPS used in most studies being approximately a million times higher than those used in human volunteer studies [288, 289], potentially making findings in mice difficult to translate to human clinical trials. A specific inhibitor of caspase-4 or caspase-5 is not clinically approved for therapeutic use. Many available drugs and therapies can target peripheral proteins such as NLRP3, caspase-1, or GSDMD [290–292]. Nevertheless, novel inhibitors of caspase-4 and/or caspase-5 may be developed by modeling similar mechanisms of action to those of bacterial virulence factors or host phospholipids. Research that elucidates the molecular basis of noncanonical inflammasome activation is expected to guide the development of therapeutics. The full spectrum of microbial and host-derived signals beyond LPS that activate caspase-4, caspase-5, and caspase-11 across different tissues and disease contexts remains poorly defined. While GBPs recruit these caspases to bacterial LPS, it remains unclear whether these caspases also engage other bacterial membrane components or how nonbacterial pathogens, such as fungi, and host-derived signals, such as oxidized phospholipids, trigger noncanonical inflammasome activation.

## NLRP6 INFLAMMASOME

NLRP6 (also known as NALP6, PYPAF5, PAN3, and CLR11.4) is highly expressed in the large and small intestine but is also expressed in the lungs, liver, kidneys, and brain of humans and mice [293–296]. NLRP6 was first identified in human cell lines as a PYRIN-containing APAF-1-like protein (PYPAF), called PYPAF5, which can activate caspase-1 and NF- $\kappa$ B [297]. Like most other NLRs, NLRP6 consists of three domains: an N-terminal PYD, a central NACHT, and a C-terminal LRR [26]. An earlier study revealed that *Nlrp6*<sup>-/-</sup> mice produced similar levels of IL-1 $\beta$  following infection with *S. Typhimurium*, *Listeria monocytogenes*, and *E. coli* as wild-type mice did [298]. Furthermore, no difference in caspase-1 activation or IL-1 $\beta$  maturation was observed in *Nlrp6*<sup>-/-</sup> bone marrow-derived macrophages infected with *S. Typhimurium* or *L. monocytogenes*, implying that NLRP6 is not involved in inflammasome signaling during infection [298]. Instead, NLRP6 was found to inhibit NF- $\kappa$ B and ERK signaling in mouse bone marrow-derived macrophages infected with *L. monocytogenes* and *Streptococcus pneumoniae* [298, 299]. However, subsequent reports revealed NLRP6-dependent IL-1 $\beta$  production in mouse bone marrow-derived macrophages infected with *S. aureus* and *S. pneumoniae* and the induction of NLRP6-dependent necroptosis and pyroptosis in macrophages and neutrophils in the lungs [299, 300]. It is also thought that NLRP6 binds to LTA from *L. monocytogenes* during infection in bone marrow-derived macrophages, triggering inflammasome activation [301]. How NLRP6 can form an inflammasome complex but also inhibit NF- $\kappa$ B and ERK in the same cell during the same infection is not known.

Several other microbial activators and inhibitors of the NLRP6 inflammasome have since been identified. Microbial metabolites, namely, taurine, histamine and spermine, either activate or inhibit NLRP6 inflammasome assembly [302] (Fig. 4). Taurine activates the NLRP6 inflammasome, leading to increased IL-18 production, whereas histamine and spermine inhibit inflammasome assembly [302]. LPS from gram-negative bacteria binds to NLRP6 monomers and triggers oligomerization with ASC [303], but LPS also initiates inflammasome-independent inhibition of NF- $\kappa$ B and ERK via NLRP6 [304], again highlighting the two facets of NLRP6 signaling. Additionally, double-stranded RNA (dsRNA) and LTA trigger NLRP6 inflammasome formation via liquid–liquid phase separation, which involves the binding of ASC to NLRP6 condensates [305]. This finding argues for a conceptually different mechanism for NLRP6 inflammasome assembly that departs from the typical oligomer formation that occurs for other inflammasomes.

The different types of PAMPs that can activate or inhibit NLRP6 activity must be considered, especially in the gastrointestinal tract, where NLRP6 expression is highest. During *C. rodentium* infection in mice, a host deubiquitinase called Cyld deubiquitinates NLRP6 through the cleavage of the K63-linked ubiquitination chain on NLRP6 [306]. This event weakens the interaction between NLRP6 and ASC, inhibiting NLRP6 inflammasome formation, which in turn limits IL-18 secretion and severe intestinal inflammation [306]. Earlier reports suggested that the NLRP6 inflammasome maintains gut homeostasis by controlling the composition of the intestinal microbiome, contributing to protection against *C. rodentium* infection in mice [293, 307]. *Nlrp6*<sup>-/-</sup> mice have an altered gut microbiome, including the expansion of two colitis-inducing pathobionts, the Prevotellaceae family [293] and *Akkermansia muciniphila* [308], and are more susceptible to DSS-induced colitis and tumorigenesis [293]. Furthermore, germ-free *Nlrp6*<sup>-/-</sup> mice colonized with conventional microbiota developed dysbiosis from 3 weeks of age [302]. However, these microbiome studies lacked littermate controls, and subsequent work has largely argued against an altered gut microbiome in littermate-controlled *Nlrp6*<sup>-/-</sup> mice [308–310].



**Fig. 4** The NLRP6 inflammasome. NLRP6 inflammasome activation is modulated by microbial and metabolic signals. Double-stranded RNA (dsRNA) from enteric viruses or lipoteichoic acid (LTA) from gram-positive bacteria directly interact with NLRP6 to undergo liquid–liquid phase separation, triggering NLRP6 inflammasome activation. In response to LTA, NLRP6 facilitates the recruitment of both caspase-1 and caspase-11 to the inflammasome complex. The microbial ligand LPS from gram-negative bacteria binds to the NLRP6 monomer, triggering NLRP6 inflammasome activation. The microbial metabolite taurine activates the NLRP6 inflammasome, whereas the metabolites histamine and spermine inhibit NLRP6 inflammasome activation

NLRP6 has been associated with other inflammatory gastrointestinal diseases, including Crohn's disease and gastrointestinal symptoms of graft-versus-host disease, and appears to be protective in the development of gastric cancer [311–315]. Epigenetic silencing of the gene encoding NLRP6 enhances cell proliferation and migration during gastric cancer [311], whereas expression of NLRP6 reduces cancer growth via direct ubiquitination and degradation of the molecular chaperone GRP78 in gastric cancer [314]. Finally, reduced NLRP6 inflammasome expression in patients with congenital large intestine conditions and Hirschsprung's disease, characterized by the absence of nerve cells in parts of the colon, suggests that NLRP6 may be protective in congenital gut diseases [316]. However, exactly how NLRP6 signaling contributes to the health of the gastrointestinal tract during development is unknown. The role of NLRP6 in noninflammasome contexts has also been demonstrated in mouse models of bacterial and viral infections, cancer, and autoinflammatory diseases involving different organs and cell types [304, 317–321]. Overall, NLRP6 remains an enigmatic NLR that plays an important role in homeostasis and disease, with both protective and damaging effects, suggesting many avenues for future studies. How NLRP6 balances its dual functions as an inflammasome sensor and as a negative regulator of NF- $\kappa$ B and ERK signaling remains unclear. It also remains unresolved whether NLRP6 is primarily activated by microbial ligands, host-derived metabolites, or damage signals and how these inputs vary across epithelial, immune, and neuronal cell types. Germ-free or

gnotobiotic mouse models with littermate controls, coupled with metabolomics to identify activating or inhibitory metabolites, could resolve the context-specific protective versus pathological functions of NLRP6.

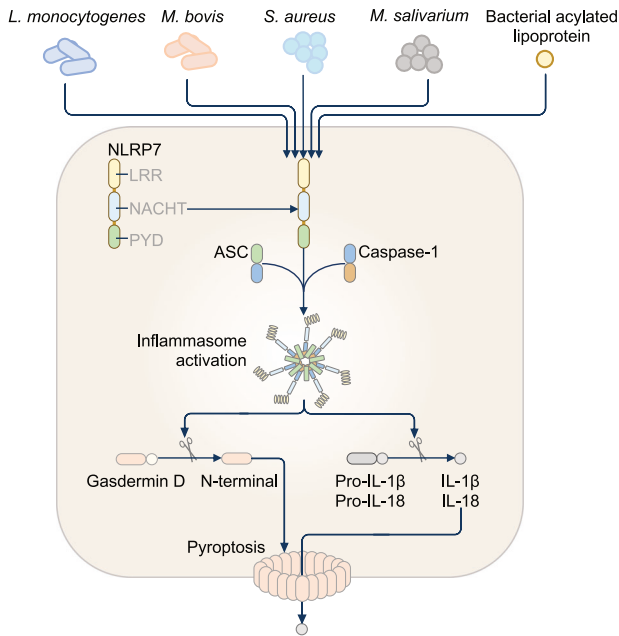
#### NLRP7 INFLAMMASOME

NLRP7 (also known as NALP7, NOD12 and PYPAF3) is a part of the reproductive NLR family [322] with predominant expression in oocytes [323] and testes [324]. NLRP7 is also expressed in cells and organs of the immune system, including spleen, thymus and bone marrow [324]. NLRP7 is only present in humans, with phylogenetic studies suggesting that NLRP7 evolved from a gene duplication event in primates, resulting in the genes encoding NLRP2 and NLRP7 [325].

Similar to most NLRP inflammasome-forming proteins, NLRP7 carries an N-terminal PYD, an NACHT, and a C-terminal LRR [26]. Evidence emerged to suggest NLRP7 can form an inflammasome complex. An siRNA screen in human macrophages identified NLRP7 as a sensor of bacterial lipopeptides, including di-acylated and tri-acylated lipopeptides [326]. Whether NLRP7 interacts directly with these lipopeptides or is activated in response to host cellular perturbations triggered by lipopeptides during bacterial infection is unknown. Following exposure to lipopeptides, NLRP7 undergoes a conformational shift, forming a high-molecular-weight inflammasome complex with ASC and pro-caspase-1, leading to the release of IL-1 $\beta$  and IL-18 [326]. Silencing of the gene encoding NLRP7 in human macrophages leads to a reduction in IL-1 $\beta$  and IL-18 release during infection with the bacterium *S. aureus* or *L. monocytogenes*, suggesting that NLRP7 activation mediates an inflammasome response [326] (Fig. 5). NLRP7 inflammasome formation has also been observed in THP-1 macrophages during infection with the bacterium *Mycobacterium bovis* [327], and in amnion epithelial cells stimulated with fibroblast-stimulating lipopeptide from the bacterium *Mycoplasma salivarium* [328]. The oligomerization of NLRP7 during inflammasome formation is mediated by its NACHT, such that the ATP-binding Walker A motif within the NACHT is required for ATP binding, hydrolysis and self-association of NLRP7 [329]. Introducing mutations into this motif, particularly GKT to AAA, impairs inflammasome responses to *S. aureus* infection and acylated lipopeptides [329].

Evidence also suggests that NLRP7 can inhibit inflammasome activation and signaling. For example, NLRP7 competitively interacts with pro-caspase-1 to inhibit NLRP3 inflammasome activation [324]. This observation was confirmed by reduced IL-1 $\beta$  release in THP-1 cells expressing the N-terminal fragment of NLRP7 [324]. The anti-inflammatory functions of NLRP7 also affect other inflammatory proteins and pathways. Indeed, NLRP7 interacts with NF- $\kappa$ B regulatory proteins, such as Fas-associated factor 1, in HEK293 cells to restrain inflammatory responses mediated by NF- $\kappa$ B [324]. However, this anti-inflammatory function of NLRP7 might be dependent on cell types because silencing the gene encoding NLRP7 in THP-1 macrophages, fibroblasts, and primary macrophages did not affect cytokine-induced and LPS-induced NF- $\kappa$ B activation [297, 327, 329, 330]. The pro-inflammatory and anti-inflammatory roles of NLRP7 may also be explained by the presence of multiple functional isoforms differing in the number of LRR generated by alternative splicing events [326, 331–333]. However, further work is needed to verify the functions of these NLRP7 isoforms. The mechanistic switch enabling NLRP7 to trigger anti-inflammatory functions or inflammasome activities also requires further investigation.

Mutations in the gene encoding NLRP7 are typically found within the LRR [334] and affect placental development and early pregnancy [335]. A mutation in the NACHT has also been found in patients with a type of molar pregnancy disorder called hydatidiform mole [336]. Furthermore, genetic variants of NLRP7 have also



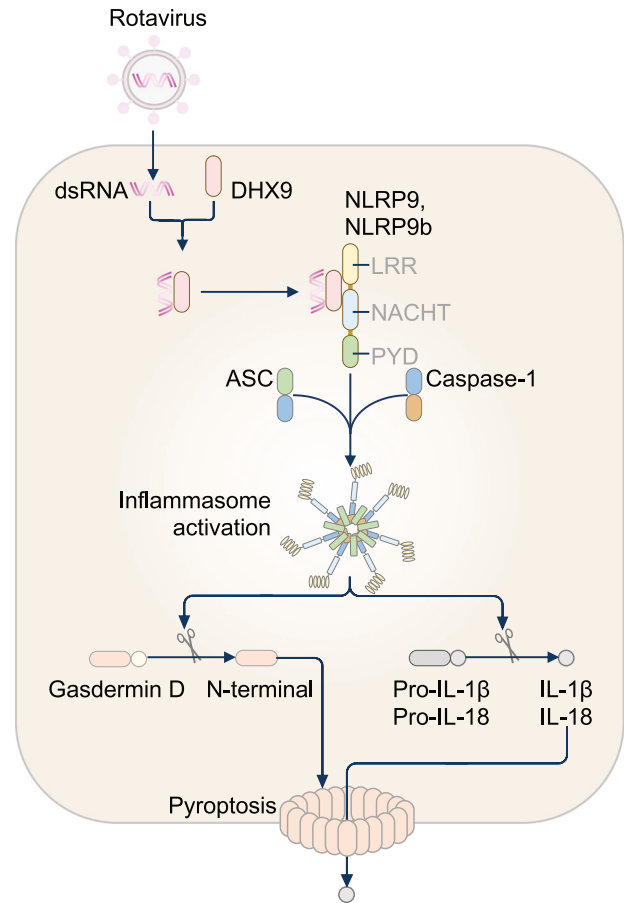
**Fig. 5** The NLRP7 inflammasome. In human macrophages, the NLRP7 inflammasome is triggered by infections with *Listeria monocytogenes*, *Mycobacterium bovis*, *Staphylococcus aureus*, and *Mycoplasma* species. NLRP7 also detects acylated lipoproteins from *Mycoplasma*, engaging ASC and caspase-1 to assemble an active inflammasome complex

been found in patients with ulcerative colitis and lung cancer [337–339]. How these disease-associated NLRP7 variants affect inflammasome activation, reproductive cell functions, and/or other pathologies is not clear. It is also unclear whether these mutations drive the dual functions of NLRP7 or whether these functions are governed by isoform diversity or cell type.

### NLRP9 INFLAMMASOME

NLRP9 (also known as NALP9, NOD6 and PAN12) is an under-explored member within the NLR family. NLRP9 is predominantly expressed in oocytes, ovaries and testes in humans, mice, and bovines [322, 340–343], suggesting a putative role in reproductive organs. Human NLRP9 is encoded on chromosome 14, which also carries NLRP2, NLRP4, NLRP5, NLRP7, NLRP8, NLRP11, and NLRP13, which are also expressed in reproductive organs [322]. This chromosomal collocation highlights a potential series of tandem duplication events in the evolutionary emergence of this group of NLRs [325]. A single gene encodes NLRP9 in humans, whereas three isoforms encode NLRP9a, NLRP9b, and NLRP9c in mice [325]. Human NLRP9 and the mouse NLRP9 isoforms have a conserved PYD, NACHT, and LRR domain arrangement, similar to most members of the NLR family [26]. Human NLRP9 PYD exists as a monomer in solution, adopting an antiparallel six-helical bundle fold [344–346]. However, the structural details of the remaining NLRP9 domains remain to be resolved.

Beyond the reproductive system, human NLRP9 and mouse NLRP9b are also strongly expressed in intestinal epithelial cells and contribute to anti-viral defense [347]. In mice, NLRP9b recognizes viral dsRNA indirectly by acting as an adaptor protein to the RNA-binding helicase DHX-9. DHX-9 binds directly to viral dsRNA and, when complexed with NLRP9b, enables the formation of the NLRP9b inflammasome [347] (Fig. 6). This inflammasome triggers the release of IL-18 and GSDMD-dependent pyroptosis in intestinal epithelial cells and restricts infection by the dsRNA virus rotavirus. Indeed, conditional deletion of NLRP9b in the mouse intestine leads to increased susceptibility to rotavirus [347]. Unlike



**Fig. 6** The NLRP9 inflammasome. The mouse NLRP9b inflammasome is activated in intestinal epithelial cells in response to rotavirus infection. Following infection, rotavirus double-stranded RNA (dsRNA) is detected by the RNA sensor DEAH-box helicase (DHX) 9, which directly interacts with NLRP9b to promote NLRP9b inflammasome activation. In human kidney embryonic kidney (HEK) 293T cells, human NLRP9 interacts with dsRNA, DHX9 and ASC

human NLRP9, mouse NLRP9b does not appear to interact with ASC, suggesting the presence of subtle structural differences between human NLRP9 and mouse NLRP9b [347]. Notably, the PYD of human NLRP9 does not undergo self-oligomerization or nucleate ASC specks [346], despite interacting with ASC in HEK293T cells infected with rotavirus [347]. This may be due to repulsive charge inversions within the PYD interfacing residues of NLRP9, which hinder the interactions between strands of the PYD necessary for self-oligomerization [345, 346]. It is also likely that oligomerization is mediated by another NLRP9 domain or additional binding partners, which also facilitate ASC binding.

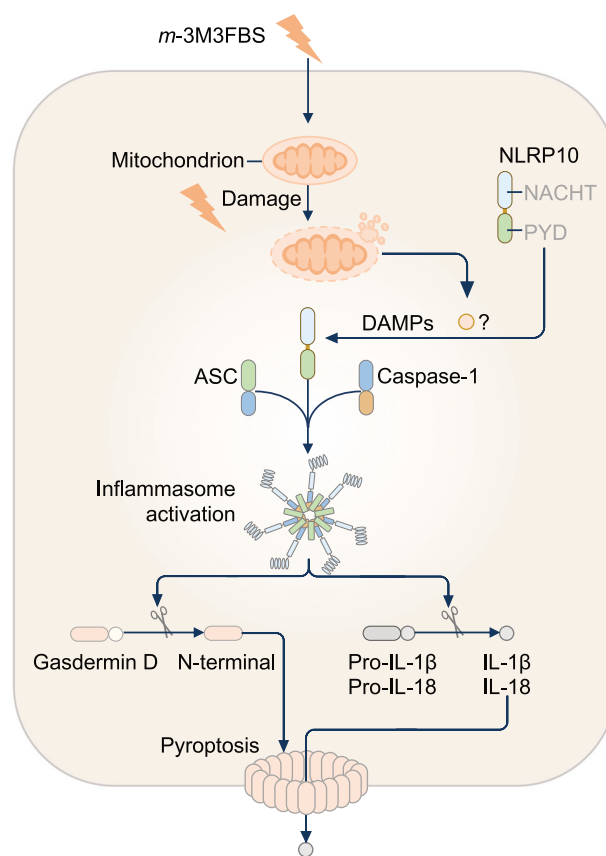
Whether NLRP9 can also sense endogenous self-dsRNA and trigger subsequent inflammasome formation is not known. However, self-derived dsRNA species generated through dysregulated epigenetic control of transposable elements, alterations in RNA modifications, genotoxic stress, and mitochondrial stress response [348] could be potential activators of NLRP9. Porcine NLRP9 interacts with the intermediate filament vimentin and induces the production of type I interferons in enterocytes stimulated with synthetic dsRNA poly (I:C) [349]. As vimentin is associated with the accumulation of endogenous dsRNA in fibroblasts within human islet preparations and is also highly expressed in astrocytes within demyelinated multiple sclerosis lesions [350], crosstalk between vimentin, NLRP9 and endogenous dsRNA could contribute to a breach in self-tolerance underpinning

Type I diabetes, multiple sclerosis and other autoimmune diseases. Mutations in NLRP9 have already been found in patients with multiple sclerosis [351] but also in patients with Alzheimer's disease [352] and colon cancer [353]. Investigating how these mutations cause NLRP9 dysfunction and contribute to neurodegeneration, autoimmunity, and cancer could provide deeper insights into the broader role of this inflammasome sensor. Although NLRP9 has an emerging role in antiviral defense, it remains unclear whether the function of NLRP9 is restricted to the intestine or extends to other anatomical sites. Whether NLRP9 senses other RNA viruses or endogenous RNA ligands remains unknown. Immunoprecipitation and crosslinking of RNA of different origins and structural features could verify the ligand-binding repertoire of NLRP9, extending its relevance beyond rotavirus infection and revealing broader roles in diseases.

### NLRP10 INFLAMMASOME

NLRP10 (previously known as CLR11.1, PAN5, PYNOD, NALP10, and NOD8) is a newly identified inflammasome sensor [354, 355]. The genes encoding human, mouse, and rat NLRP10 carry only two exons encoding NACHT and PYD, such that NLRP10 lacks the LRR typically found in other NLRs [26]. Human and mouse NLRP10 share 55.5% amino acid sequence identity; human and rat NLRP10 share 55.9%; and mouse and rat NLRP10 share 91.5% amino acid sequence identity [356]. NLRP10 is expressed across most organs in both humans and mice, including the brain, colon, heart, kidney, liver, skeletal muscle, skin, small intestine, and testis [295, 356, 357]. In humans, NLRP10 is more abundant in the colon, liver, muscles, and small intestine, whereas in mice, NLRP10 has the highest expression in the colon, kidney, and testis [295]. Earlier investigations using an overexpression system revealed that NLRP10 inhibited ASC aggregation and caspase-1-dependent cleavage of IL-1 $\beta$  [356, 357], suggesting that NLRP10 has an inhibitory function. Further investigations yielded conflicting results concerning whether NLRP10 is proinflammatory or anti-inflammatory. Some studies suggest a proinflammatory role for NLRP10 in *S. flexneri* infection [358] and skin hypersensitivity [359], whereas other studies revealed an anti-inflammatory role in *Mycobacterium tuberculosis* infection [360], endotoxic shock [357], and fungal and parasitic infections [361, 362]. These context-dependent roles could imply that the function of NLRP10 is highly cell type- and stimulus-specific. NLRP10 was originally thought to initiate the adaptive immune response in mice by triggering dendritic cell migration [363]. However, this purported function of NLRP10 was instead caused by the cytoskeletal protein DOCK8, owing to the presence of a coincidental *Dock8* mutation in the *Nlrp10*<sup>-/-</sup> mice used in the study [364].

Subsequent studies revealed that NLRP10 has inflammasome-activating effects on primary differentiated human keratinocytes and mouse intestinal epithelial cells [354, 355] (Fig. 7). Upon stimulation with the phospholipase C activator *m*-3M3FBS in differentiated keratinocytes, NLRP10 is recruited to destabilized mitochondria [355]. This mitochondrial localization promotes the assembly of the NLRP10 inflammasome complex, resulting in caspase-1 activation, GSDMD cleavage, and the secretion of IL-1 $\beta$  and IL-18 [355]. Structurally, both NACHT and PYD are necessary for the ability of NLRP10 to function as an inflammasome sensor in response to mitochondrial damage induced by *m*-3M3FBS [354, 355]. Furthermore, the Walker A and B motifs within the NACHT, which mediate ATP binding, are important for NLRP10 inflammasome assembly [354, 355]. Notably, in HEK293 cells expressing NLRP10 carrying an atopic dermatitis-associated mutation, the R243W variant [365], ASC speck formation was abolished following *m*-3M3FBS stimulation, indicating that R243W is a loss-of-function variant that impairs inflammasome assembly [355]. NLRP10 inflammasome activation has also been reported in mouse colonic organoids and in mice treated with DSS [354].



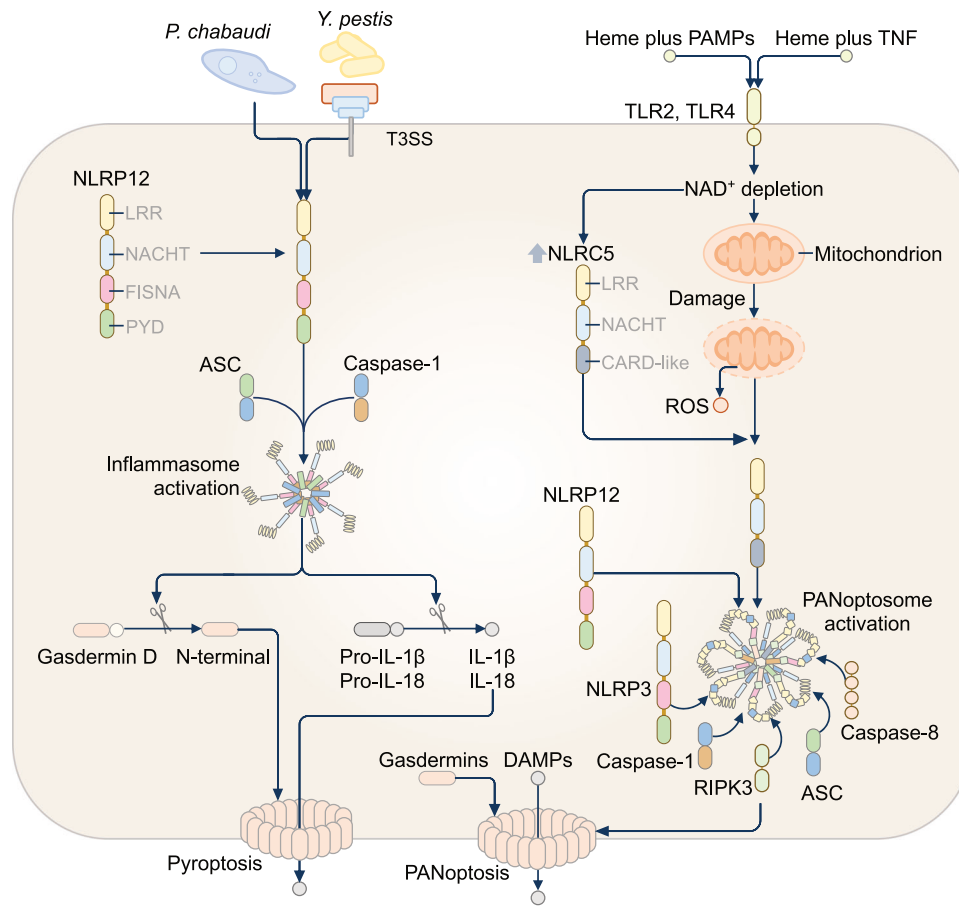
**Fig. 7** The NLRP10 inflammasome. NLRP10 detects mitochondrial damage caused by the chemical *m*-3M3FBS and assembles an inflammasome complex. Additionally, the proinflammatory agent dextran sodium sulfate (not shown) induces NLRP10 inflammasome formation in mouse colonic epithelial cells through an unknown mechanism

Given that NLRP10 deficiency promotes skin inflammation in humans and exacerbates intestinal inflammation in mice, therapeutic strategies aimed at restoring or enhancing NLRP10 function may represent promising anti-inflammatory approaches.

While AIM2 can directly bind to cytosolic mitochondrial DNA released from damaged mitochondria, NLRP10 does not appear to interact with mitochondrial DNA [354, 355]. In immortalized mouse macrophages depleted of mitochondrial DNA in which the gene encoding AIM2 was replaced with the gene encoding human or mouse NLRP10, ASC speck formation and caspase-1 activation can be triggered following stimulation with *m*-3M3FBS [355]. In addition, the NLRP3-specific inhibitor MCC950 did not reduce IL-1 $\beta$  secretion or ASC speck formation in *m*-3M3FBS-stimulated mouse macrophages [355]. These findings imply that inflammasome activation occurs independently of NLRP3, AIM2 and mitochondrial DNA. The precise ligand(s) of NLRP10 are not yet known, but it is possible that NLRP10 may not bind to any ligands and instead recognizes mitochondrial-specific damage signals or perturbations. Furthermore, the molecular mechanisms guiding the recruitment of NLRP10 to damaged mitochondria and how disease-associated variants such as R243W disrupt the function of NLRP10 are important areas for investigation.

### NLRP12 INFLAMMASOME

NLRP12 (also known as MONARCH-1, NALP12, PYPAF7 and RNO) was first identified in the human leukemic cell line HL60 [366]. Human NLRP12 is predominantly expressed in myeloid cells, such as macrophages, neutrophils, monocytes, and immature dendritic



**Fig. 8** The NLRP12 inflammasome. In mouse macrophages, the NLRP12 inflammasome is activated following infection with *Yersinia pestis* or *Plasmodium chabaudi*. The combination of the heme-containing component of hemoglobin with pathogen-associated molecular patterns (PAMPs) or the cytokine TNF activates Toll-like receptors (TLR2 and TLR4), leading to depletion of cytoplasmic NAD<sup>+</sup>. NAD<sup>+</sup> loss upregulates the innate immune sensor NLRC5 and induces mitochondrial stress, resulting in reactive oxygen species (ROS) production and the assembly of a PANoptosome complex containing NLRP12, NLRC5, NLRP3, ASC, caspase-1, caspase-8, and RIPK3. This complex drives PANoptosis, a lytic inflammatory cell death pathway mediated by gasdermins, which disrupt the plasma membrane and release damage-associated molecular patterns (DAMPs)

cells [367]. NLRP12 functions as an inhibitor of inflammation [368–370], an initiator of inflammasome [371, 372], or for scaffolding the PANoptosome [373]. Earlier studies suggest that NLRP12 suppresses canonical and noncanonical NF- $\kappa$ B pathways [368–370], or colocalizes with ASC to inhibit [374] or activate inflammasomes [371, 372]. The first evidence that NLRP12 can assemble a physiological inflammasome complex is in response to the bacterial pathogen *Y. pestis* [372]. The *Y. pestis* Type III Secretion System (T3SS) can activate the NLRP12 inflammasome in mouse macrophages, resulting in the secretion of IL-1 $\beta$  and IL-18 [372]. *Nlrp12*<sup>-/-</sup> mice exhibit decreased IL-1 $\beta$  and IL-18 secretion, rendering them more susceptible to *Y. pestis* infection compared to wild-type mice [372] (Fig. 8). A further study has shown that NLRP12, in synergy with NLRP3, mediate caspase-1-dependent release of IL-1 $\beta$  and pyroptosis in mouse splenic macrophages and dendritic cells in response to the parasite *Plasmodium chabaudi* [371] (Fig. 8). In contrast to these studies, another study has shown that ectopically expressed human NLRP12 interacts with human NLRP3 in HEK293T cells, leading to the inhibition of the NLRP3 inflammasome [374]. The PYD of NLRP12 can also form a heterotypic interaction with the inhibitory protein of NF- $\kappa$ B signaling, FAF-1 [375, 376], which might provide another mechanism by which NLRP12 inhibits pro-inflammatory responses. The switch in mechanisms between pro-inflammatory functions and anti-inflammatory functions by NLRP12 remains to be resolved.

The precise microbial ligands from *Y. pestis* or *P. chabaudi* that activate NLRP12 are not known. However, a recent study has potentially shed light on the role of NLRP12 in sensing PAMPs and DAMPs. In response to heme in the presence of TNF or activators of TLRs, such as LPS, PAM3CSK4 or R848, the transcription factor IRF1 induces the expression of NLRP12 [373]. NLRP12 is required to drive the assembly of an NLRP12 PANoptosome complex containing NLRP12, NLRP3, ASC, caspase-1, caspase-8, and RIPK3 [373]. This PANoptosome mediates lytic inflammatory cell death and IL-1 $\beta$  and IL-18 secretion [373]. Furthermore, NLRC5 has been identified as another NLR that is part of this protein complex, functioning as a sensor of both NAD<sup>+</sup> depletion and ROS production induced by LPS and heme [377]. NLRC5 and NLRP12 can directly interact with one another and facilitate the recruitment of other PANoptosome complex components [377] (Fig. 8). Genetic deletion of NLRP12 in mice causes a reduction in acute kidney damage and lethality in hemolytic disease [373], suggesting a pathological role of NLRP12. Addressing whether *Y. pestis* or *P. chabaudi* infection can activate the NLRP12-NLRC5 PANoptosome could reveal a potential mechanism of transition from the inflammasome scaffold to a PANoptosome scaffold. *Y. pestis* encodes a heme-protein acquisition system that allows the bacterium to use heme as a source of iron [378], raising the possibility that heme could be released during *Y. pestis* infection, activating NLRP12. Furthermore, malaria caused by *P. chabaudi* infection results in the release and accumulation of oxidized heme

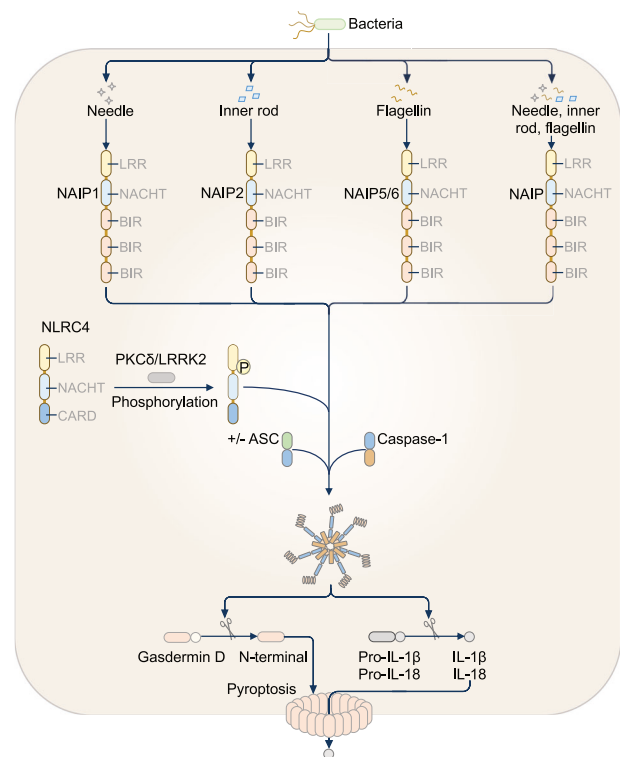
[379] that might be sufficient to drive NLRP12 activation. Given that NLRP12 has been implicated in both inflammasome and PANoptosome signaling, what mechanisms govern the shift from potentially beneficial inflammasome activity to pathological PANoptotic signaling could clarify the context-specific outcomes of NLRP12 activation.

### NAIP-NLRC4 INFLAMMASOME

NLRC4 (also known as CARD12, CLAN, CLAN1 and IPAF) was first identified by a search of genes with sequence similarity to caspase-1 [380]. In humans, NLRC4 is expressed in monocytes, monocyte-derived macrophages, neutrophils, peripheral blood mononuclear cells, and intestinal immune, epithelial and stromal cells, whereas in mice it is found in BMDMs, dendritic cells, neutrophils, intestinal epithelial cells, astrocytes, microglia, and B and T lymphocytes [381, 382]. NLRC4 carries an N-terminal CARD, a NACHT, and a C-terminal LRR. The first evidence that NLRC4 forms an endogenous inflammasome complex came from a study showing that NLRC4 induces caspase-1 activation in BMDMs infected with the bacterium *S. Typhimurium* [383]. Subsequent studies found that *S. Typhimurium* strains lacking flagellar components FljC and FljB cannot robustly activate NLRC4 in wild-type BMDMs [384, 385]. Furthermore, transfection of *S. Typhimurium* flagellin into wild-type BMDMs leads to inflammasome activation [384, 385]. *L. pneumophila* flagellin was also subsequently found to induce the activation of the NLRC4 inflammasome [386–389], firmly establishing NLRC4 as a bona fide sensor of bacterial flagellin. Other virulence factors from bacteria with structures and/or functions similar to flagellin can also activate NLRC4. These protein factors include Type III secretion system components PrgJ from *S. Typhimurium*, Mxil from *S. flexneri*, PscI from *P. aeruginosa*, and EprJ and EsI from *E. coli* [390].

As no direct interaction between NLRC4 and bacterial flagellin was reported, it was speculated that additional proteins may act as direct sensors of flagellin that drive NLRC4 activation. During this time, mouse NLR family apoptosis inhibitory protein 5 (NAIP5), one of the seven mouse NAIP paralogs [391], is known to contribute to host resistance to *L. pneumophila* infection and restrict bacterial replication in macrophages by detecting cytosolic flagellin [392, 393]. A conserved C-terminal region of flagellin was identified as the critical domain recognized by mouse NAIP5, which triggers pyroptosis and IL-1 $\beta$  release in macrophages [394]. Notably, flagellin-deficient *L. pneumophila* mutants evade NAIP5- and caspase-1-mediated restriction in mice [395, 396]. These findings established mouse NAIP5 as a cytoplasmic sensor of bacterial flagellin capable of initiating inflammasome-mediated host defense. The molecular connection between NAIPs and NLRC4 became clearer with the discovery that NAIPs are receptors of flagellin and T3SS components that activate the NLRC4 inflammasome [397, 398] (Fig. 9). Mouse NAIP1 and NAIP2 directly bind the T3SS needle and inner-rod proteins, respectively, while mouse NAIP5 and mouse NAIP6 directly bind flagellin [397, 398]. The functions of mouse NAIP3, NAIP4, and NAIP7 remain to be defined. Unlike mice, humans express a single NAIP that appears to be functionally analogous to murine NAIP1 in sensing T3SS needles [399, 400]. Later studies revealed that human NAIP can also sense flagellin and inner-rod proteins [401, 402] (Fig. 9).

Both human and mouse NAIPs contain a central NACHT with subdomains: helical domain 1 (HD1), helical domain 2 (HD2), and the winged helix domain (WHD) [381]. In addition to NACHT, NAIPs feature a C-terminal LRR and an N-terminal baculoviral IAP repeat (BIR) domain [381]. Cryo-EM structures have elucidated how ligand binding to NAIP drives NAIP–NLRC4 assembly [403–407]. Binding of the *S. Typhimurium* inner rod protein PrgJ to an inactive mouse NAIP2 triggers the formation of a disk structure comprising one mouse NAIP2 monomer and ten mouse



**Fig. 9** NAIP–NLRC4 inflammasome. The NAIP–NLRC4 inflammasome is activated by bacterial flagellin and components of the type III secretion system (T3SS). In mice, distinct NAIP proteins recognize flagellin, needle, and rod proteins, whereas in humans, a single NAIP protein detects all these ligands. Phosphorylation of NLRC4 by the protein kinase PKC $\delta$  and leucine-rich repeat kinase 2 (LRRK2) promotes NAIP–NLRC4 complex formation and the recruitment of caspase-1, with or without ASC, to assemble the inflammasome

NLRC4 monomers [403, 407]. This activated disk structure exposes the CARD of mouse NLRC4, enabling NLRC4 to recruit caspase-1 to form an inflammasome [403, 407]. In the case of the mouse NAIP5–FljC complex, a “trap-and-lock” mechanism sequesters the flagellin D0 domain within the NAIP5 hydrophobic pocket, which is stabilized by the NAIP5 insertion domain located between HD2 and the WHD [406]. An alternative model suggests that a wide-open conformation of mouse NAIP5 has a fully accessible nucleating surface, which recruits NLRC4 [405]. Upon ligand binding, the WHD undergoes a 20° rotation, leading to a steric clash with inactive NLRC4 [405]. This event leads to the transition of NLRC4 from an inactive state to an active state [405]. Subsequent studies demonstrated that, unlike murine NAIPs with discrete ligand-binding surfaces, human NAIP is activated via ligand-induced tightening of its lasso-like motif, a loop formed by the last eight C-terminal residues of human NAIP that thread through the insertion domain and pull it toward the human NLRC4 LRR, stabilizing the complex through additional hydrogen bonds [404]. Specifically, the *Bacillus thailandensis* T3SS needle protein directly interacts with the baculovirus IAP repeat 1 domain of human NAIP, forming a stable needle–NAIP binary complex [404]. This complex induces the conformational change required for human NAIP to recruit and activate NLRC4, thereby assembling the NAIP–NLRC4 inflammasome complex [404]. The findings that ligand-bound NAIPs initiate NLRC4 oligomerization clarify the cooperative relationships between these proteins in inflammasome assembly.

NLRC4 activation is regulated in a context-dependent manner by posttranslational modifications, particularly phosphorylation at S533 [408] (Fig. 9). When mouse BMDMs are infected with *S.*

Typhimurium at a multiplicity of infection (MOI) of 1, the kinases PKC- $\delta$  and LRRK2 phosphorylate NLRC4 at S533 [408]. Mutation of this residue to alanine (S533A) or pharmacological inhibition of PKC- $\delta$  by the nonselective PKC inhibitors staurosporine and K-252a reduces NLRC4 oligomerization and IL-1 $\beta$  release [408], indicating that phosphorylation at this site is required for inflammasome activation. Host-derived retrotransposon RNAs also activate the NLRC4 inflammasome in a manner dependent on S533 phosphorylation [409]. However, other findings complicate this model [410, 411]. NLRC4 is phosphorylated at S533 in the inactive mouse NLRC4 monomer [403], suggesting that phosphorylation may be necessary but not sufficient for NLRC4 activation. Furthermore, when a higher infection dose of *S. Typhimurium* at an MOI of 20 is used, NLRC4 activation occurs regardless of S533 phosphorylation [411], and genetic deletion of PKC- $\delta$  does not affect inflammasome activation in response to either *S. Typhimurium* or *S. flexneri* [410].

The interplay between NLRC4 and other inflammasome sensors is essential for mounting effective immune responses against pathogenic and sterile stimuli. Earlier investigations into inflammasome signaling revealed that NLRC4 and NLRP3 have overlapping and cooperative functions in the host defense against *Salmonella* infection [156]. Subsequent mechanistic studies further revealed that NLRC4 and NLRP3 are physically recruited together into a single macromolecular inflammasome complex during *Salmonella* infection [157, 412]. The NLRC4 inflammasome can also recruit other proteases, such as caspase-8, facilitating a coordinated signaling event that enhances the maturation of IL-1 $\beta$  or prevents actin polymerization to limit the intracellular bacterial burden [413, 414]. Importantly, this functional interplay extends to sterile inflammatory processes within the central nervous system. The lipid lysophosphatidylcholine, a DAMP derived from the mammalian plasma membrane and implicated in neurodegenerative diseases and demyelination, activates both NLRC4 and the NLRP3 inflammasome in microglia and astrocytes [415]. Compared with wild-type controls, mice lacking both NLRC4 and NLRP3 exhibit reduced astrogliosis [415], underscoring the contribution of these inflammasome sensors to sterile neuroinflammation. Although the NLRC4–NLRP3 axis is well established, whether NLRC4 interacts with additional inflammasome sensors to drive disease remains an open question.

Aberrant activation of NLRC4 has been implicated in other inflammatory disorders and cancer. For example, gain-of-function mutations of NLRC4 cause or enhance multiple autoinflammatory diseases, including enterocolitis [416], recurrent macrophage activation syndrome [417], and familial cold autoinflammatory syndrome [418]. In allergic asthma, NLRC4 is activated in response to protease allergen-induced release of High mobility group box 1, a protein involved in coordinating cellular stress response [419]. Indeed, upon administration with *Aspergillus* protease ovalbumin, the NLRC4 inflammasome promotes asthma with increased airway hyperresponsiveness and IL-1 $\beta$  secretion in the bronchoalveolar lavage fluid of mice [419]. The functional role of NLRC4 in cancer appears to be highly context dependent. The NLRC4 inflammasome promotes obesity-associated breast cancer progression by promoting angiogenesis and increased expression of vascular endothelial growth factor [420]. In contrast, the NLRC4 inflammasome prevents the high-fat diet-induced growth of colon cancer-derived liver metastasis [421]. In colitis-associated colorectal cancer, the NLRC4 inflammasome has been shown to prevent the development of tumorigenesis [422]. However, other studies using the same mouse model have challenged this conclusion and reported that the NLRP3 inflammasome, instead of NLRC4 inflammasome, is responsible for preventing the development of colorectal tumors [206, 214]. Differences in the findings from these studies may be due to variations in gut microbiome communities among experimental mouse groups, as changes in the gut microbiome profile can substantially influence the progression

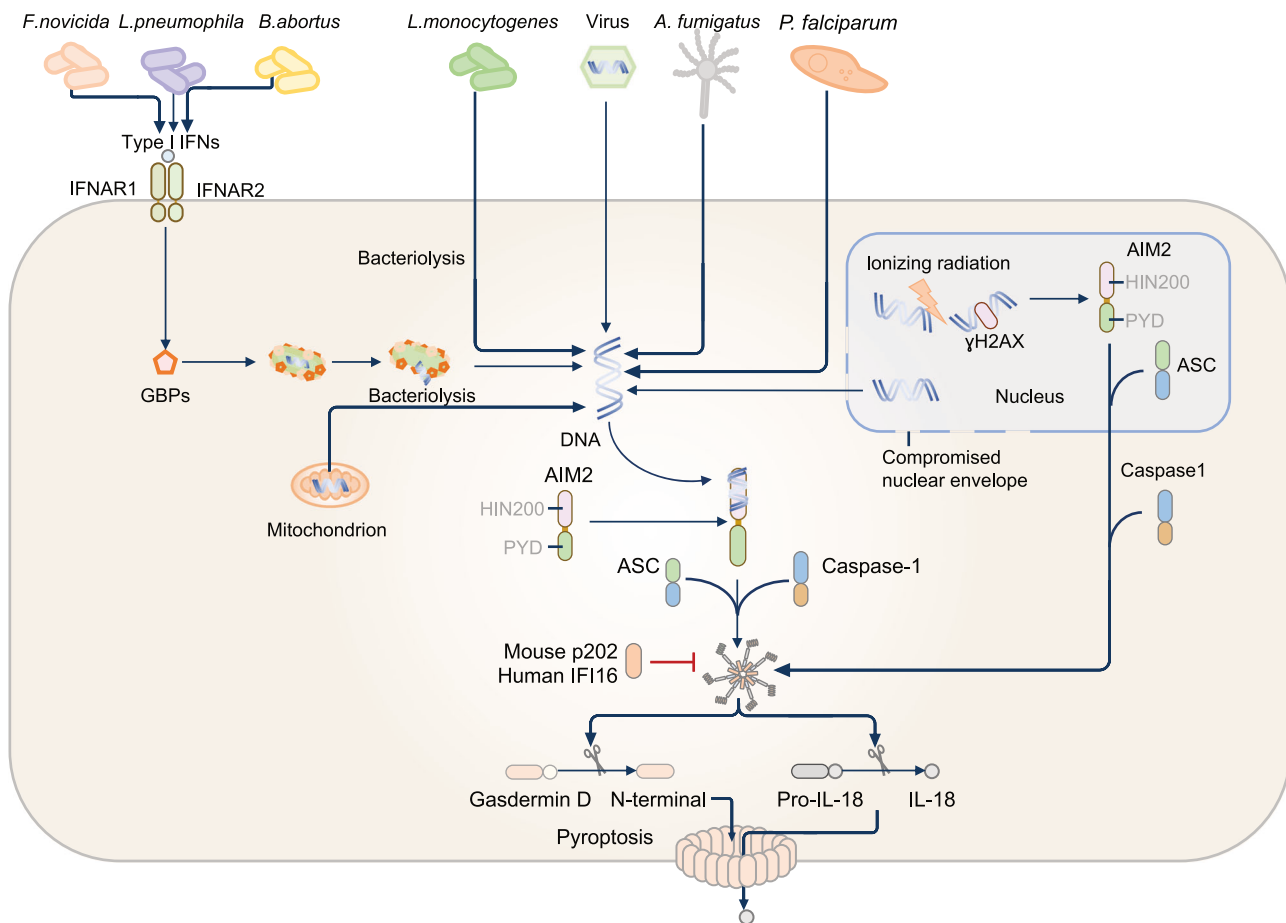
of intestinal cancer and other diseases [423, 424]. The use of littermate-controlled groups is an optimal approach to standardize the gut microbial communities [425]. Under littermate-controlled conditions, NLRC4 has an inflammasome-independent function by interacting with the ATR-ATRIP DNA damage complex to maintain genomic stability and suppress tumor development [382].

Current approaches to therapeutically target NLRC4 are still in their infancy, but emerging insights from structural and mechanistic studies highlight several promising directions. Given that NLRC4 plays a role in promoting or limiting disease processes, precise modulation of NLRC4 function is essential. Small molecules that enhance or disrupt the interaction between NLRC4 and its interacting proteins represent a promising strategy to target NLRC4-mediated pathways in contexts such as cancers, infectious diseases, and autoinflammatory disorders. Therapeutic modulation of NLRC4 posttranslational modifications, such as phosphorylation, may hold potential and provide context-dependent fine-tuning of inflammasome activity in disease settings. Furthermore, selective targeting of specific NLRC4–NAIP interactions in disease contexts could offer more precise therapeutic strategies than global inhibition of the inflammasome.

### AIM2 INFLAMMASOME

AIM2 (also known as Gm1313 and Irf210) is an inflammasome sensor protein that binds to cytosolic double-stranded DNA (dsDNA) [426–429]. AIM2 consists of a C-terminal hematopoietic interferon-inducible nuclear (HIN) protein with a 200 amino acid repeat (HIN200) domain and an N-terminal PYD [430, 431]. In the resting configuration, AIM2 PYD associates with the HIN200 domain through electrostatic interactions [431]. The AIM2 HIN200 domain binds dsDNA independently of sequence specificity through electrostatic interactions between the positively charged oligonucleotide/oligosaccharide-binding (OB) folds of the HIN200 domain and the negatively charged phosphate groups on the DNA backbone [430]. This sequence-independent recognition mechanism allows AIM2 to recognize dsDNA derived from pathogens and/or pathogen-induced or sterile-induced damage to the host cell. Once the HIN200 domain interacts with dsDNA, the AIM2 PYD can bind other AIM2 PYD and ASC PYD through hydrophobic interactions, triggering inflammasome responses [431]. Evidence also suggests that AIM2 PYD drives both AIM2 filamentation and dsDNA binding but not autoinhibition of the resting AIM2 protein [432].

AIM2 is activated during various scenarios in which dsDNA is released from pathogens. Bacterial infections of *Francisella tularensis* [257, 433–440] and *L. monocytogenes* [441–444], viral infections of herpes simplex virus [445] and pseudorabies virus [446], fungal infection of *A. fumigatus* [182], and protozoal infections of *Plasmodium berghei* [447, 448] and *Plasmodium falciparum* [448] are among the microbial triggers that can activate AIM2 (Fig. 10). In some cases, the activation of AIM2 leads to the recruitment of the cytosolic sensors Pyrin and Z-DNA-binding protein 1 (ZBP1) into the same signaling complex, in addition to ASC and caspase-1, which are typically observed within the inflammasome [445, 449]. AIM2 is expressed at a basal level in the resting state and can be further upregulated by type I interferons [450]. In response to bacterial infection, host interferon-inducible GTPases, including GBPs and the immunity-related GTPase IRGB10, are also globally upregulated in macrophages by type I interferons [255, 451]. Both GBPs and IRGB10 associate with pathogen-containing vacuoles and/or cytoplasmic bacteria, leading to membrane disruption [256, 257, 265, 266, 440]. During *Francisella novicida* infection, mouse GBP1, GBP2, GBP3, and GBP5 are recruited to the surface of the bacterial membrane [440]. A highly hydrophobic stretch within mouse GBP1, GBP1<sup>28–67</sup>, has been identified as an antimicrobial region that directly targets *F. novicida* for bacterial killing [440]. GBPs further recruit IRGB10, which also targets cytoplasmic *F. novicida* [257], where GBPs and



**Fig. 10** The AIM2 inflammasome. Human and mouse AIM2 inflammasomes are activated in response to microbial or self-DNA. Cytosolic bacteria such as *Francisella novicida*, *Legionella pneumophila* and *Brucella abortus* induce the production of type I IFNs that signal through IFNAR receptors, which drive the expression of guanylate-binding proteins (GBPs) and disrupt bacterial membranes, releasing DNA into the cytosol and facilitating AIM2 activation. In addition, *Listeria monocytogenes* undergoes bacteriolysis, releasing bacterial DNA into the cytoplasm to activate AIM2. DNA viruses such as mouse cytomegalovirus and fungal pathogens such as *Plasmodium berghei* and *Plasmodium falciparum* can activate AIM2 independently of type I IFN signaling. AIM2 also detects radiation-induced damage to host DNA, which is marked by  $\gamma$ H2AX, in the nucleus. Furthermore, AIM2 recognizes host DNA in the cytoplasm following damage to the nucleus or mitochondria. Following the detection of DNA, AIM2 recruits ASC and caspase-1 to form an active inflammasome complex. AIM2 can be inhibited by several host factors, including p202 in mice and IFN- $\gamma$ -inducible protein 16 (IFI16) in humans

IRGB10 collectively compromise bacterial membrane integrity, mediating the release of bacterial dsDNA [257, 437, 438, 440]. Mouse GBP2, GBP3, and GBP5 also promote AIM2 inflammasome activation in macrophages infected with *Brucella abortus* [452]. It is possible that mitochondrial ROS, which are induced during *B. abortus* infection [453], may damage either the bacterial or the mitochondrial membrane that drives the cytosolic release of dsDNA. In other cases, *L. monocytogenes* undergoes autobacteriolysis in the macrophage cytoplasm, which leads to bacterial dsDNA release and AIM2 inflammasome activation [442]. Among bacterial pathogens, the AIM2 inflammasome plays a protective role in mice infected with *F. tularensis*, *B. abortus*, *Mycobacterium tuberculosis* or *S. aureus*, resulting in a reduced bacterial burden and/or disease severity [257, 434, 435, 437, 440, 452–458]. In contrast, the AIM2 inflammasome can also induce excessive inflammation in response to *L. monocytogenes* infection, leading to increased bacterial burden in the liver and reduced survival of mice [459] (Fig. 10).

In addition to its role in bacterial pathogens, AIM2 is also activated during infection with DNA and RNA viruses [460]. For DNA viruses, activation of the AIM2 inflammasome is usually triggered by viral genomic DNA [461, 462]. In the case of HBV, the virus enters the phagolysosome in human monocytes, leading to

the release of viral DNA in the host cytoplasm [461]. During human papillomavirus infection, viral dsDNA colocalizes with ASC specks and activates the AIM2 inflammasome in the cytoplasm of human keratinocytes [462]. Among DNA viruses, the AIM2 inflammasome has been shown to play protective roles in mouse models of herpes simplex virus-1 and pseudorabies virus-1 infection, leading to viral clearance [445, 446]. The importance of the AIM2 inflammasome during infection with DNA viruses is further demonstrated by mechanisms in which viruses directly block AIM2, with the tegument protein pUL83 from human cytomegalovirus and VP22 from herpes simplex virus-1 interacting with and inhibiting AIM2 [463, 464].

Notably, AIM2 can also sense infections caused by RNA viruses [465–468]. Although the mechanisms of AIM2 activation induced by RNA viruses are poorly understood, RNA viruses may induce mitochondrial or nuclear damage in mammalian cells, resulting in the release of host dsDNA for AIM2 recognition. Indeed, the IAV proteins M2 and PB1-F2 can damage mitochondrial membranes to trigger cytosolic mitochondrial DNA release [177, 469]. The AIM2 inflammasome, depending on the experimental conditions, plays either protective or detrimental roles during influenza infection. In mice intranasally infected with 40,000 plaque-forming units (PFUs) of IAV PR8, activation of the AIM2 inflammasome leads to

increased survival and secretion of IL-1 $\beta$  in the lungs [465]. In contrast, for mice intranasally infected with 40–8000 PFU of IAV PR8, AIM2 promotes lethality [466].

Aberrant DNA release from damaged or dying mammalian cells can also drive sterile inflammation and cancer development [470–474]. Sterile damage induced by the protease inhibitor nelfinavir [475] and the pollutant perfluoroalkyl substances [476] mediates cytosolic dsDNA release from the nucleus and mitochondria, respectively, driving the activation of the AIM2 inflammasome. In the context of cancer, AIM2 either inhibits or promotes tumorigenesis in an inflammasome-dependent or inflammasome-independent manner [471, 472, 477–504]. Most studies describe an inflammasome-independent role of AIM2 in suppressing tumor growth, such as in colorectal cancer [471, 472, 477–480], breast cancer [487–490], cervical carcinoma [484], gastric cancer [485], hepatocellular carcinoma [491, 492], osteosarcoma [486], and renal carcinoma [481–483]. Other studies indicate that AIM2 enhances tumor progression in non-small cell lung cancer [493], lung adenocarcinoma [494, 495] and squamous cell carcinoma [496–498]. Furthermore, AIM2 interacts with the ubiquitin-conjugating enzyme Ube2i to facilitate sumoylation-based suppression of the type I interferon response, limiting kidney inflammation in systemic lupus erythematosus [505]. In the context of an inflammasome-dependent function, AIM2 can be activated by circulating dsDNA in the serum of mice treated with the carcinogen diethylnitrosamine, which promotes inflammasome-mediated inflammation and hepatocellular carcinoma progression [499]. Furthermore, the AIM2 inflammasome enhances non-small cell lung cancer by promoting the entry of cancer cells into the G2/M phase [500]. Nanoparticle-mediated delivery of the gene encoding AIM2 into renal cell carcinoma tissue also promotes caspase-1 activity and IL-1 $\beta$  secretion, leading to inhibition of tumor growth in mice [501]. Owing to both tumor-promoting and tumor-suppressive roles, pharmacological intervention targeting AIM2 activity must be finely controlled for therapeutic purposes.

Therapeutic targeting of AIM2 is a subject of future research because activation of the AIM2 inflammasome is detrimental to many autoimmune and inflammatory diseases [506–518]. The development of AIM2 inhibitors is ongoing. However, no clinically approved drugs are currently available. The synthetic oligodeoxynucleotide A151, via its TTAGGG motif and phosphorothioate backbone, can bind the AIM2 HIN200 domain, block dsDNA binding, and prevent AIM2 inflammasome formation [519]. Additional candidates, such as the seed extract of the *Cornus officinalis* plant [520] and the chemical compounds J114 [521] and 4-sulfonic calixarenes [522], have been reported to inhibit AIM2 inflammasome activation. However, off-target effects have been described for these drugs [519–522], indicating that newer candidates must be developed. Inspirations may be drawn from how AIM2 is inhibited in mammalian cells. AIM2 is constitutively ubiquitinated and undergoes proteasomal degradation [523]. In the presence of dsDNA, the deubiquitinating enzyme USP21 binds to and deubiquitinates AIM2, thereby preventing its degradation and increasing its protein stability [523]. Inhibiting USP21 may be used to degrade AIM2. Furthermore, AIM2 can be blocked by other interferon-inducible HIN-containing proteins, such as mouse p202 and human IFI16- $\beta$  [524, 525]. Mouse p202 contains a HIN2 domain that interacts with and blocks the AIM2 HIN200 domain [524]. Human IFI16- $\beta$  is structurally similar and functionally equivalent to mouse p202, which also exerts an inhibitory effect on AIM2 [525]. Although AIM2 is a well-characterized cytosolic dsDNA sensor, the mechanisms controlling its stability, such as ubiquitination and deubiquitination by USP21 or other regulators, including p202 or IFI16- $\beta$ , require further investigation.

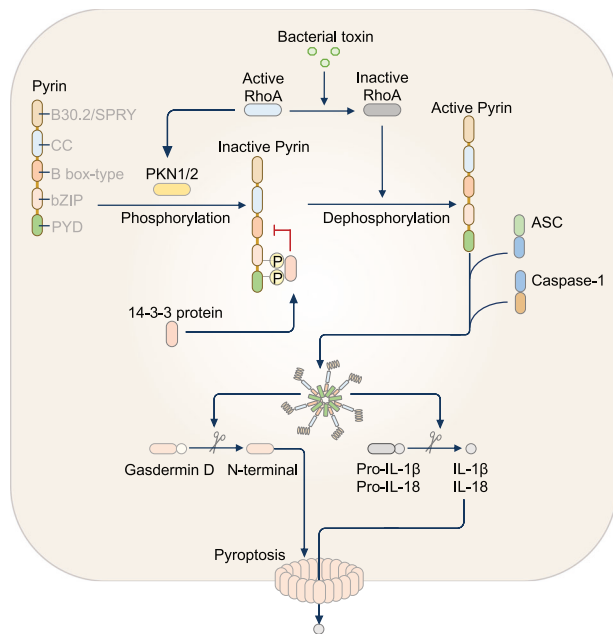
### PYRIN INFLAMMASOME

Pyrin (also known as marenostriin, MEFV, MEF and TRIM20) is encoded by the *MEFV* gene and is expressed in cells of the innate

immune system, including neutrophils, monocytes, dendritic cells, granulocytes, and eosinophils, in humans [526, 527]. Pyrin was named after the Greek word for fever, Pyretós, owing to the association between *MEFV* variants and familial Mediterranean fever (FMF), the most common noninfectious genetic fever in the world [528]. Pyrin is a member of the TRIM family of proteins, although it is considered an incomplete TRIM protein because of the absence of an E3 ubiquitin ligase domain [529, 530]. Instead, human Pyrin has a five-domain structure consisting of an N-terminal PYD, a bZIP transcription factor domain, a B-box domain, a coiled-coil domain, and a C-terminal B30.2 domain (also known as the SPRY domain) [531]. Mouse Pyrin has a similar domain structure but lacks the B30.2 domain [26].

Whether Pyrin is a genuine inflammasome sensor has been debated for many years. An earlier yeast two-hybrid screen revealed that the PYD of human Pyrin interacts with ASC [532], suggesting that human Pyrin either has a role in inflammasome formation or inflammasome inhibition. In human THP-1 macrophages, siRNA-mediated knockdown of the gene encoding Pyrin led to increased release of IL-1 $\beta$  in response to LPS [533], suggesting that Pyrin may act as a negative regulator of the inflammasome. Similarly, compared with those from wild-type mice, peritoneal macrophages from *Mefv*<sup>-/-</sup> mice presented increased NLRP3-dependent IL-1 $\beta$  release [534], further supporting a role for Pyrin as a negative regulator of the inflammasome. However, peritoneal macrophages from knock-in mice expressing mouse Pyrin carrying a human B30.2 domain with FMF-associated mutations underwent constitutive caspase-1 activation and ASC dependent, NLRP3-independent IL-1 $\beta$  release [535]. These findings complicated interpretations of Pyrin as a putative negative regulator of inflammasomes or as a bona fide inflammasome sensor. Further clarification of the role of Pyrin in inflammasome biology revealed that Pyrin triggers ASC-dependent caspase-1 activation and IL-1 $\beta$  release in human mononuclear cells infected with the gram-negative bacterium *Burkholderia cenocepacia* [536]. This initial study establishes Pyrin as a putative inflammasome sensor of an infectious trigger. How Pyrin senses *B. cenocepacia* infection in mammalian cells was unknown until a subsequent study linking Pyrin and Rho-GTPase modifications [537] (Fig. 11).

Rho-GTPases drive cytoskeletal remodeling, including actin and microtubule dynamics [538], by switching between an active GTP-bound state and an inactive GDP-bound state [539]. Several bacterial pathogens, including *B. cenocepacia* and *Clostridium difficile*, disrupt actin cytoskeleton dynamics either during entry into or during intracellular replication within mammalian cells [536, 537, 539]. This process is mediated by certain bacterial toxins and effector proteins, including TecA from *B. cenocepacia*, TcdA and TcdB of *C. difficile*, Vops from *Vibrio parahaemolyticus*, IbpA from *H. somni*, and ADP ribosylating C3 toxin from *Clostridium botulinum*. These toxins can modify the switch-1 region of Rho subfamily proteins and activate pyrin [537]. Compared with wild-type BMDMs, *Mefv*<sup>-/-</sup> BMDMs cannot undergo caspase-1 cleavage and pyroptosis following stimulation with TecA, TcdB, Vops, IbpA or C3 toxin [537]. Furthermore, TcdB, which is defective in glucosyl transferase activity, does not activate pyrin because the glycosylation event at the switch-1 region of RhoA necessitates the inactivation of RhoA [537]. These findings indicate that Pyrin prevents disruptions in RhoA functions triggered by certain bacterial toxins [537]. A recent study identified a novel endogenous regulator of Rho GTPases in the context of bacterial infection. The cell division cycle 42–165 aa (CDC42–165aa), a protein encoded by the circular RNA circCDC42, was found to promote pyrin inflammasome activation and pyroptosis in macrophages during *Klebsiella pneumoniae* infection by inhibiting the activity of the Rho GTPase CDC42 [540]. This discovery further reinforces the role of Rho family GTPase perturbation as a unifying upstream signal for Pyrin-mediated innate immune responses.



**Fig. 11** Pyrin inflammasome. Under basal conditions, human and murine pyrin proteins are phosphorylated by the RhoA effector kinase PKN1/2 and bind to 14-3-3 proteins, maintaining them in an inactive state. Bacterial toxins, including *Clostridium difficile* TcdA and TcdB, inhibit RhoA activity and downstream PKN1/2-mediated phosphorylation, resulting in pyrin dephosphorylation and dissociation from 14-3-3 proteins. Activated pyrin then recruits ASC and caspase-1 to assemble a functional inflammasome complex

Subsequent studies further clarified the mechanisms by which Pyrin detects RhoA inactivation (Fig. 11). Under homeostatic conditions, the Rho-dependent kinases PKN1 and PKN2 (herein PKN1/2) mediate Pyrin phosphorylation at Ser208/242 in humans and at Ser205/241 in mice [541, 542]. Phosphorylated Pyrin then binds to 14-3-3 signaling proteins, maintaining Pyrin in an inactive state [541, 543]. A mutation at the S242R phosphorylation site of human Pyrin attenuates the binding between Pyrin and 14-3-3, resulting in increased ASC speck formation in HEK 293T cells and increased IL-1 $\beta$  release in THP-1 cells [544]. Furthermore, bacterial toxins, such as TcdB, covalently modify RhoA by glucosylation, locking RhoA in its inactive GDP-bound state. Since PKN1 and PKN2 require active GTP-bound RhoA for their catalytic activity, inactivation of RhoA abolishes PKN1/2 function, leading to Pyrin dephosphorylation [541, 542]. This event disrupts the binding between Pyrin and 14-3-3 proteins, possibly leading to a conformational change in Pyrin. These findings highlight the critical role of Pyrin phosphorylation in maintaining Pyrin in an inhibitory state. Importantly, *Yersinia* species have evolved mechanisms to avoid Pyrin activation, such that the *Yersinia* effector protein YopM promotes PKN1/2-mediated Pyrin phosphorylation and prevents Pyrin inflammasome activation [545, 546]. The importance of this regulatory mechanism is underscored by findings that ancient FMF-associated Pyrin mutations may have conferred resistance to *Y. pestis* during historic plague pandemics by enabling heightened inflammasome activation [547].

The assembly of the Pyrin inflammasome is thought to occur at the microtubule organizing center and requires dynein adaptor histone acetyltransferase 6 (HDAC6) and the aggresome processing pathway, which is a cellular pathway that disposes of misfolded proteins [548]. In immortalized BMDMs, HDAC6 contributes to caspase-1 activation, IL-1 $\beta$  secretion, and cell death following TcdB stimulation [548]. However, in the U937 human

monocytic leukemia cell line overexpressing Pyrin, HDAC6 is not required for IL-18 secretion and cell death in response to the Pyrin activator and bile acid derivative BAA473 [549], suggesting that the role of HDAC6 in Pyrin activation may be cell-type- and trigger specific. Notably, perturbations in cytoskeletal dynamics can also drive Pyrin-dependent inflammation. For example, a mutation in the actin depolymerization cofactor WDR1 results in systemic autoinflammation in mice [543]. Deletion of the gene encoding Pyrin in *Wdr1* mutant mice leads to decreased serum IL-18 levels compared with those in *Wdr1* mutant mice, which carry a functional Pyrin [543]. A further study suggested that Pyrin activation may be facilitated by the lysosomal regulator complex component LAMTOR1 positioned on the membrane of late endosomes or lysosomes [550]. Compared with wild-type BMDMs, mouse BMDMs lacking LAMTOR1 have an impaired ability to secrete IL-1 $\beta$  following stimulation with TcdB [550]. It is possible that cell types, organelles, and Pyrin triggers converge to drive inflammasome activation and pathological inflammation, underscoring the potentially adaptable mechanisms to different physiological threats. These mechanisms may also explain why Pyrin appears to respond to AOM-DSS in the large intestine of mice, leading to the release of IL-18, maintenance of intestinal barrier integrity, and protection against colitis-associated colorectal cancer [551].

Mutations in the gene encoding Pyrin cause several autoinflammatory disorders. FMF is the most common clinical manifestation arising from mutations in Pyrin, with most of these mutations occurring in the B30.2 domain [544, 552–554]. Blood mononuclear cells from patients with FMF carrying mutant Pyrin show impaired binding between mutant Pyrin and 14-3-3 and between mutant Pyrin and PKN1/2 [542]. However, the structural details of how mutations in the B30.2 domain abrogate the Pyrin–14-3-3 interaction and prevent Pyrin phosphorylation are not known. It is plausible that B30.2 mutations may induce conformational changes that either reduce accessibility to key phosphorylation sites or disrupt autoinhibition, resulting in Pyrin self-oligomerization and inflammasome activation. Recent work also suggests that mutations in the central helical scaffold consisting of coiled-coil alpha helices, in addition to the B30.2 domain, can differentially modulate inflammasome activity, revealing more nuanced Pyrin regulation than previously appreciated [555]. In addition to FMF, Pyrin mutations have been found in patients with Pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND), Pyrin-associated dominant disease (PADD), pyrogenic arthritis, pyoderma gangrenosum and acne (PAPA), mevalonate kinase deficiency (MKD), and autoinflammatory periodic fever, immunodeficiency and thrombocytopenia (PFIT) [542, 556–558]. In these disorders, Pyrin mutations disrupt the interaction between Pyrin and its inhibitory regulators, destabilize the autoinhibitory conformation of Pyrin, or interfere with Rho-GTPase signaling. The net result is aberrant Pyrin activation and excessive IL-1 $\beta$  production, with IL-1 $\beta$  serving as an inflammatory mediator [542, 556–558].

The molecular and genetic basis of Pyrin activation has led to the development of therapeutic strategies, particularly in FMF. Patients with FMF are treated primarily with the anti-inflammatory alkaloid colchicine, which prevents microtubule polymerization, disrupts inflammasome activation, and suppresses IL-1 $\beta$  release [559]. However, in FMF patients who are resistant to colchicine, IL-1-blocking therapies, such as the recombinant IL-1 receptor antagonist anakinra or the anti-IL-1 $\beta$  monoclonal antibody canakinumab, have proven effective at suppressing Pyrin inflammasome-driven inflammation and achieving complete remission of febrile attacks [560, 561]. Future strategies may include the development of small molecules that stabilize Pyrin autoinhibition, for example, by enhancing PKN1/2-mediated phosphorylation of Pyrin or strengthening the binding between Pyrin and 14-3-3. Since Pyrin can detect perturbations in

Rho-GTPases [537], further investigations into whether Pyrin can detect Rho-GTPase alterations in cancers and neurological conditions, where Rho-GTPase dysregulation is frequently observed [562–564], could enhance our understanding of Pyrin and guide the development of targeted interventions.

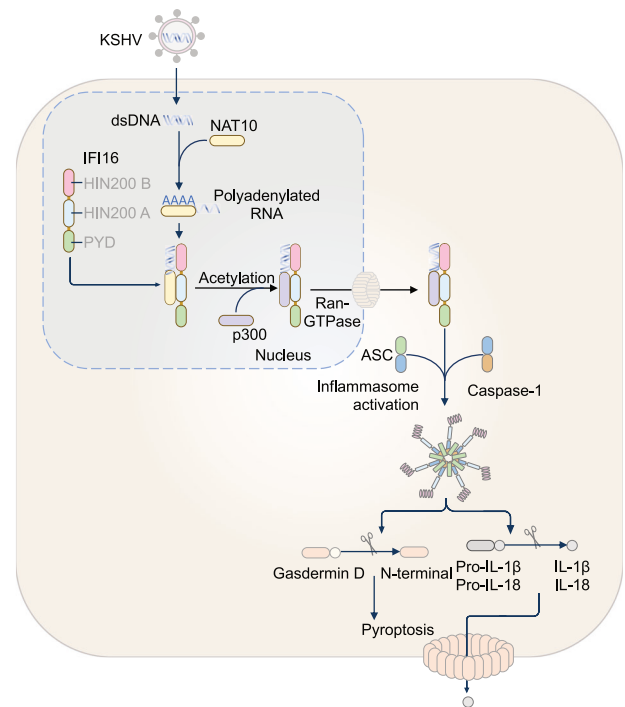
### IFI16 INFLAMMASOME

Interferon gamma-inducible protein 16 (also known as IFI16 or IFNGIP1) is a member of the ALR or PYHIN protein family and plays a role in DNA sensing, inflammasome activation, and interferon signaling [565]. IFI16 is found in humans, and the closest ortholog of IFI16, called p204, is found in mice [566]. Multiple cell types express IFI16, including peripheral blood monocytes, T cells, CD34<sup>+</sup> myeloid precursors, and epithelial cells [567]. Both IFI16 and p204 carry an N-terminal PYD and two C-terminal HIN-200 domains, HINa and HINb, which recognize DNA through oligonucleotide-binding folds [568–570]. IFI16 can bind to DNA fragments of at least 60 bp but optimally binds 150 bp fragments [430, 570]. The HINb domain of IFI16 has a positively charged concave surface that facilitates binding to both strands of dsDNA at the dsDNA backbone, whereas HINa only interacts with one strand at a time, allowing it to bind both ssDNA and dsDNA [569, 571, 572]. In contrast, both HIN domains of mouse p204 bind to DNA via a crosslinker connecting two oligonucleotide binding folds [573].

IFI16 is localized in the nucleus, nucleolus, nucleoplasm, and cytoplasm [429, 574–582]. These subcellular localizations determine the role of IFI16 in recognizing cytosolic and nuclear DNA, inducing IFN- $\beta$  production or inflammasome formation [429, 574–582]. In the cytoplasm, IFI16 senses Vaccinia viral DNA and interacts with STING to increase IFN- $\beta$  production [570]. In the nucleus, IFI16 can sense HSV-1 DNA to induce the production of type I interferons [583, 584]. In HEK293T cells, mutations in residues within the IFI16 HINa domain involved in DNA binding increase IFN- $\beta$  release, whereas deletion of HINb attenuates IFN- $\beta$  expression, suggesting that the complementary domains cross-control IFI16-mediated production of type I interferons [569]. In addition to driving the production of type I interferons, IFI16 can form an inflammasome in the nucleus in response to viral DNA during Kaposi sarcoma herpes virus (KSHV) infection [583]. KSHV encodes a long noncoding RNA known as polyadenylated nuclear RNA, which undergoes acetylation by interacting with N-acetyltransferase (NAT10) [584]. This interaction promotes IFI16 mRNA acetylation via NAT10, increasing IFI16 translation and enhancing DNA sensing [584]. Following recognition of KSHV DNA, IFI16 is also acetylated by the histone acetyl transferase p300 and translocates from the nucleus to the cytoplasm. This translocation is mediated by Ran-GTPase, a GTP-binding protein that controls the movement of other proteins across the nuclear envelope [583]. In the cytoplasm, the PYD of IFI16 interacts with ASC to form an inflammasome [583, 585] (Fig. 12). However, the precise molecular mechanisms governing IFI16 activation, such as acetylation, are unclear. Additionally, how IFI16 activity is restricted to viral DNA and not host DNA within the nucleus requires further exploration. Investigating the potential role of mouse p204 in the formation of an inflammasome may also provide insights into the physiological functions of this pathway. Site-directed mutagenesis of IFI16 acetylation sites, chromatin immunoprecipitation (ChIP) with viral versus host DNA, and comparative studies using murine p204 knockout or human IFI16 knock-in models may clarify the precise mechanisms by which IFI16 senses nucleic acids and drives antiviral immunity.

### CARD8 INFLAMMASOME

Caspase recruitment domain-containing protein 8 (CARD8, DACAR, KIAA0955 or NDPP1) is a human inflammasome sensor

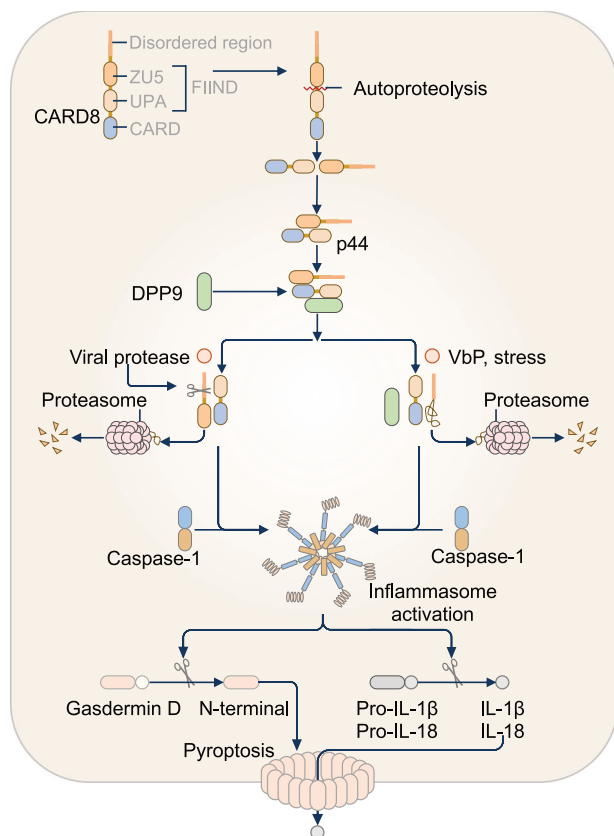


**Fig. 12** The IFI16 inflammasome. Kaposi's sarcoma-associated herpesvirus (KSHV) DNA is sensed by interferon gamma-inducible protein 16 in the nucleus. The polyadenylated nuclear RNA of KSHV binds to N-acetyltransferase (NAT) 10, increasing acetylation and increasing the expression of IFI16. Additional acetylation by histone acetyltransferase p300 enables translocation to the cytoplasm via the Ras-related nuclear protein guanosine triphosphate (Ran GTPase), where IFI16 binds viral DNA and assembles an inflammasome

protein that shares structural homology with human NLRP1 [586] and is expressed predominantly in T cells [587, 588]. No homologs of CARD8 are found in mice [587, 588]. CARD8 consists of a CARD and an FIIND comprising two subdomains called ZU5 and UPA [586]. The structural similarity between CARD8 and NLRP1 initially suggested that CARD8 may function as an inflammasome sensor.

Like NLRP1, CARD8 can be activated by the chemical inhibitor Val-BoroPro, which inhibits the proteolytic enzymes DPP8 and DPP9 [589]. Under homeostatic conditions, CARD8 undergoes autoproteolysis, releasing two fragments: an N-terminal fragment consisting of the FIIND ZU5 subdomain and a 160-amino acid disordered region and a C-terminal fragment consisting of the FIIND UPA subdomain and CARD [590]. These fragments remain noncovalently associated and together form a p44 subunit comprising ZU5, UPA and CARD, which bind the proteolytic enzyme DPP9 [589, 591]. This interaction with DPP9 sequesters the UPA region necessary for CARD8 oligomerization, keeping CARD8 inactive [589, 591]. However, upon Val-boroPro-induced stress, the disordered N-terminal region and ZU5 subdomain are targeted for degradation by the 20S proteasome through an unknown mechanism [591]. This degradation releases the C-terminal UPA–CARD fragment, which can no longer be restrained by DPP9. In the absence of DPP9-mediated restriction, the UPA–CARD region is set free to facilitate CARD8 oligomerization and inflammasome formation [591] (Fig. 13).

The CARD8 inflammasome is activated in response to a range of viral infections and contributes to the pathogenesis of disease development [592–595]. For example, CARD8 activation and subsequent pyroptosis in CD4<sup>+</sup> T cells infected with HIV-1 drive the depletion of the CD4<sup>+</sup> T-cell population. The binding between HIV-1 proteases and CARD8 during viral entry [596] or reactivation



**Fig. 13** CARD8 inflammasome. Under resting conditions, CARD8 undergoes autoproteolysis and remains inactive through binding to dipeptidyl peptidase (DPP) 8 or 9. Viral proteases from human immunodeficiency virus (HIV) type 1 and Coxsackievirus B3 cleave the disordered N-terminal region of CARD8, releasing the C-terminal fragment, which recruits caspase-1 to assemble the CARD8 inflammasome. The chemical Val-BoroPro or cellular stress disrupts binding to DPP8/9, exposing the N-terminal fragment for proteasomal degradation and liberating the C-terminal fragment to initiate inflammasome assembly

from latency [595] in CD4<sup>+</sup> T cells causes proteolysis of the CARD8 N-terminus, leading to the production of an unstable Neo-terminus, which is targeted for proteasomal degradation [595]. The subsequent release of a bioactive UPA-CARD fragment results in the formation of an inflammasome complex with caspase-1, leading to the pyroptosis of CD4<sup>+</sup> T cells and low CD4<sup>+</sup> T-cell counts [595]. Similarly, the 3CL<sup>pro</sup> protease from coronaviruses, including SARS-CoV-2 [594], and the 3C protease from the endocarditis-causing virus Coxsackievirus B3 [593] activate CARD8 by cleaving the CARD8 N-terminal region. This sensing of viral protease activity may be mediated by specific amino acid residues found in human CARD8 but not in other nonhuman primates [594]. For example, CARD8 proteins from nonhuman primates are incapable of sensing HIV-1 protease activity because of the absence of a human-specific F59-F60 motif located within the N-terminal region of FIIND [596], suggesting that polymorphisms of CARD8 found in different host species may explain their susceptibility or resistance to certain viruses.

Other triggers, including unfolded protein response inducers and reductive stress, have also been shown to activate CARD8 by accelerating the degradation of the N-terminal fragment [51, 52]. Additionally, the inhibition of aminopeptidases such as M24B prolidase and Xaa-Pro aminopeptidase-1 by the small-molecule inhibitor CQ31 has been shown to activate CARD8 [597]. CQ31 treatment results in the accumulation of endogenous proline-containing peptides, including Xaa-Pro dipeptides, which inhibit

DPP8/9 enzymatic activity and activate CARD8 [597]. As a selective CARD8 activator, CQ31 may serve as a valuable tool for elucidating CARD8 mechanisms and offers therapeutic potential for modulating CARD8 activity to kill virus-infected cells.

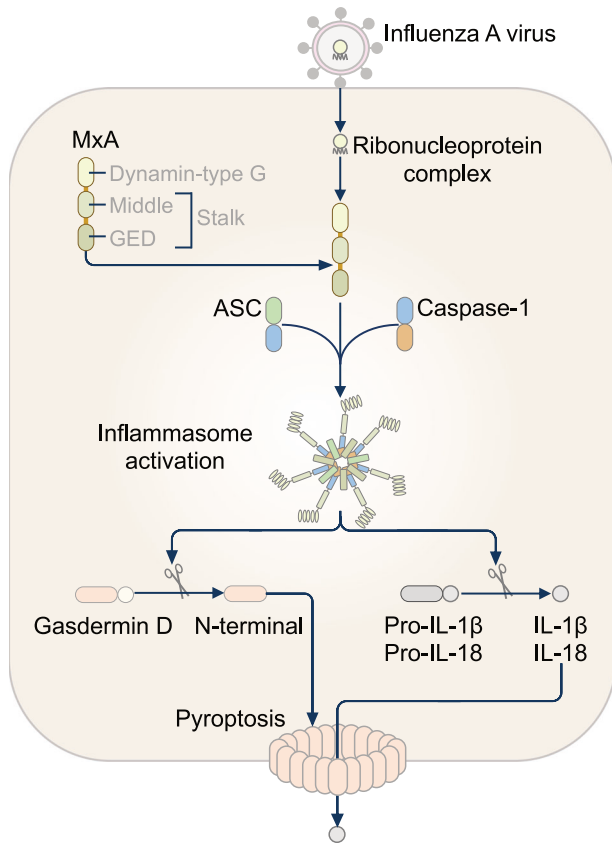
In addition to responses to viral infections, CARD8 signaling has been associated with autoimmune, neurodegenerative and cardiovascular diseases. CARD8 mutations have been found in patients with Alzheimer's disease [598], IBD [599], and rheumatoid arthritis [600]. Heterozygous variants of CARD8 in combination with genetic variants of NLRP3 have been found to increase the risk of ischemic stroke [601]. Future studies characterizing the dysregulated functions of these disease-associated mutations in CARD8 could further reveal opportunities to therapeutically modulate CARD8 activity.

### MXA INFLAMMASOME

The Myxovirus resistance (also known as Mx) proteins are a family of antiviral proteins with pivotal roles in orchestrating innate immune responses against DNA viruses such as hepatitis B virus (HBV) and RNA viruses such as IAV and vesicular stomatitis virus (VSV) [602]. These interferon-inducible proteins are recognized for their ability to impede the viral replication cycle [602–605]. Human Myxovirus resistance protein A (MxA, also known as MX1 and IFI-78K) has been identified as an inflammasome sensor protein that is activated in response to IAV infection [606]. In respiratory epithelial cells, MxA can interact with the ribonucleoprotein complex of IAV, which comprises the viral nucleoprotein and viral polymerase [606]. The nucleoproteins of other viruses, such as vesicular stomatitis virus (VSV) and human parainfluenza virus, are also recognized targets of human MxA [602], but whether MxA senses these nucleoproteins or common structures and/or motifs within nucleoproteins and drives inflammasome activation is not known. The GTPase domain (also known as the dynamin-type G domain) of MxA was found to bind the PYD of ASC, triggering ASC oligomerization [606]. This interaction leads to the formation of an MxA inflammasome complex, leading to the secretion of IL-1β in respiratory epithelial cells infected with IAV [606] (Fig. 14). In cell types that do not express MxA, such as human polymorphonuclear blood monocytes (PBMcs), inflammasome activation in response to IAV infection relies on NLRP3 [606].

C57BL/6J mice do not express functional Mx1 or Mx2 proteins. Compared with conventional C57BL/6J mice, which lack functional Mx1 and Mx2, C57BL/6J mice genetically engineered to carry the entire human Mx locus are more effective at controlling avian IAV infections [606]. Genetic deletion of caspase-1 and caspase-11 in the genetically engineered C57BL/6J mouse strain leads to increased susceptibility to avian IAV infection, highlighting the protective roles of MxA, caspase-1 and caspase-11 in mice. In another mouse model, the C57BL/6J mouse strain, which had been backcrossed to carry functional Mx1, presented increased resistance to human IAV infection [607]. In this mouse genetic background, the role of inflammasomes has been revealed by the genetic deletion of caspase-1 and caspase-11 in *Mx1<sup>-/-</sup>Tlr7<sup>-/-</sup>Mavs<sup>-/-</sup>* mice. Compared with inflammasome-competent *Mx1<sup>-/-</sup>Tlr7<sup>-/-</sup>Mavs<sup>-/-</sup>* mice, these mice exhibit enhanced protection against human IAV infection and prolonged survival [607], indicating that inflammasomes are detrimental. These findings emphasize that inflammasome functions can either be beneficial or detrimental to the host, depending on the genetic background of the mouse strains used and, in part, whether these mice carry functional Mx proteins.

Mutations in MxA have been found in colon cancer [608], lung cancer [609], and ovarian cancer [610]. In addition, the GTPase domain of MxA is required to inhibit the motility and invasiveness of human prostate carcinoma cells [611], but whether this mechanism is dependent on inflammasome signaling is not known. Therefore, a more in-depth exploration of the molecular



**Fig. 14** The MxA inflammasome. Myxovirus resistance protein A (MxA) contains a dynamine-type GTPase domain, a middle domain, and a GTPase effector domain that forms a stalk-like structure. During influenza A virus infection, the GTPase domain of MxA binds to the pyrin domain of an apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), promoting inflammasome activation

mechanisms of MxA function could increase the number of available molecular targets for pharmacological intervention. For example, determining whether MxA recognizes common structural motifs within viral nucleoproteins or specific features of IAV may clarify the breadth of its sensing ability.

### CONCLUDING REMARKS

The molecular mechanisms governing inflammasome activation has enabled a more in-depth understanding of innate immune responses and their roles in disease pathogenesis. Since the discovery and conceptualization of the inflammasome complex, studies have revealed how different inflammasome sensors are triggered in response to a plethora of PAMPs, DAMPs, and exogenous danger signals. Molecular and structural studies have provided important insights into the activation mechanisms of well-characterized inflammasome sensors. Emerging evidence has also clarified the regulation of inflammasome sensors by unconventional or novel mechanisms. Future research on posttranslational modifications, downstream effects of inflammasome-mediated pyroptosis, and wider implications of inflammasome-mediated immune responses beyond infectious diseases, such as in cancer and autoinflammatory and neurodegenerative diseases, are likely areas of active research. Mechanistic and structural studies on the lesser characterized inflammasomes remain an important area for investigation. The inflammasome field has also evolved to a point that fundamental discoveries have been translated to clinical trials of inflammasome inhibitors

in humans, particularly in the light of many new and emerging small-molecule NLRP3 inhibitors. These inhibitors will provide hope to patients with aberrant activation of NLRP3 inflammasome, including those with a genetic basis, but inhibitors for most inflammasome sensors are not currently available.

Several critical areas require further research to fully elucidate the role of inflammasomes in health and disease. The context-dependent nature of inflammasome activation, such as the effects of cell types and diseases, remains an active area of inquiry. Moreover, the coordination and potential synergy or antagonism between different inflammasome sensors are poorly understood. For instance, how inflammasome sensor proteins cooperate or compete within a single cell and between different cells to modulate a holistic host response is not known. The revelation of the PANoptosome concept, in which multiple inflammasome components, cytosolic sensors, caspases, and RIPKs aggregate into a single protein complex, represents an example of molecular integration between immune signaling pathways. Further biological insights surrounding these PANoptosomes might fill current knowledge gaps on immune sensing cooperativity and offer new targets for therapeutic development. In conclusion, while substantial efforts have been made to understand the molecular complexities of inflammasome sensors, much remains to be answered. The crosstalk between sensors, the context-dependent nature of activation, molecular mechanisms governing inflammasome signaling, and the identification and clinical development of specific inhibitors are all important focal points as the field moves forward.

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## AUTHOR CONTRIBUTIONS

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## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

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