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STATE-OF-THE-ART REVIEW

Structural mechanisms of inflammasome assembly

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Inflammasomes are supramolecular signaling complexes that activate a subset of caspases known as the inflammatory caspases, an example of which is caspase **1**. Upon stimulation by microbial and damage-associated signals, inflammasomes assemble to elicit the first line of host defense via the proteolytic maturation of cytokines interleukin-1β and interleukin-18, and by induction of pyroptotic cell death. Inflammasome assembly requires activation of an upstream sensor, a downstream effector and, in most cases, an adaptor molecule such as apoptosis-associate speck-like protein containing a caspase recruitment domain (ASC). Depending on whether ASC is required, inflammasomes can be categorized into ASC-dependent and ASC-independent inflammasomes. Here, we review current understandings of the structures of inflammasomes, as probed using traditional structural methods, as well as biochemical, biophysical and single-molecule methods. The key structural scaffold for inflammasome assembly is composed of filaments of Pyrin domains and caspase recruitment domains (CARD) in the sensor, adaptor and effector components. Nucleated polymerization appears to govern the ordered assembly process from activation of a Pyrin domain-containing sensor such as AIM2 by dsDNA or NLRP3 by extracellular particulates, to recruitment of the Pyrin domain and CARD-containing adaptor ASC, and finally to activation of CARD-containing caspase **1**. The underlying filamentous architecture of inflammasomes and the cooperativity in the assembly may explain the 'all-or-none' response in inflammasome activation. Inflammasomes are tightly regulated by a number of cytosolic inhibitors, which may change the morphology and assembly kinetics of inflammasomes. Biochemical and cellular studies suggest that Pyrin domain and CARD filaments possess prion-like properties in propagating inflammasome activation within and between cells.

Introduction

Inflammasomes are elaborate supramolecular complexes formed in the cytosol that play critical roles in eliciting innate immune responses against pathogens and other damage-associated signals [1]. Most canonical inflammasomes consist of a sensor component, an adaptor component and an effector component such as caspase **1**, and possibly caspase **11** (mouse) and caspases **4** and **5** (human). Upstream sensor components, after

Abbreviations
ALR, AIM2-like receptor; ASC, apoptosis-associate speck-like protein containing a caspase recruitment domain; CARD, caspase recruitment domain; DD, death domain; EM, electron microscopy; GBP5, guanylate-binding protein 5; IL, interleukin; NLR, NOD-like receptor; PYD, Pyrin domain.

which the names of the inflammasomes are often designated, detect danger signals and recruit adaptors, which in turn recruit and activate the caspase effectors. The most common adaptor is apoptosis-associate speck-like protein containing a caspase recruitment domain (ASC), which defines ASC-dependent inflammasomes. Activated caspases proteolytically process pro-interleukin-1β (pro-IL-1β) and pro-IL-18. The matured IL-1β cytokines activate the IL receptor-signaling cascade, which converges with Toll-like receptor signaling through a common adaptor, MyD88, to stimulate the transcription of many pro-inflammatory and antiviral genes [2].

Canonical inflammasomes assemble upon the activation of two classes of sensor molecules: NOD-like receptors (NLRs) and AIM2-like receptors (ALRs). The human genome encodes 22 NLRs [3], but only NLRP1 [4], NLRP3 [5,6], NLRP6 [7], NLRP7 [8], NLRP12 [9] and the NAIP/NLRC4 complex [10,11] have been reported to assemble into their respective inflammasomes. These essential sensor proteins are composed of an effector domain such as the caspase recruitment domain (CARD), Pyrin domain (PYD) or baculoviral IAP repeat domain for downstream interactions, a nucleotide-binding and oligomerization domain NACHT, and a leucine-rich repeats domain (Fig. 1). Both NACHT and leucine-rich repeats may be important for auto-inhibition of the sensor proteins in the absence of stimulation [12]. ALRs comprise a PYD domain and a dsDNA-binding domain HIN200, and are simpler than NLRs. The assembly of inflammasomes relies on homotypic interactions between component proteins. In ASC-dependent inflammasomes, upstream sensor proteins, such as NLRP3 and AIM2, recruit ASC via interactions between their PYDs. ASC then interacts with caspase **1** via CARD/CARD interactions. Therefore, the bipartite nature of ASC represents the core structure of these inflammasomes. The NAIP/NLRC4/caspase **1** complex is a representative ASC-independent inflammasome, in which NAIP is the sensor and NLRC4 is the adaptor, although both NAIP and NLRC4 belong to NLRs by domain definition (Fig. 1) [13]. NLRC4 contains a CARD and can directly recruit and activate caspase **1**.

Until recently, the structural model of an NLR inflammasome was based on caspase-activating apoptosomes, in which the sensor proteins human Apaf-1, *Drosophila* Dark or *Caenorhabditis elegans* CED-4 share a domain organization similar to NLRs [14–16]. The crystal structure of CED-4 revealed a donut-shaped octameric assembly of the NACHT domain [17]. The heptameric and octameric electron microscopy (EM) structures of human Apaf-1 and *Drosophila* Dark showed oligomerization of the NACHT domain into disk-like structures that are characteristic of AAA+ ATPases [18–20]. NLRP1 has been previously shown by low-resolution EM analysis to oligomerize into a disk-like structure, likely through the NACHT domain [4]. For ALRs, the crystal structure of the AIM2 HIN200 domain in complex with dsDNA revealed the molecular basis for sequence-independent recognition of cytosolic viral nucleic acids [21]. However, limited structural information was available on any full ternary inflammasome complexes in either the

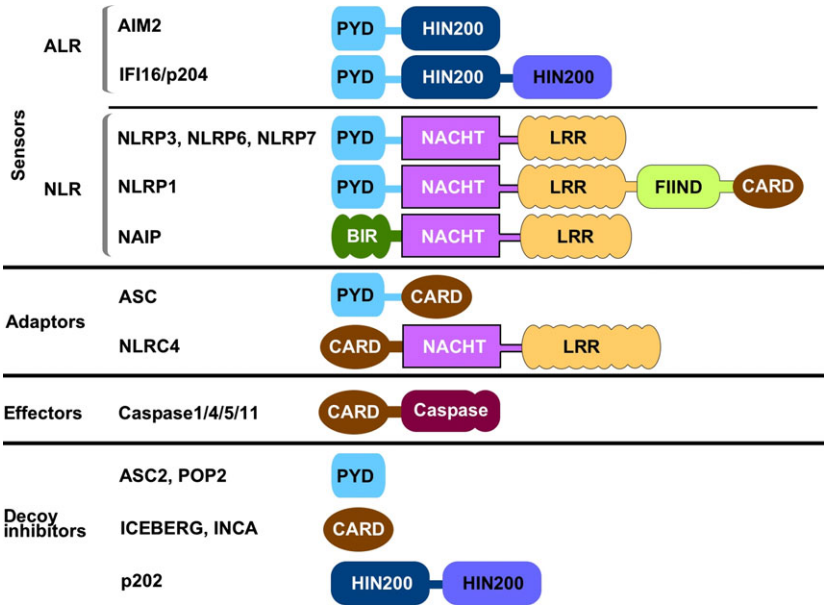

Fig. 1. Domain organization of common inflammasome component proteins. The NLR protein NLRC4 is often classified as a sensor. Here, we categorize it as the adaptor for NAIP because NAIP directly senses bacterial proteins and NLRC4 links activated NAIP to caspase **1**.

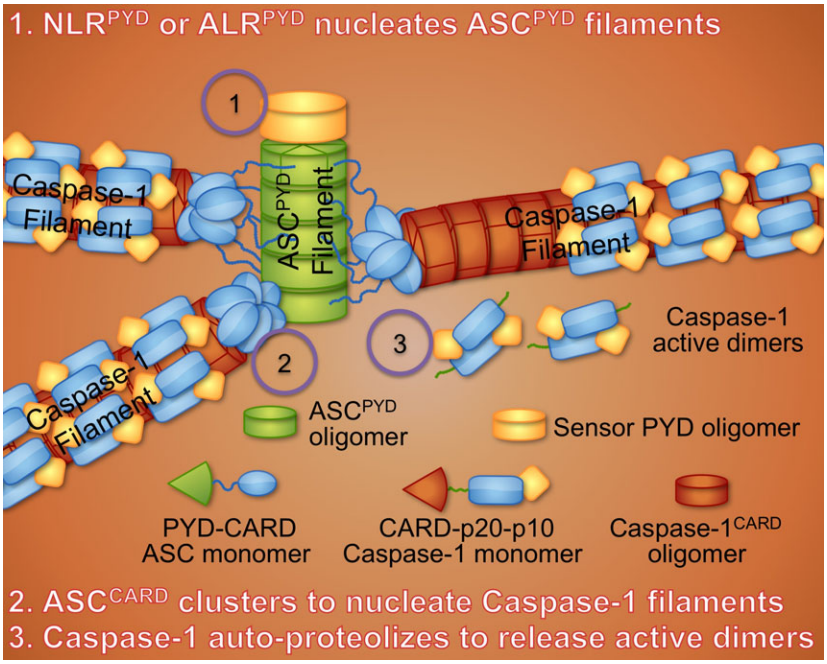

Fig. 2. Two-step nucleated polymerization mechanism for the assembly of ASC-dependent inflammasomes.

NLR or the ALR family. We now have a much better understanding on both the structural organization and the assembly mechanism of inflammasomes, which extend significantly from the apoptosome-based model [22–24]. In this Minireview, we summarize recent structural and mechanistic findings that provide a unified assembly mechanism of inflammasomes. This mechanism centers on PYD and CARD filament formation. In essence, the main oligomerization platform for caspase **1** activation resides in the PYD and CARD filaments, in contrast to the NACHT-mediated ring-like platform for caspase activation in apoptosomes.

Nucleated polymerization of PYD and CARD in inflammasome assembly

Many inflammasomes have been shown to cluster into micron-sized puncta or specks [8,25–28]. When ASC was first characterized using biochemical methods, the micron-sized structure was found to be resistant to Triton-X 100, implying its unusual stability [26]. In macrophages such as human THP-1 cells, a single cytosolic punctum containing the upstream sensors (NLRs or ALRs), ASC and downstream caspases is generated upon inflammasome activation [29]. Pyroptosis-incapable HEK293T cells overexpressing ASC also form a similar structure [30], indicating that ASC is a major determinant of biophysical properties and cellular functions in inflammasomes. The kinetic properties of ASC aggregation have been shown to generate a rapid 'all-or-none' response [31].

Our recent biochemical and structural insights point to a two-step nucleation–polymerization mechanism for the assembly of ASC-dependent inflammasomes [22] (Fig. 2). First, it is demonstrated *in vitro* that a sub-stoichiometric amount of the upstream sensor protein AIM2 or NLRP3 nucleates filament formation of fluorescently labeled monomeric ASC^{PYD} in a fluorescence polarization assay through PYD/PYD interactions [22]. These data corroborated previous observations on ASC aggregation that initial speck formation (nucleation) greatly enhanced further addition of soluble ASC molecules to the speck (polymerization) [31]. In the AIM2/ASC^{PYD} or the NLRP3/ASC^{PYD} complex, the sensor protein AIM2 or NLRP3 localizes at the end of each ASC filament, as shown by EM [22]. Second, clustered ASC could nucleate the formation of caspase $\mathbf{1}^{CARD}$ filaments through CARD/CARD interactions [22]. Filament formation of caspase **1** leads to a dramatically increased local concentration to induce dimerization and dimerization-mediated allosteric changes to allow caspase **1** auto-processing and activation.

Reconstituted ternary AIM2/ASC/caspase **1** inflammasomes showed star-shaped structures, in which multiple filaments protrude radially from a single central hub, as visualized by EM [22]. In this complex, AIM2 is sub-stoichiometric to ASC, and ASC is further sub-stoichiometric to caspase **1**, suggesting amplifications of signal transduction from the sensor to the adaptor, and then from the adaptor to the effector. Gold labeling experiments reveal that the center of the stars

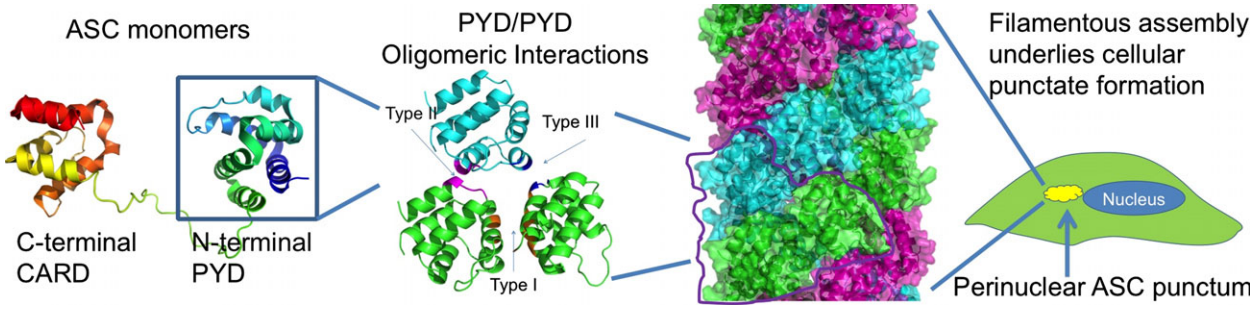

Fig. 3. Structures underlying the filamentous assembly of ASC-dependent inflammasomes. (Left to right) Full-length ASC monomer NMR structure, homotypic interaction between ASC^{PYD} molecules, cryo-EM structure of the three-start helical assembly of ASC^{PYD} filaments, and perinuclear location of an ASC-containing punctum.

contains AIM2 and ASC, whereas the main filament bodies are caspase **1**. *In vitro*, these star-shaped structures can further aggregate over time to filamentous spheres of hundreds of nanometers to microns in size, as if balls of yarn [22]. Cellular EM experiments suggest that the single ASC punctum formed in cells resembles the highly aggregated *in vitro* reconstituted ternary inflammasome complexes [22], although the details of a cellular ASC punctum await higher resolution elucidation. Collectively, these studies begin to bridge the gap between observed ASC-dependent inflammasomes *in vitro* and in cells, and to afford an aggregation mechanism of inflammasome assembly that is consistent with the observed 'all-or-none' response kinetics [22,31].

Structure of the ASC^{PYD} filament

The adaptor molecule ASC plays a pivotal role in connecting stimulation to assembly of inflammasomes, by providing multiple interaction surfaces through its N-terminal PYD and C-terminal CARD. Both PYD and CARD belong to the death domain (DD) superfamily and possess a characteristic six-helix bundle fold. They reside in proteins important for immune signaling, such as apoptosis, necrosis, and in the case of inflammasomes, cytokine maturation and pyroptosis. These domains mediate protein–protein interactions by bringing together partners with compatible interfaces to form high-order oligomeric signalosomes, which are important for proximity-driven activation of the kinase and caspase domains in the interaction partners [32]. Previous structural studies have indicated that each DD family member contains up to six interfaces to achieve such interactions through a helical assembly mechanism [33]. These interfaces are classified into three types of asymmetric interactions as in the PIDD DD/RAIDD DD complex [34], the Fas DD/FADD DD complex [35] and the MyD88 DD/IRAK4 DD/IRAK2 DD complex [36].

Until recently, the self-aggregation tendencies of full-length (FL) ASC, as well as its individual domains PYD and CARD, made it difficult to study ASC assembly mechanisms structurally. The monomeric structures of ASC^{FL} and ASC^{PYD} have been solved using NMR in highly acidic conditions, under which the self-association was abolished [37,38]. The monomeric structures provided a framework for understanding the interaction surfaces using extensive mutagenesis [39], in which a large number of charged and hydrophobic residues in ASC^{PYD} were shown to be important for filament formation of GFP-tagged ASC^{PYD} in transfected Cos7 cells.

We recently extended these earlier efforts by solving the cryo-EM structure of the ASC^{PYD} filament at a near atomic resolution (Fig. 3), an undertaking that was facilitated by recent advancement in both instrumental and computational methods in cryo-EM [22]. We found that ASC^{PYD} filaments have helical symmetry, which was utilized for structure determination by way of an iterative helical real-space reconstruction [40]. In the ASC^{PYD} filament, ASC^{PYD} subunits pack densely in a spiral with an axial rise of 14.0 Å and a right-handed rotation of 53° to create a cylinder-like structure. The filament structure has threefold symmetry when looking down the helical axis and is hollow along the helical axis, with inner and outer diameters of ~ 20 and ~ 90 Å, respectively. The hollow space inside the filament is probably for structural architecture only and has no obvious functional consequences.

The ASC^{PYD} filament structure revealed in molecular detail three types of asymmetric interfaces in PYD/PYD interactions that are similar to the conserved interfaces in DD interactions [33]. The Type I interaction was formed by helices α1/α4 of one subunit and α2/α3 of the adjacent in-strand subunit, burying

~ 880 Å^2 of surface area. The Type II interaction was mediated by residues in the α4–α5 corner of one subunit and the α5–α6 corner of another subunit in the adjacent strand. Lastly, residues in the α2–α3 corner of one subunit interacted with those in the α1–α2 corner of another subunit in the adjacent strand to form the Type III interface. Types II and III interfaces were less extensive than the Type I interface, burying ~ 540 and 360 Å^2 of surface area, respectively. Although similar in overall architecture, significant differences exist in the relative orientations between each PYD/PYD interaction pair and their DD/DD interaction counterpart, suggesting both structural conservation and variability.

Structure-based mutagenesis confirmed the importance of these ASC interfacial residues in filament formation *in vitro* and in ASC-induced IL-1β processing [22]. The data also confirmed previous extensive mutagenesis studies on the requirement for ASC^{PYD} filament formation in cells [39]. Presuming that the nucleators of ASC^{PYD} such as AIM2^{PYD} and NLRP3^{PYD} adopt the same helical assembly, we predicted that AIM2 and NLRP3 residues are important for ASC interaction. Mutating these residues compromised the ability of AIM2 and NLRP3 to nucleate ASC^{PYD} filaments, confirming the validity of the predicted interactions [22].

Unified structural model of ASC-dependent inflammasome formation

The recent structural information led us to propose a two-step nucleated polymerization model for the assembly of ASC-dependent inflammasomes [22]. After stimulation reaches its threshold, the corresponding sensor protein oligomerizes to form a cluster of PYD, which serves to recruit monomeric ASC and nucleate the ASC^{PYD} filament. Conformational changes in ASC^{PYD} upon recruitment to the filament may dictate a directional polymerization of ASC [22,38]. Because the 23-residue interdomain linker between the PYD and CARD of ASC is flexible, as shown by the NMR structure of the monomeric full-length ASC [37], the ASC^{PYD} filament might then act as a platform for the flexibly linked CARD to cluster and to nucleate the caspase **1** filament by a homotypic CARD/CARD interaction. Structural information is currently lacking for ASC^{CARD} and caspase **1**^{CARD} in their monomeric and filamentous forms. Future studies may reveal the molecular basis for ASC-mediated caspase **1** filament formation. In these two nucleated polymerization steps, ASC acts as the core protein for assembling ASC-dependent inflammasomes.

Inflammasome assembly is highly regulated. First, inflammasome component proteins are auto-inhibited in the absence of stimulation. Ligand stimulation overcomes auto-inhibition to allow inflammasomes to form. Second, spatial separation of NLRP3 and ASC into different compartments in the cell provides another regulatory role in inflammasome activation. For example, ASC is found in the mitochondria, cytosol and nucleus, whereas NLRP3 is located mainly in the endoplasmic reticulum [41]. Upon stimulation, mitochondria approach the endoplasmic reticulum at the perinuclear region, promoting an interaction between NLRP3 and ASC, and inducing inflammasome assembly and activation [41].

ASC-independent inflammasomes

Recent structural and biochemical studies on the NAIP/NLRC4 inflammasome have provided insights into the assembly mechanism of ASC-independent inflammasomes (Fig. 4). The crystal structure of NLRC4 lacking the N-terminal CARD revealed an auto-inhibited state brought about by intramolecular interactions in the NACHT and leucine-rich repeats [12]. It is suggested that upon interaction with activated NAIP proteins, NLRC4 opens to form a structure similar to the Apaf-1 apoptosome. Using negative-stain EM, reconstituted mouse NAIP5/mouse NLRC4 complex showed a disk-like assembly consisting of 11 or 12 protomers, upon stimulation with a flagellin fragment of *Salmonella typhimurium* [16].

We propose that ASC-independent inflammasomes such as NAIP/NLRC4/caspase **1** also form filamentous structures through CARD/CARD interactions. In this scenario, the oligomeric NAIP/NLRC4 complex brings the CARD of NLRC4 into proximity to nucleate caspase **1** filaments and mediate caspase activation. It is likely that from each disk-like NAIP5/NLRC4 complex, multiple caspase **1** filaments may emanate, resulting in a star-shaped structure like that of ASC-dependent inflammasomes. Alternatively, if each disk-like NAIP5/NLRC4 complex can only induce the formation of one caspase **1** filament, then the overall morphology of the ternary complex may be more linear. Further structural studies are required to resolve the question.

Recent studies suggest that although ASC is not an absolute requirement for the NAIP/NLRC4 inflammasome, it can be recruited to the inflammasome and is responsible for formation of the single large punctum in cells [27]. NLRC4 has been shown to carry out a redundant role as the NLRP3 inflammasome during *Salmonella* infection by recognizing distinct bacterial

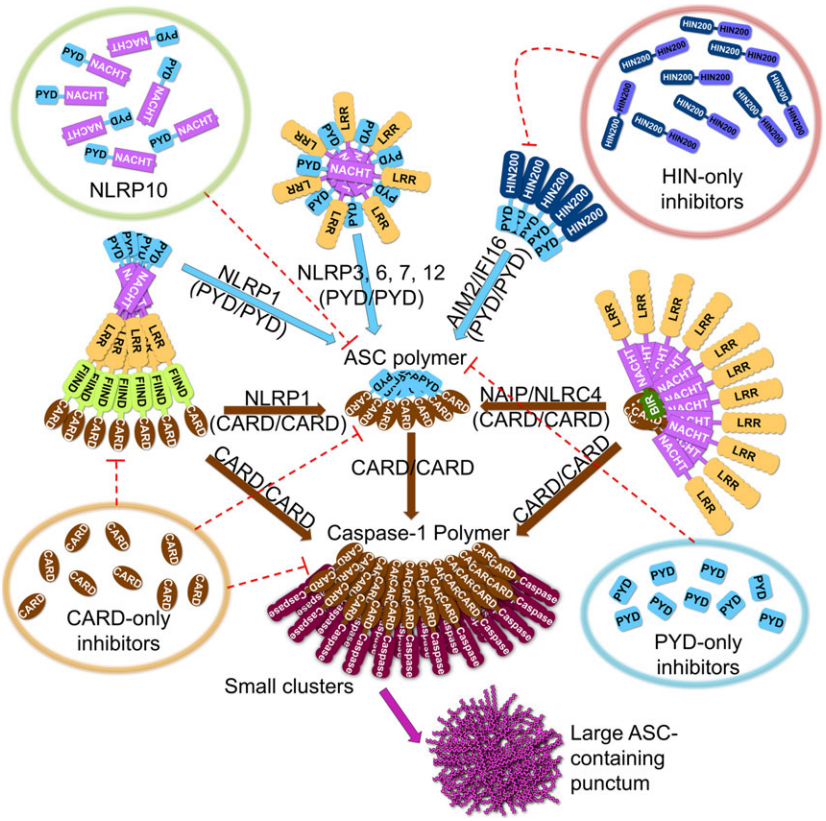

Fig. 4. Interaction architecture and potential regulation of different types of inflammasomes.

triggers [25]. Interestingly, NLRC4 is recruited together with NLRP3 to the same inflammasome punctum via ASC [27], probably through CARD/CARD co-polymerization among NLRC4, ASC and caspase **1**. The data suggest that ASC may be the key molecule that holds together individual inflammasome complexes such as the star-shaped entities seen *in vitro* to generate the single punctum in cells, via currently unknown mechanisms. Another ASC-independent inflammasome is formed by mouse NLRP1b, which lacks the N-terminal PYD necessary for ASC recruitment. Similarly, ASC is required for NLRP1b punctum formation and pyroptosis, but was dispensable for caspase **1** activation and IL-1β secretion [42]. These data suggest that pyroptosis and cytokine secretion may be distinct cellular outcomes, and formation of small, microscopic inflammasome complexes is sometimes sufficient for caspase **1** activation without causing pyroptotic cell death.

Inflammasome activators

Unlike ALRs, which are activated via dsDNA binding to their HIN200 domains, the direct activators for most NLRs, with the exception for NAIP/NLRC4 inflammasomes, remain illusive. For NLRP3, an extensively studied inflammasome, a large number of triggers has been identified, including perturbations of intracellular Ca^{2+}, K^+ and reactive oxygen species levels, extracellular ATP, and particulates such as uric acid crystals and cholesterol crystals [43–45]. However, it is unclear whether any of these stimuli directly induce NLRP3 activation. Based on the crystal structure of an auto-inhibited NLRC4 [12], which has a similar domain organization to NLRP3, any direct stimuli of NLRP3 would need to overcome this auto-inhibition to allow the NACHT domain to oligomerize. We contemplate two potential mechanisms for NLRP3 activation. First, it has been reported that K^+ efflux is a common trigger of NLRP3 activation [45]. Biochemically, the measured intracellular ionic strength changes may cause structural alterations to promote opening of the domains as well as recruitment of ASC because of the highly charged nature of the PYD/PYD interactions [22]. Second, NLRP3 may not directly sense the perturbations, but instead use interacting proteins. Cellular and animal studies have implicated DHX33 and guanylate-binding protein 5 (GBP5) as NLP3 binders [5,46]. The DEAD domain of DHX33 appears to interact with the NACHT

domain of NLRP3, which is a region responsible for NLRP3 oligomerization [46]. GBP5, by contrast, interacts with NLRP3 through its PYD domain. Using protein engineering, a tetrameric GBP5 mutant successfully stimulated NLRP3–ASC oligomerization, mostly likely through the promotion of PYD clustering [5]. With either possibility of NLRP3 activation, further studies are required to shed light on the underlying molecular mechanisms.

Inflammasome regulators

NLRs with inhibitory functions, PYD- and CARD-only regulators, and non-canonical effector caspases provide additional complexity to the assembly and morphology of inflammasomes [47] (Fig. 4). The exact mechanism of inhibition by NLRs with a proposed inhibitory role remains unclear. Pyrin and NLRP7 have been proposed as positive or negative regulators in contradictory studies [8,48–50]. NLRP10, which lacks the C-terminal leucine-rich repeats domain, was found to have an inhibitory role, probably by sequestration of activator NLRs [51–54]. PYD-only proteins including ASC2 [55] and POP2 [56], and CARD-only proteins including COP1 [57], INCA [58] and ICEBERG [57,59], may also inhibit inflammasome assembly by sequestration. These studies suggest that inflammasome with a permutation of different components and regulators may manifest in a multitude of morphology depending on the activation signal.

Prion-like properties of inflammasomes and the component proteins

Recent studies pointed to the prion-like properties of two signaling filaments, $MAVS^{CARD}$ and ASC^{PYD} [23,60]. Genetic and biochemical approaches showed that these domains are functional prion-like aggregates in yeast, even though both CARD and PYD possess the six-helix DD superfamily structural fold rather than an amyloid scaffold. Similar to our observation of nucleated filament formation, the ASC^{PYD} filament can convert inactive ASC into the active, filamentous form, as in prion-like structural conversion. The exciting finding is that in mammalian cells, ASC-containing specks of inflammasomes are released after pyroptosis into the extracellular space where they promote further IL-1β processing and are engulfed by macrophages to induce inflammasome activation in the recipient cells [61,62]. Therefore, the filamentous higher order inflammasome signaling complexes also implicate previously unexpected mechanisms of signal transduction between cells, and may be functionally important for the host immune system.

Concluding remarks

Structural information on inflammasomes has been unraveled in recent years, but there remain questions to be addressed. One challenge regards the cellular architecture of inflammasomes in intact cells, which would require super-resolution light microscopy and cellular electron microscopy imaging. Another challenge is on the activated conformation of sensor proteins or sensor/adaptor complexes such as the NAIP5/NLRC4 complex. For most inflammasomes, the direct activation ligands or signals are unknown, with the exception of AIM2 and NAIPs that are activated by intracellular dsDNA and bacterial components, respectively. Finally, the nature of the supramolecular assemblies of inflammasomes and their functional implications are only beginning to be elucidated, which might transform how we think about inflammasome biology as well as signal transductions in general.

Author contribution

Alvin Lu drafted the manuscript, and Hao Wu revised and approved the final version.

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