

# Structural Mechanisms in NLR Inflammasome Assembly and Signaling

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**Abstract** Inflammasomes are multimeric protein complexes that mediate the activation of inflammatory caspases. One central component of inflammasomes is nucleotide-binding domain (NBD)- and leucine-rich repeat (LRR)-containing proteins (NLRs) that can function as pattern recognition receptors (PRRs). In resting cells, NLR proteins exist in an auto-inhibited, monomeric, and ADP-bound state. Perception of microbial or damage-associated signals results in NLR oligomerization, thus recruiting inflammatory caspases directly or through the adaptor molecule apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). The assembled NLR inflammasomes serve as dedicated machinery to facilitate the activation of the inflammatory caspases. Here, we review current understanding of the structures of NLR inflammasomes with an emphasis on the molecular mechanisms of their assembly and activation. We also discuss implications of the self-propagation model derived from the NAIP–NLRC4 inflammasomes for the activation of other NLR inflammasomes and a potential role of the C-terminal LRR domain in the activation of an NLR protein.

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## 1 Introduction

In the evolutionary “arms race,” vertebrates from the primitive lamprey to humans have developed an arsenal of weapons conferring protection against invading pathogens, including bacteria, viruses, fungi, and parasites (Boehm et al. 2012; Flajnik and Du Pasquier 2004; Kimbrell and Beutler 2001). The first line of host defense, the innate immune system in mammals, relies on recognition of evolutionarily conserved pathogen components (pathogen-associated molecular patterns, PAMPs) or endogenous danger-associated molecular patterns (DAMPs) by an array of germline-encoded pattern recognition receptors (PRRs) (Janeway and Medzhitov 2002; Medzhitov and Janeway 1997). PRR-mediated signaling cascades get the innate immune system in gear, including production of antimicrobial proteins (Mukherjee and Hooper 2015), secretion of cytokines, and pyroptosis (Takeuchi and Akira 2010).

Thus far, several types of PRRs have been identified, including toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs), absent in melanoma 2 (AIM2)-like receptors (ALRs), and nucleotide-binding domain (NBD)- and leucine-rich repeat (LRR)-containing proteins (NLRs). Activation of some NLRs or ALRs often results in the formation of high molecular weight cytosolic protein complexes, termed inflammasomes, that serve as a platform for recruitment of the pro-inflammatory caspases (Lamkanfi and Dixit 2014; Martinon et al. 2009; von Moltke et al. 2013). Recruitment of the pro-inflammatory caspases by the complexes in many cases is through the adaptor molecule apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). Among the pro-inflammatory caspases, caspase-1 is best-characterized, and its activation by inflammasomes leads to proteolytic processing of pro-interleukin 1 $\beta$  (pro-IL-1 $\beta$ ) and pro-IL-18, and cell death.

Multiple inflammasomes have been identified, denoted by the sensor PRR protein within the inflammasome, such as AIM2 inflammasome (Burckstummer et al. 2009; Fernandes-Alnemri et al. 2009; Hornung et al. 2009; Roberts et al. 2009), NLRP1 inflammasome (Martinon et al. 2002), NLRP3 inflammasome (Agostini et al. 2004), and NAIP–NLRC4 inflammasome (Kofeod and Vance 2011; Mariathasan et al. 2004; Zamboni et al. 2006; Zhao et al. 2011). Other NLR family members including NLRP2 (Minkiewicz et al. 2013), NLRP6 (Elinav et al. 2011), NLRP7 (Khare et al. 2012), NLRP12 (Vladimer et al. 2012), and the ALR protein interferon gamma-inducible protein 16 (IFI16) (Kerur et al. 2011; Unterholzner

et al. 2010) may also assemble into inflammasomes, but more studies are needed to confirm their *in vivo* roles. More recently, a non-canonical inflammasome formed by direct recognition of cytosolic lipopolysaccharide (LPS) by caspase-11 in mice (caspase-4 and caspase-5 in human) was found to mediate pyroptotic cell death (Hagar et al. 2013; Kayagaki et al. 2013; Shi et al. 2014).

During the past few years, rapid progress has been made toward structural and biochemical understanding of inflammasomes including their activation and regulation mechanisms. Several excellent reviews have summarized structural studies of the ALR inflammasomes (Shaw and Liu 2014; Xiao 2015). In this review, we focus on the recent structural elucidations of NLR inflammasome assembly and signaling.

## 2 Overviews of NOD-Like Receptors

NLR family members are typically characteristic of a tripartite structure comprising a varied N-terminal effector domain, a central nucleotide-binding and oligomerization domain (NOD), and a C-terminal LRR domain. Depending on the N-terminal effector domain, NLR family can be further divided into several subfamilies, such as NLRC with an N-terminal caspase recruitment domain (CARD), NLRP with an N-terminal pyrin domain (PYD), and NAIP with three tandem N-terminal baculovirus inhibitor of apoptosis (BIR) protein repeat domains. The NOD module, which can be further divided into an NBD and a helical domain 1 (HD1) followed by a winged helical domain (WHD), is an ADP-/ATP-binding motif conserved in the signal transduction ATPases with numerous domains (STAND) subfamily, including the apoptotic protein apoptotic peptidase-activating factor-1 (Apaf-1) in mammals (Danot et al. 2009). Similar tripartite structural organization is also present in the plant disease resistance proteins (NLR-type receptors, also known as R proteins) (Maekawa et al. 2011; Jones and Dangl 2006). There are 22 NLR family members in humans and at least 34 in mice. In unchallenged cells, NLR proteins are maintained inactive through their C-terminal LRR domains, as several LRR-truncated NLRs were constitutively active in inducing downstream signaling (Hu et al. 2013; Kofoed and Vance 2011; Poyet et al. 2001). Consistently, unchecked inflammasome activation has been shown to associate with many severe auto-inflammatory diseases (Davis et al. 2011; Lamkanfi and Dixit 2012; Strowig et al. 2012; Wen et al. 2012). Activation of an NLR protein is believed to accompany with the exchange of ADP for ATP (Danot et al. 2009; Duncan et al. 2007).

NAIP–NLRC4, NLRP1, and NLRP3 are subsets of the NLR family which are best understood for their ability to assemble into inflammasomes. The NAIP–NLRC4 inflammasome plays a critical role in host defense against facultative intracellular pathogens such as *Salmonella typhimurium*, *Shigella flexneri*, and *Legionella pneumophila* (Lamkanfi and Dixit 2012). NAIPs with seven homologs (Naip1–7) in mice and one in humans are responsible for the direct recognition of PAMPs from these pathogens. In mice, Naip5 and Naip6 act as an intracellular

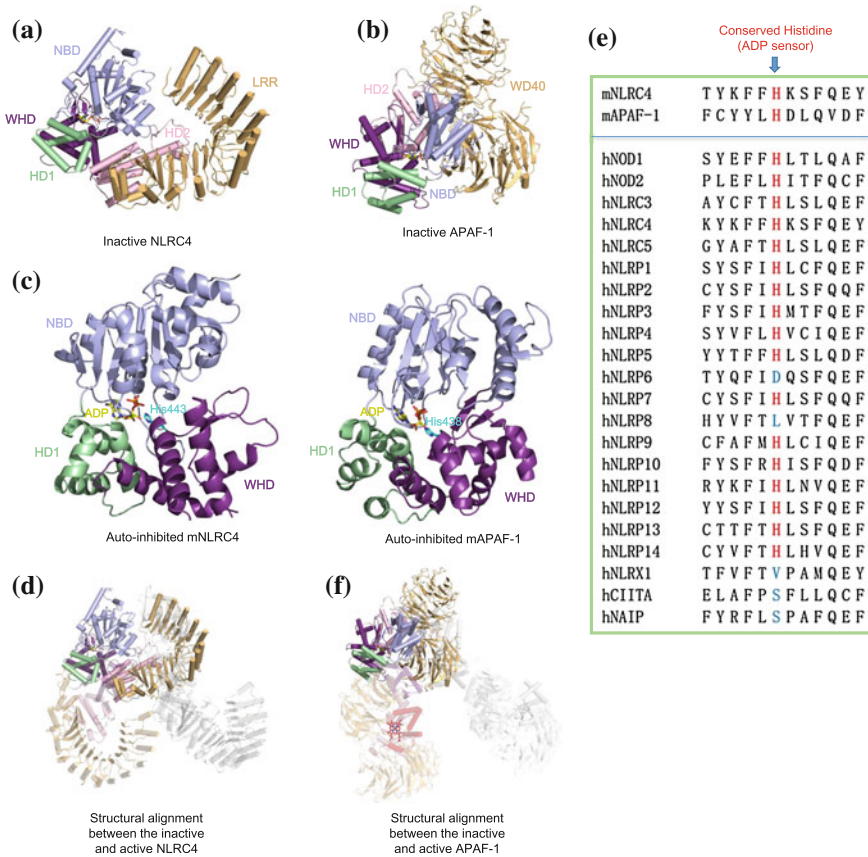
sensor for bacterial flagellin, whereas Naip1 and Naip2 recognize the needle protein and the rod protein from the type III secretion system (T3SS) of bacteria, respectively (Franchi et al. 2006; Kofoed and Vance 2011; Lightfield et al. 2008; Miao et al. 2006, 2010; Yang et al. 2013; Zhao et al. 2011). Upon recognition by their cognate NAIP proteins, these bacterial components stimulate NAIP–NLRC4 association, resulting in assembly of the NAIP–NLRC4 inflammasomes (Halff et al. 2012; Kofoed and Vance 2011; Zhao et al. 2011).

NLRP1 is the first PRR shown to form inflammasomes (Martinon et al. 2002), though the physiological activation signals were initially unknown. Humans have a single *NLRP1* gene, while mice have three paralogs (*Nlrp1a*, *Nlrp1b*, and *Nlrp1c*) exhibiting high allelic variations. The *Nlrp1b* inflammasome is a major component of host defense against *Bacillus anthracis*, mediating inflammatory response to the anthrax lethal toxin (LeTx) (Boyden and Dietrich 2006; Moayeri et al. 2010; Terra et al. 2010). LeTx is a two-component toxin composed of protective antigen (PA) and lethal factor (LF). LF is a metalloprotease, and its activity is required for the activation of *Nlrp1b* inflammasome by cleaving a position at the N-terminal side of a responsive rat *Nlrp1b* allele (Chavarria-Smith and Vance 2013; Fink et al. 2008; Hellmich et al. 2012; Levinsohn et al. 2012).

The NLRP3 inflammasome can be activated by many stimuli, including components from bacterial, viral, and fungal pathogens (Dostert et al. 2008; Eisenbarth et al. 2008; Ichinohe et al. 2009; Joly and Sutterwala 2010; Kanneganti et al. 2006; Mariathasan et al. 2006; Martinon et al. 2006). It is widely accepted that the activation of NLRP3 inflammasome requires two agonist-induced signals with one priming NF- $\kappa$ B-mediated expression of NLRP3 and inflammatory cytokines (Bauernfeind et al. 2009), and the other one triggering the activation and assembly of NLRP3 inflammasome. Given the large diversity of the agonists, it is believed that all these agonists may cause some converged effects in hosts monitored by the NLRP3 inflammasome (Lamkanfi and Dixit 2014; Tschopp and Schroder 2010). But recent studies appear to support potassium efflux as a common step essential for NLRP3 inflammasome activation induced by many stimuli (He et al. 2016; Munoz-Planillo et al. 2013; Petrilli et al. 2007).

### 3 Auto-Inhibition Mechanism of NLRC4

Structural studies of mouse *Nlrc4* in its active and inactive forms provide insight into the autoinhibition mechanism of NLR family protein (Hu et al. 2013, 2015; Zhang et al. 2015). As observed in other STAND members (Danot et al. 2009), the auto-inhibited *Nlrc4* (Hu et al. 2013) is monomeric and ADP-bound (Fig. 1a). The structure of *Nlrc4*<sup>NOD</sup> largely resembles that of Apaf-1<sup>NOD</sup> (Fig. 1b, c) (Chai and Shi 2013; Reubold et al. 2011; Riedl et al. 2005). All the ADP-interacting residues of *Nlrc4* are from the conserved NBD and HD1 except *Nlrc4*<sup>H443</sup> from the WHD that forms a single hydrogen bond with the  $\beta$ -phosphate group of the bound ADP (Fig. 1c). Structural comparison between the inactive (crystal structure) and active



**Fig. 1** Auto-inhibition mechanism of NLRC4. **a** The overall structure of auto-inhibited Nlrc4<sup>ΔCARD</sup> (PDB code: 4KXF) shown in cartoon. Color codes for domains are indicated. **b** The overall structure of auto-inhibited Apaf-1 (PDB code: 3SFZ) shown in cartoon. Color codes for domains are indicated. The NBD domains of Nlrc4 and Apaf-1 are shown in the same orientation in **a** and **b**. **c** Histidine from the WHD of Nlrc4 and Apaf-1 interact with the bound ADP in the auto-inhibited state. The bound ADP molecules are shown in yellow and stick, and the side chains of the histidine are shown in cyan and stick. **d** Structural alignment between the inactive Nlrc4 and active Nlrc4. The NBD of auto-inhibited Nlrc4 (in cartoon) is aligned with that of one active Nlrc4 protomer (in transparent cartoon) from a lateral dimer (EMDB code: EMD-3141). The other protomer is shown in gray and transparent cartoon. **e** Most NLR proteins have a conserved histidine residue (highlighted in red) as the ADP sensor. **f** Structural alignment between the inactive Apaf-1 and active Apaf-1. The NBD of auto-inhibited Apaf-1 (in cartoon) is aligned with that of one active Apaf-1 protomer (in transparent cartoon) from a lateral dimer (PDB code: 3JBT)

(cryo-EM) Nlrc4 (Hu et al. 2015; Zhang et al. 2015) showed that its WHD, HD2, and LRR as a whole undergo striking structural re-organization relative to the NBD and HD1 on activation (Fig. 1d). This observation indicates that the hydrogen bond formed between Nlrc4<sup>H443</sup> and ADP is specific for the inactive Nlrc4. Disruption of the specific interaction is expected to favor conformational changes in the WHD.

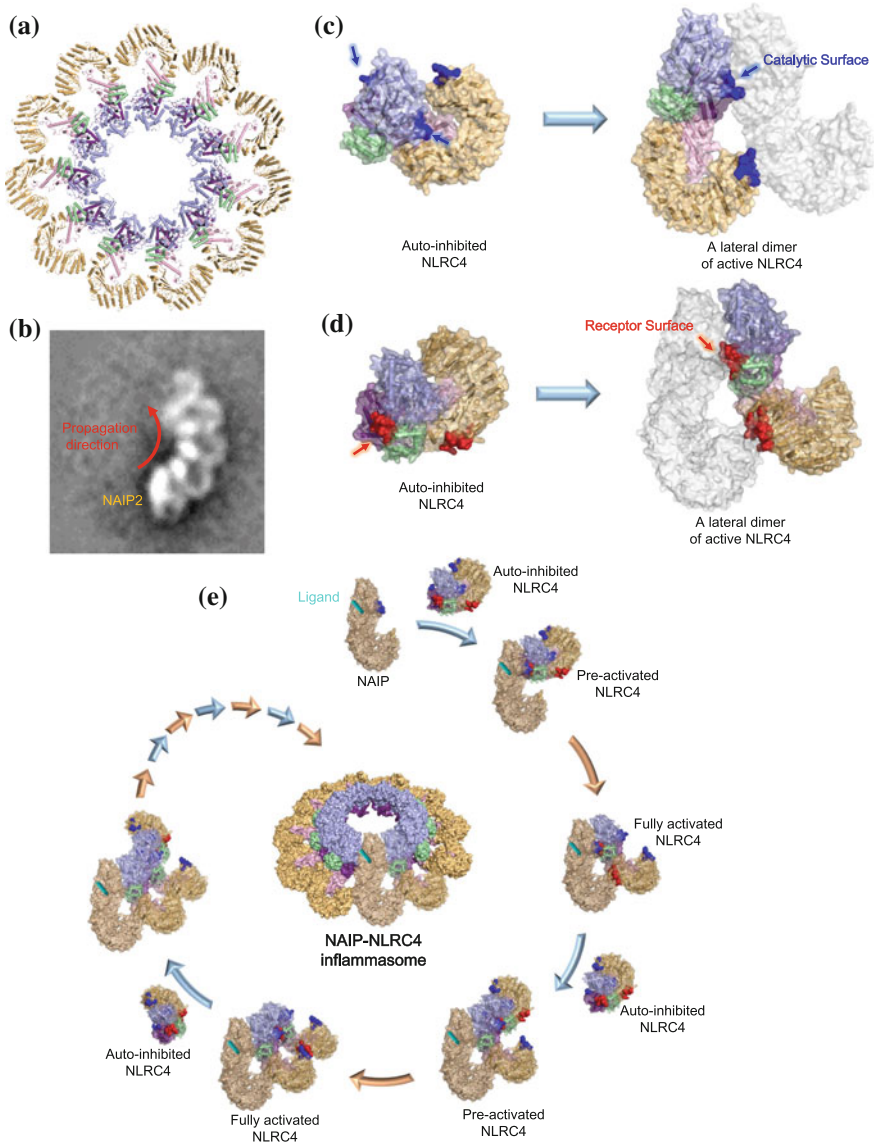
On the other hand, attenuation of ADP binding caused by loss of the hydrogen bond would promote the ATP-binding activity of Nlrc4 because of a stable ratio between ADP and ATP in cells. Both of the effects would facilitate the activation of Nlrc4. Indeed, the Nlrc4<sup>H443L</sup> mutation led to ligand- and Naip-independent activation of Nlrc4 in 293T cells (Hu et al. 2013). In strong support of a role played by the single hydrogen bond in Nlrc4 autoinhibition, the NLRC4<sup>H443P</sup> mutation was recently identified in patients with the familial cold auto-inflammatory syndrome (FCAS) characterized by the constitutive activation of the NAIP–NLRC4 inflammasomes (Kitamura et al. 2014). Furthermore, mice expressing this Nlrc4 mutant also developed dermatitis and arthritis caused by excessive IL-1 $\beta$ -mediated production of IL-17A from neutrophils (Kitamura et al. 2014). Nlrc4<sup>H443</sup> is highly conserved in most of the NLRs from both mammals (Fig. 1e) and plants, suggesting a conserved role played by the conserved histidine in the autoinhibition of NLR proteins. Consistent with this idea, mutations of the “MHD” motif (“H” is equivalent to Nlrc4<sup>H443</sup>) in plant NLRs also resulted in their constitutive activation (Takken et al. 2006; van der Biezen and Jones 1998; Williams et al. 2011). In fact, the histidine is also conserved in the apoptotic protein Apaf-1 and forms a hydrogen bond with the bound ADP (Fig. 1c). But further studies are needed to test whether this residue is important for Apaf-1 autoinhibition.

In the cryo-EM structures (Hu et al. 2015; Zhang et al. 2015), lateral dimerization of Nlrc4 is largely mediated by packing of one side of the NBD from one protomer against the opposite side of the NBD from the other protomer (Fig. 1d), as observed in the Apaf-1 apoptosome (Fig. 1f) (Zhou et al. 2015). Structural superposition of one protomer from the lateral dimer with the inactive Nlrc4 showed that the LRR domain of an inactive Nlrc4 occupies the position of the other protomer (Fig. 1d). This observation indicates the LRR domain keeps Nlrc4 in inactive by sequestering it in a monomeric state. Interestingly, the two C-terminal WD40 domains of the inactive Apaf-1 also sterically occlude the monomeric Apaf-1 from oligomerization (Fig. 1f). HD2, also known as the arm domain, exists in all the mammalian NLRs and some other STAND proteins. Packing against one side of NBD in the closed form of Nlrc4 (Fig. 1a), HD2 is positioned to completely overlap with WHD in the active form of Nlrc4 that interacts with the HD1 from an adjacent protomer (Fig. 1d). Thus, HD2 exerts its inhibitory effects by steric blockage of both the WHD-HD1 and NBD-NBD interfaces in the oligomeric Nlrc4. In summary, several domain–domain interactions that may act cooperatively or even synergistically are involved in Nlrc4 autoinhibition. Given the striking effect generated by the Nlrc4<sup>H443L</sup> or Nlrc4<sup>H443P</sup> mutation (Hu et al. 2013; Kitamura et al. 2014), perturbation of the hydrogen formed between this residue and ADP could be a critical step to initiate Nlrc4 activation.

## 4 Mechanism of NAIP–NLRC4 Inflammasome Activation and Assembly

Two recent cryo-EM studies by our and Wu's groups provide the first glimpse of the mechanism underlying NAIP–NLRC4 inflammasome activation and assembly (Hu et al. 2015; Zhang et al. 2015). Biochemical studies from both groups showed that the Naip2–Nlrc4 complex induced by PrgJ (a rod protein of type III secretion system from *Salmonella typhimurium*) was substoichiometric with a dominating amount of Nlrc4. Further study using NTA-nanogold labeling EM indicated that only one Naip2 molecule was incorporated into the complex. In the cryo-EM structures, the complex formed a wheel-like architecture containing 10 or 11 protomers (Fig. 2a), reminiscent of the structure of Apaf-1 apoptosome (Yuan and Akey 2013; Zhou et al. 2015). Thus, the stoichiometry between Naip2 and Nlrc4 in the complex is 1:9 or 1:10. In addition to the wheel-like structures, the complex containing Naip2/5 and the CARD-deleted Nlrc4 (Nlrc4<sup>ΔCARD</sup>) also formed unclosed and twisted structures with variable protomers (Fig. 2b). For each type of the partially oligomerized particles, 2D class averages showed that the protomer at one end differed from the remaining copies, indicating that it corresponds to Naip2. This observation further supports the data from the nanogold labeling study and indicates an unidirectional propagation process for the assembly of the wheel-like structures.

Capturing of the partial oligomers starting with Naip2 suggests that Nlrc4 is capable of self-activation for assembly of the wheel-like structures. This conclusion is further supported by structural and biochemical data (Hu et al. 2015; Zhang et al. 2015). As discussed above, one oligomerization surface of Nlrc4 is completely blocked by the C-terminal LRR domain (Fig. 1d). This oligomerization surface (catalytic surface; Fig. 2c) is therefore activation-created because it can be formed only after structural re-organization during the Nlrc4 activation. In contrast, the other oligomerization surface is largely solvent-exposed and pre-exists in the inactive Nlrc4 (Fig. 2d). The two oligomerization surfaces are clearly discernible in the partially oligomerized structures with Naip2 contacting the receptor surface of Nlrc4 (Fig. 2b). These data led to a model of Nlrc4 self-activation in which Nlrc4, once activated by Naip2/5, unmarks its catalytic surface to interact with the solvent-exposed receptor surface of an inactive Nlrc4, consequently activating its. This would result in self-propagation of the active conformation of Nlrc4 and eventual assembly of the wheel-like structures (Fig. 2e). This model predicts that an Nlrc4 mutant with an impaired catalytic surface would not be able to self-activate, but can still be activated by a Naip protein because of its intact receptor surface. In strong support of this conclusion, the Nlrc4<sup>R288A</sup> mutation located at the catalytic surface abolished Nlrc4-mediated caspase-1 activation, but the mutant protein still formed a flagellin-induced heterodimer with Naip5 (Hu et al. 2015). Furthermore, the mutation terminated oligomerization of wild-type Nlrc4 induced by flagellin and Naip5, forming partial oligomers as observed with the PrgJ–Naip2–Nlrc4<sup>ΔCARD</sup> complex (Hu et al. 2015). Further evidence for the model comes from the



observation that the constitutively active Nlrc4<sup>H443L</sup> mutant activated the wild-type Nlrc4 independent of Naip2/5 (Hu et al. 2015).

The self-activation model explains why only one Naip molecule is incorporated into the Naip–Nlrc4 inflammasomes. A Naip protein is needed to activate Nlrc4, exposing the catalytic receptor of Nlrc4 to initiate self-activation. Both the wheel-like and unclosed structures demonstrated that Naip2 occupies an equivalent position to Nlrc4, suggesting that Naip2 can use a similar mechanism to that of



◀ **Fig. 2** Assembly of NAIP–NLRC4 inflammasome through self-propagation. **a** The overall structure of oligomerized Nlrc4<sup>ACARD</sup> (EMDB code: EMD-3141) shown in cartoon. Structural domains are labeled in the same color as indicated in Fig. 1a. **b** A representative 2D class average image of the partially oligomerized PrgJ–Naip2–Nlrc4<sup>ACARD</sup> complex using negative-stain EM. The protomer at one end corresponding to Naip2 is indicated. The *red arrow* indicates the propagation of the partial oligomerized complex. **c** Structural remodeling creates the catalytic surface for oligomerization during the activation of Nlrc4. Structures are shown in surface, and the catalytic surface is shown in *blue* and indicated by *arrows*. The region involved in the LRR–LRR interaction is also shown in *blue*. **d** The receptor surface is largely solvent-exposed and pre-exists in the inactive Nlrc4. Structures are shown in surface, and the receptor surface is shown in *red* and indicated by *arrows*. The region involved in the LRR–LRR interaction is also shown in *red*. **e** A schematic diagram for the ligand-induced and self-propagated activation mechanism of NAIP–NLRC4 inflammasome

Nlrc4 self-activation for the activation of Nlrc4. Indeed, the residues from the catalytic surface of Nlrc4 are highly conserved among all the Naip members (Hu et al. 2015; Zhang et al. 2015). Mutations of the critical residues from the catalytic surface of Naip5 abrogated flagellin-induced interaction with Nlrc4, contrasting with Nlrc4<sup>R288A</sup> from the catalytic surface of Nlrc4. In contrast, those from the receptor surface of Nlrc4 are highly variable in the Naip proteins. Thus, the receptor surface from a Naip protein does not match the catalytic surface of Nlrc4 or its own. This ensures that Naip members can neither self-oligomerize nor be further recruited into an existing Naip–Nlrc4 complex by an activated Nlrc4, resulting in incorporation of only one Naip molecule into one Naip–Nlrc4 inflammasome.

## 5 A Positive Role of the C-Terminal LRR Domain in the Activation of NAIP–NLRC4 Inflammasomes?

As mentioned above, the receptor surface of a Naip protein does not match the catalytic surface of Nlrc4. Then an ensuing question is how the Naip–Nlrc4 inflammasomes are closed to form wheel-like structures? The underlying mechanism remains unclear, but the Nlrc4<sup>CARD</sup> clearly has an important role in this process, because its deletion resulted in the formation of unclosed oligomers that were not found with the full-length Nlrc4 (Hu et al. 2015; Zhang et al. 2015). The Nlrc4<sup>CARD</sup> contributes to the closure of the inflammasomes likely through the formation of a circular structure formed in the hub of the wheel-like structure (Hu et al. 2015). Additionally, the ligands of a Naip could also have a role in the closure of the Naip–Nlrc4 inflammasomes. Clearly, higher-resolution structures of Naip–Nlrc4 inflammasomes are expected to address this question.

Available structural, biochemical, and cell-based data strongly support the notion that the C-terminal LRR domain is important to keep an NLR autoinhibited. Unexpectedly, however, the Nlrc4<sup>LRR</sup> and Naip2<sup>LRR</sup> also contribute to the assembly of Naip–Nlrc4 inflammasomes through electrostatic complementarity between two consecutive LRRs (Fig. 2c, d). This is in contrast to the WD40

domains of Apaf-1 in the formation of the apoptosome (Fig. 1f). It currently remains unknown whether the LRR–LRR interaction has a role in the full activation of the Naip–Nlrc4 inflammasomes. But the NOD module, while important to mediate Nlrc4 oligomerization, is not sufficient for assembly of the wheel-like structures of Naip–Nlrc4 inflammasomes as evidenced by the data from Nlrc4<sup>CARD</sup> deletion (Hu et al. 2015; Zhang et al. 2015). Structurally, other domains, if presented to a proper position, could serve a similar role to an LRR domain in sequestering an NLR protein in a monomeric state, as only marginal interactions are formed between Nlrc4<sup>LRR</sup> and Nlrc4<sup>NBD</sup> in an inactive Nlrc4 (Fig. 1a). Mechanistically, the horse-shoed LRR domain appears optimal to act as a structural fold for closure of the inflammasomes because of its curvature. In agreement with this possibility, a more recent study showed that fusion of a 76-kDa protein to the C-terminus of Naip5 resulted in the formation of a helical structure of the flagellin–Naip5–Nlrc4 complex (Diebold et al. 2015). Thus, it appears that the Naip<sup>LRR</sup> and Nlrc4<sup>LRR</sup> might have a dual role in the full activation of the Naip–Nlrc4 inflammasomes. Interestingly, a similar role has been proposed for the PYD of AIM2, which can exert its auto-inhibitory effect in the resting state of AIM2 and also stabilize the dsDNA-binding conformation of AIM2 via intermolecular PYD–PYD interaction upon activation (Xiao 2015).

A potential role of the Nlrc4<sup>LRR</sup> in inflammasome activation is not necessarily contradictory with the observation that its deletion led to constitutively active Nlrc4 in caspase-1 activation (Hu et al. 2013; Kofoed and Vance 2011; Poyet et al. 2001). One plausible reason for this may be that the Nlrc4 mutant is only partially active because some of the mutant protein is unable to assemble into wheel-like structures required for caspase-1 activation. Quantitative assays of the caspase-1 activating activity of wild-type Nlrc4 and the mutant would be useful to test this possibility. Alternatively or additionally, it is also conceivable that unclosed oligomers formed by the Nlrc4 mutant can still bring caspase-1 molecules into proximity for activation. This is particularly possible in the presence of ASC, because overexpression of the Nlrc4<sup>CARD</sup> alone induced ASC into filaments to activate caspase-1 (Cai et al. 2014; Zhang et al. 2015). Whether this is associated with ASC-promoted caspase-1 activation by the Naip–Nlrc4 inflammasomes (Broz et al. 2010) remains unknown. But many interesting questions could be raised by this possibility. For example, what are the functional differences between the closed and unclosed Naip–Nlrc4 inflammasomes? Is it a possible way of regulating the activity of Naip–Nlrc4 inflammasomes *in vivo*?

## 6 Activation of Other NLR Inflammasomes

A number of single gain-of-function mutations in Nlrc4 including the disease-related ones have been identified (Canna et al. 2014; Kitamura et al. 2014; Romberg et al. 2014). All these mutations were mapped to destabilize the inactive conformation of Nlrc4, resulting in the activation of caspase-1. The

self-propagation property of Nlrc4 suggests that a small population of fully activated Nlrc4 molecules resulting from the mutations can induce assembly of immunoactive Nlrc4 homo-oligomers by activating the mutation-destabilized inactive Nlrc4. A similar mechanism in principle could be utilized by the gain-of-function and disease-associated mutations for the activation of NLRP3 inflammasome (Schroder and Tschopp 2010). Consistently, modeling studies suggested that some of the NLRP3 mutations directly lead to relief of autoinhibition and constitutive activation of NLRP3 (Albrecht et al. 2003; Chai and Shi 2013). However, further studies are required to determine whether NLRP3 or other immune NLR proteins also possesses self-propagation activity. But such an activity would endow them with high sensitivity to PAMPs or DAMPs and efficiency for signaling, allowing quick response of hosts to invading pathogens. The NOD module should be responsible for the self-propagation activity of Nlrc4, as the Nlrc4<sup>ΔCARD</sup> mutant still formed Naip-induced oligomers with multiple Nlrc4 s. Given the highly conserved NOD among NLRs, it appears reasonable to assume that other NLRs may also have a similar activity to Nlrc4. Naips and Apaf-1, however, are known not to be the case. The two buried oligomerization surfaces in an inactive Apaf-1 rule out the possibility of self-activation. No structural information is available for a Naip protein yet. But it is expected to follow the conserved oligomerization mode of the STAND proteins, in which one side of the NBD from the one protomer stacking against the opposite side of the NBD from the other protomer in a lateral dimer. Structure-based sequence alignment showed that the two oligomerization surfaces of a Naip protein do not match each other for homo-oligomerization, thus precluding it from homo-oligomerization.

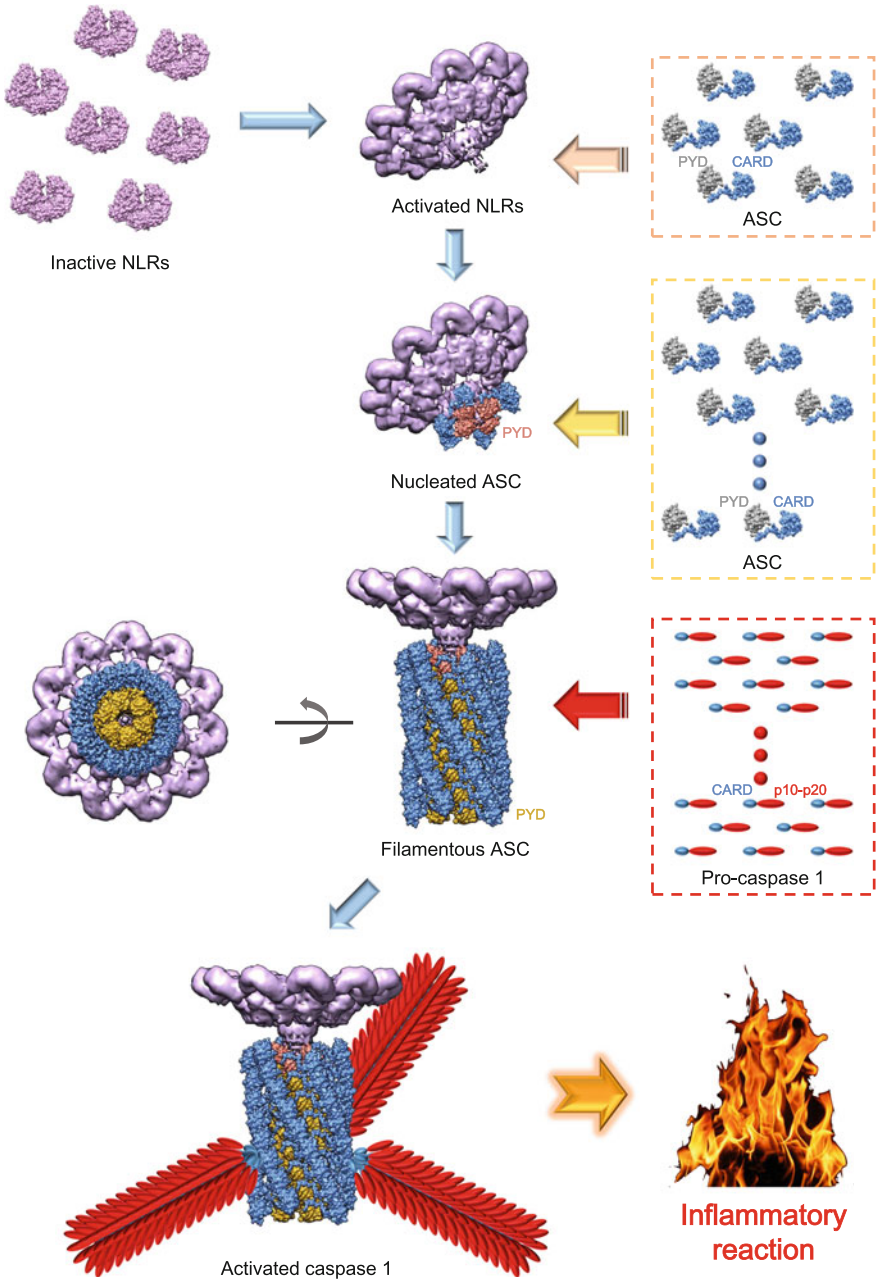
The self-propagation activity of Nlrc4 is induced by a Naip protein. However, other inflammasomes containing two different NLRs have not been biochemically demonstrated. Thus, for an NLR to self-propagate, either an as-yet identified paired NLR(s) similar to Naips is needed or the single NLR can act as both the sensor and the self-propagating protomer. To probe the former possibility, sequence alignment could provide useful information, as homology between Naips and Nlrc4 is important for their heterodimerization. Some tangent evidence from the studies of Nlrp1b activation seems consistent with the latter possibility. Data from several studies indicated that mouse and rat Nlrp1b sense the protease activity of lethal toxin by acting as substrates to activate the Nlrp1b inflammasome (Chavarria-Smith and Vance 2013; Hellmich et al. 2012; Levinsohn et al. 2012). Cleavage of Nlrp1b by LeTx is required for caspase-1 activation in non-macrophage cells, and a strict correlation between cleavage sensitivity and the activation of Nlrp1b inflammasome has been observed (Chavarria-Smith and Vance 2013). However, only a very small fraction of Nlrp1b was cleaved within 90 min, and at this time point, mouse macrophages showed robust immune responses (Boyden and Dietrich 2006; Chavarria-Smith and Vance 2013; Muehlbauer et al. 2007). While several mechanisms can be formulated to explain these results, self-activation would be the most straightforward one.

## 7 Insights into the Assembly of ASC-Dependent Inflammasomes

ASC as a core component of inflammasomes contains an N-terminal PYD and C-terminal CARD (de Alba 2009). The ASC<sup>PYD</sup> can interact with those from almost all the PYD-containing inflammasomes, and the ASC<sup>CARD</sup> can recruit the downstream pro-caspase-1 through homotypic CARD–CARD interactions (Poyet et al. 2001). In the CARD-containing inflammasomes, such as the Naip–Nlr4 and NLRP1b inflammasomes, the CARDS from them are believed to mediate the direct recruitment of pro-caspase-1 for activation. In the absence of ASC, the Naip–Nlr4 and NLRP1b inflammasomes induce caspase-1-mediated pyroptosis, but interestingly fail to induce caspase-1 auto-proteolysis (Broz et al. 2010; Van Opdenbosch et al. 2014). In the presence of ASC, Nlr4-mediated caspase-1 proteolytic processing is significantly promoted (Broz et al. 2010).

Recent studies provide significant insight into the mechanism underlying assembly of ASC-dependent inflammasomes (Fig. 3) (Cai et al. 2014; Lu et al. 2014; Sborgi et al. 2015). The upstream sensors NLRs or ALRs form a high-order oligomer upon the recognition of their ligands, leading to the clustering of their PYDs or CARDS (Hu et al. 2015; Jin et al. 2012; Morrone et al. 2015; Zhang et al. 2015). The clustered PYDs or CARDS can serve as a platform to recruit the adaptor ASC via PYD–PYD or CARD–CARD interactions and nucleate the self-propagated filament formation of monomeric ASC<sup>PYD</sup>. Structural studies of the ASC<sup>PYD</sup> filament showed a cylinder-like structure with the ASC<sup>PYD</sup> packing densely in a spiral (Lu et al. 2014; Sborgi et al. 2015). The ASC<sup>CARD</sup>, which localizes at the outer layer of the ASC<sup>PYD</sup> filament, forms another platform to nucleate the formation of caspase-1 filaments through CARD–CARD interactions (Lu et al. 2014). The highly polymerized caspase-1 could further lead to the proximity-induced caspase-1 auto-processing, resulting in the full activation of ASC-dependent inflammasomes. Consistently, the reconstituted AIM2<sup>PYD</sup>–ASC–caspase-1 ternary complex showed a one-by-one increased stoichiometry, with AIM2<sup>PYD</sup> under stoichiometric to ASC and ASC under stoichiometric to caspase-1 (Lu et al. 2014). EM studies of the AIM2–ASC complex revealed star-shaped structures in which AIM2<sup>PYD</sup> and ASC form the central hub, whereas caspase-1 forms multiple filaments extended radially from the center (Lu et al. 2014). These results support the observations ASC formed micron-sized punctum or specks associated with upstream sensors and downstream caspases upon inflammasome activation (Masumoto et al. 1999) and a rapid “all-or-none” response generated by ASC oligomerization.

**Fig. 3** Schematic diagram for the assembly of ASC-dependent inflammasomes. ▶ Upstream-sensing proteins (NLRs or AIM2) oligomerize upon activation, forming a platform to recruit the adaptor protein ASC. The nucleated ASC can promote the ASC filament formation through a self-propagated manner, leading to the clustering of the CARD of ASC, which can further promote caspase-1 filament formation and proximity-induced activation



## 8 Concluding Remarks

Although significant progress has been made toward inflammasomes during the past few years, our understanding on their assembly and activation is far from being complete. At present, only a few inflammasomes have been successfully purified or reconstituted using homogeneous recombinant proteins, which is an important step in understanding the structural and functional mechanism of inflammasomes. Ligand-induced oligomerization of an NLR is widely believed to be an important step for the formation of NLR inflammasomes. Future structural studies are required to investigate whether a wheel-like structure is also formed in other NLR inflammasomes. A few NLRs have been shown to directly recognize their cognate ligands, but the underlying structural mechanisms are largely unknown. A recent study demonstrated that the structural determinants for the recognition of bacterial PAMPs by Naips lie in the central NOD module rather than the C-terminal LRR domain as previously hypothesized (Tenthorey et al. 2014). Whether this holds true with ligand recognition by other NLRs is unknown. Moreover, the possibility still remains that the LRR also has a role in ligand recognition by Naips. Recent studies showed that a member of NIMA-related kinases (NEK7) interacts with NLRP3 and acts as an essential component downstream of potassium efflux for NLRP3 inflammasome assembly and activation, which interestingly do not require the kinase activity of NEK7 (He et al. 2016; Schmid-Burgk et al. 2016; Shi et al. 2015). How the interaction with NEK7 activates NLRP3 remains unknown. ATP binding was shown to be important for the activation of several NLRs, such as NLRP3 (Duncan et al. 2007), human NLRP1 (Faustin et al. 2007), and NOD1/2 (Strober et al. 2006), but mutation of the ATP-binding motif in mouse *Nlrp1b* resulted in constitutive activation of the *Nlrp1b* inflammasome (Liao and Mogridge 2013). Therefore, the differential roles of ATP in the assembly of NLR inflammasomes still remain to be further scrutinized.

Formation of filamentous structures by ASC is emerging as a general mechanism for the assembly and signal transduction of the ASC-dependent inflammasomes (Kagan et al. 2014; Lu and Wu 2015). The PYDs of an oligomerized NLR or ALR should function to seed the filaments, but the underlying structural mechanism is currently unclear. Given the high diversity of the ASC-dependent inflammasomes, it is hard to imagine that PYDs form a conserved structure in the oligomerized NLRs or ALRs for the seeding activity. Another challenge is that how ASC filaments activate caspase-1. Caspase-1 recruitment by the ASC filaments through the homotypic CARD–CARD interaction presumably brings caspase-1 molecules into close proximity for activation. But the model does not reveal the molecular mechanism underlying caspase-1 activation. The pro-inflammatory caspases, mouse caspase-11 and human caspase-4/5, directly recognize cytoplasmic LPS, forming non-canonical inflammasomes (Shi et al. 2014). Structural studies in conjunction with other biophysical approaches are needed to elucidate how these caspases recognize LPS and consequently become activated. In contrast to other NLRs, activated NOD1/2 recruits receptor-interacting serine/threonine protein kinase 2

(RIPK2) through homotypic CARD–CARD interactions, resulting in close proximity and activation of RIPK2–I $\kappa$ B kinase (IKK) (Caruso et al. 2014; Strober et al. 2006). Future studies are required to determine whether these two NLRs use similar principles to other NLR for kinase activation.

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