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Structural and mechanistic elucidation of inflammasome signaling by cryo-EM

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The innate immune system forms an evolutionarily ancient line of defense against invading pathogens and endogenous danger signals. Within certain cells of innate immunity, including epithelial cells and macrophages, intricate molecular machineries named inflammasomes sense a wide array of stimuli to mount inflammatory responses. Dysregulation in inflammasome signaling leads to a wide range of immune disorders such as gout, Crohn's disease, and sepsis. Recent technological advances in cryo-electron microscopy (cryo-EM) have enabled the structural determination of several key signaling molecules in inflammasome pathways, from which macromolecular assembly emerges as a common mechanistic theme. Through the assembly of helical filaments, symmetric disks, and transmembrane pores, inflammasome pathways employ highly dynamic yet ordered processes to relay and amplify signals. These unprecedentedly detailed views of inflammasome signaling not only revolutionize our understanding of inflammation, but also pave the way for the development of therapeutics against inflammatory diseases.

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Introduction

Innate immunity is mediated by a series of germline-encoded pattern recognition receptors (PRRs) that respond to pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs). The already identified PRR families include transmembrane proteins such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as cytosolic proteins such as RIG-I like receptors (RLRs), AIM2-like receptors (ALRs), nucleotide-binding domain (NBD) and leucine-rich repeat-containing (LRR) proteins (NLRs), and cyclic GMP-AMP synthase (cGAS) [1–3].

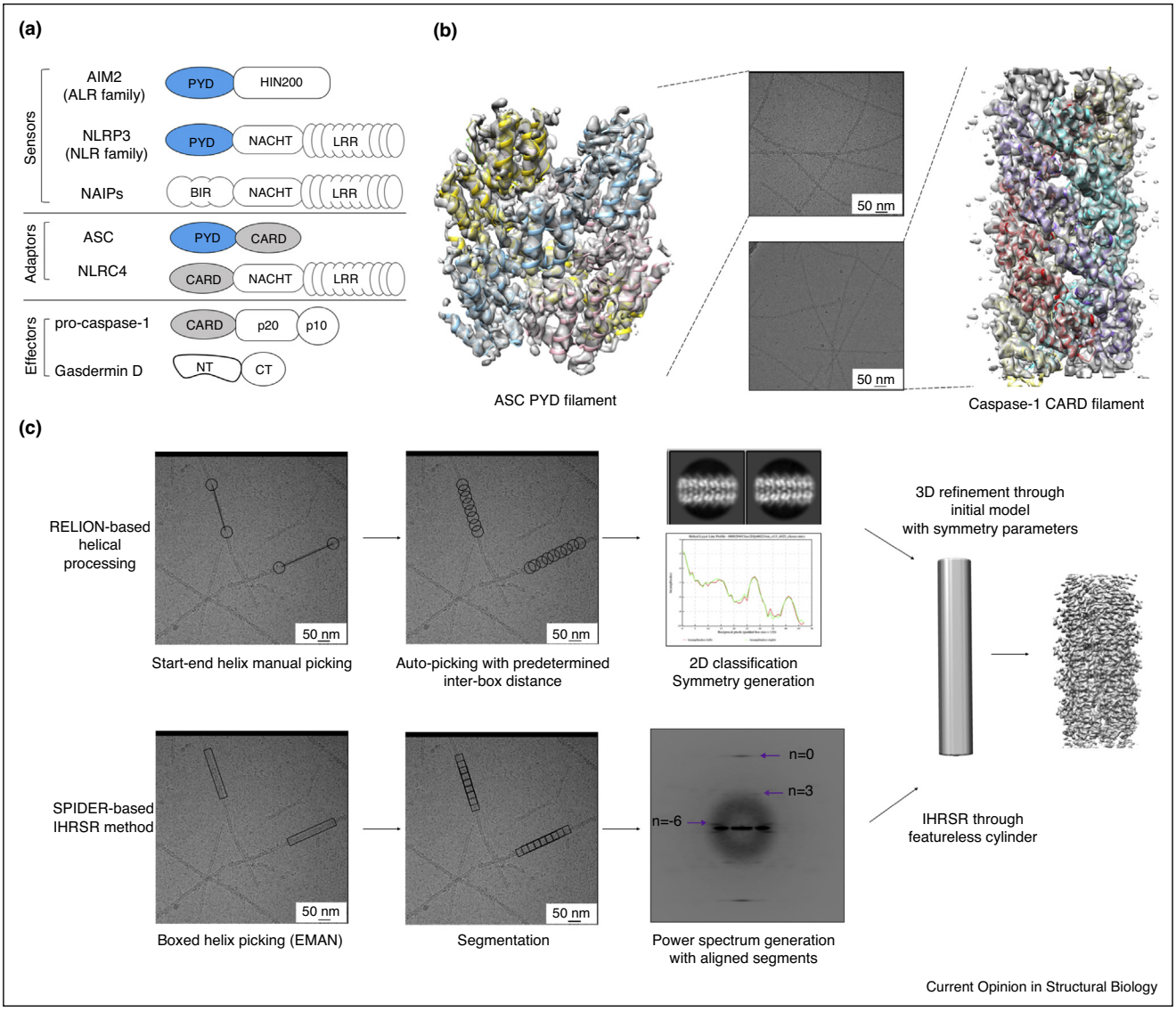
When triggered by PAMPs and DAMPs, many PRRs stimulate the downstream transcription of inflammation-related genes encoding pro-inflammatory cytokines, interferons, and antimicrobial proteins. By contrast, certain NLRs and ALRs induce inflammatory signaling through the assembly of cytosolic multimeric protein complexes known as canonical inflammasomes, which comprise the NLR and ALR sensors, adaptors such as apoptosis-associated, speck-like protein containing a caspase recruitment domain (ASC), and effectors such as caspase-1 [4,5•,6•] (Figure 1a). In addition to canonical inflammasomes, cytosolic lipopolysaccharides (LPS) from Gram-negative bacteria and oxidized phospholipids can directly engage caspase-11 to form the non-canonical inflammasome, which then activates caspase-11 [7,9,10]. Inflammasome-activated caspases promote the proteolytic maturation of cytokines interleukin-1β (IL-1β) and IL-18, and cleave effector molecule gasdermin D (GSDMD) to free its active N-terminal fragment (GSDMD-NT) from its auto-inhibitory C-terminal fragment (GSDMD-CT) [11,12] (Figure 1a). Membrane pore formation by GSDMD-NT regulates cytokine release and results in pyroptosis, a highly inflammatory form of programmed cell death [13•,14–16].

In this review, we discuss recent structural studies of inflammasome signaling using the cutting-edge cryo-EM. A characteristic of inflammasome pathways is the assembly of large macromolecular complexes, often heterogeneous and intractable to X-ray crystallography. Cryo-EM structures of these complexes have provided detailed mechanistic insights into inflammasome signaling and a new paradigm for signal transduction.

Inflammasome filaments reconstructed by cryo-EM

Widely distributed among inflammasome sensors, adaptors, and effectors, the death domain (DD) superfamily comprises protein–protein interaction domains crucial in inflammatory signaling, and consists of death domain (DD), death effector domain (DED), caspase recruitment domain (CARD), and Pyrin domain (PYD). Structurally, all family members share an antiparallel six-helix bundle architecture despite differences in their primary sequence [17,18]. For ALR and NLR PYD-containing (NLRP) inflammasomes, the PYD of ALR and NLRP sensors is responsible for recruiting the adaptor protein

Figure 1



Molecular components and filamentous structures in inflammasome signaling.
(a) Domain organization of proteins involved in inflammasome pathways. **(b)** Raw cryo-EM images and structures of ASC^{PYD} (PDB ID: 3J63) and caspase-1^{CARD} (PDB ID: 5FNA) filaments overlaid with their cryo-EM maps. **(c)** General procedures of helical processing. For data processing in RELION, in the manual picking step, black circles indicate the start-end coordinates, and black lines indicate the length of the picked filaments. In the auto-picking step, the distance between different black circles is defined as the inter-box distance, which equals the number of asymmetric units multiplied by the helical rise. Good 2D class averages are selected to generate layer line information [24••]. For the IHRSR method in SPIDER, helical filaments are picked manually using EMAN script: e2helixboxer.py [45]. In the segmentation step, the rectangle box length is determined empirically [26]. The image of the powerspectrum comes from this literature [23].

ASC, which has an N-terminal PYD and a C-terminal CARD, through homotypic PYD–PYD interactions. The C-terminal CARD of ASC then serves as a scaffold for the recruitment of downstream effector caspase-1 through CARD–CARD interactions. On the other hand, the assembly of NLR CARD-containing (NLRC) inflammasomes is ASC-independent, as NLRCs directly engage caspase-1 via CARD–CARD interactions.

Before the cryo-EM revolution, nuclear magnetic resonance (NMR) and X-ray crystallography were used to determine DD superfamily structures in inflammasomes (PDB ID: NLRP3, 2NAQ, 3QF2; NLRP1, 1PN5; NLRP4, 4EWI; NLRP7, 2KM6; NLRP10, 2DO9; and NLRP12, 2l6A), and often an acidic environment was used in these two methods to keep the proteins in a monomeric or dimeric form. Under physiological pH,

however, DD superfamily proteins often assemble into filamentous polymers. The recently resolved cryo-EM structures of ASC^{PYD} and caspase-1^{CARD} filaments reveal the molecular basis for the helical assembly in these polymers [19••,20•], thereby providing an explanation for signal amplification and threshold kinetics during inflammasome signaling (Figure 1b). The ASC^{PYD} filament displays a C3 point symmetry with each helical strand possessing a right-handed 53° twist and a 13.9 Å rise, whereas the caspase-1^{CARD} filament contains a single helical strand with a left-handed 100.2° twist and a 5.1 Å rise. Common to these DD family filaments is that they are stabilized by three types of DD interactions mediated by six complementary surfaces [19••,20•,21–23].

Successful determination of these DD filament structures mainly relied on helical processing in RELION [24••] and iterative helical real-space reconstruction (IHRSR) in SPIDER [25••] (Figure 1c). Instead of single particles, helical filaments were picked from the micrographs and segmented with an appropriate inter-box distance. In RELION, the extracted segments were 2D classified to generate averages with optimal features. Power spectra were calculated to obtain the layer lines, which were used to deduce the twist and rise of the helical object. With an initial 3D model, which can be a featureless cylinder or a simulated helical lattice with the deduced helical parameter, RELION can perform 3D classification or direct 3D refinement with local refinement of the suggested symmetry. For IHRSR, the box length for segmentation was tested empirically with consideration for the different radii of curvature of filaments in different cases [26]. The 2D classification step is dispensable, as the symmetry could be estimated by indexing the power spectra from aligned segments and then optimized iteratively from a starting cylinder model.

The discovery of filament structures formed by the DD superfamily has far-reaching biological implications. A classical picture of cell signaling is that ligand-induced conformational changes at receptors, often dimerization or trimerization, lead to the recruitment and activation of downstream molecules such as secondary messengers and enzymes. In innate immunity, the formation of higher-order complexes via homotypic protein–protein interactions suggests a novel and more mechanistically complex mode of signal transduction. Consistent with this higher-order signaling hypothesis, biomolecules can form punctate aggregates, exhibit cooperativity during oligomerization, and display threshold behaviors in cellular response [27]. It is possible that higher-order assembly represents a general signaling mechanism employed in biological processes beyond inflammasome activation and innate immunity.

Cryo-EM structure of the NAIP-NLRC4 inflammasome

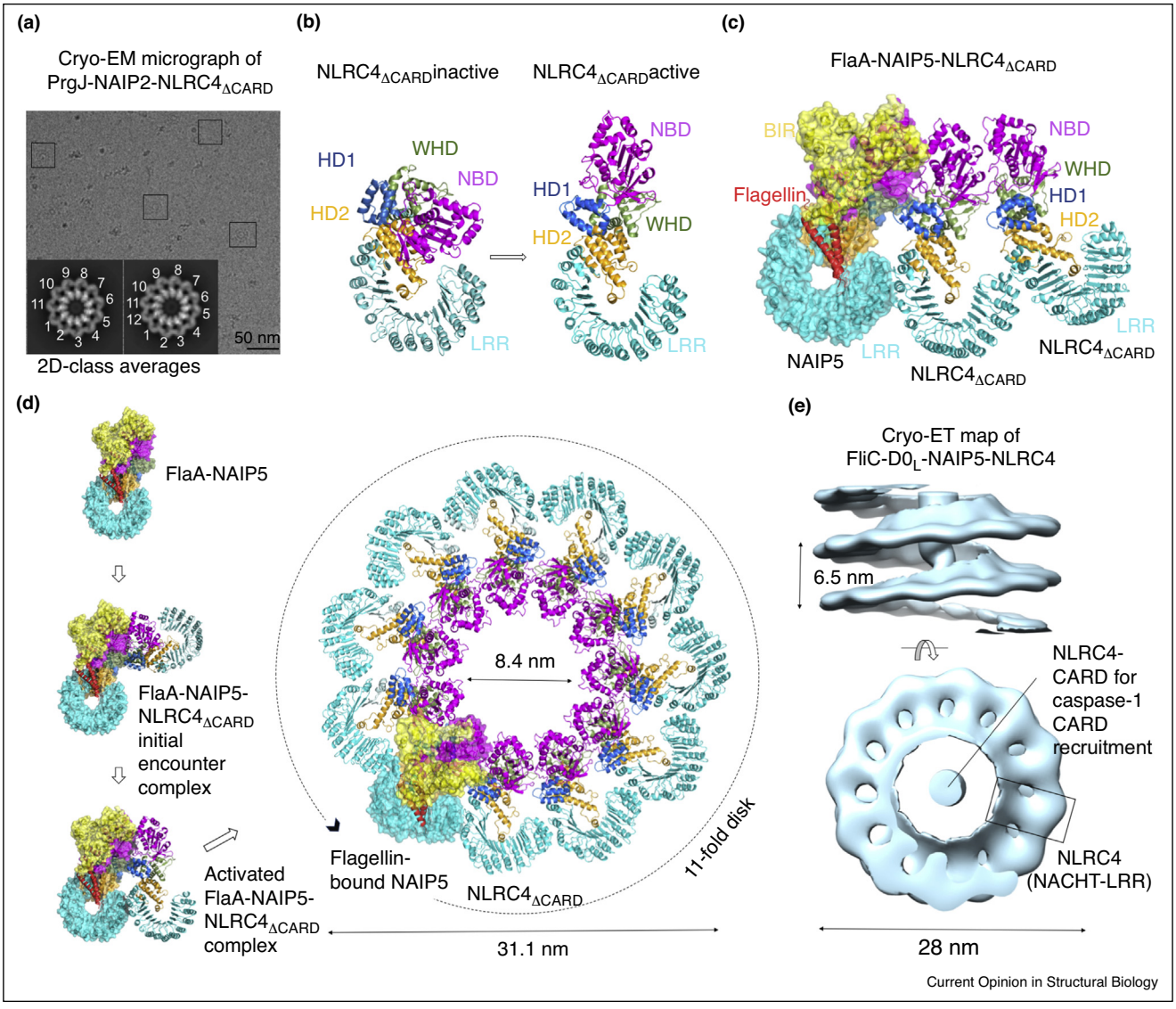
An important characteristic of inflammasome signaling is the switch from auto-inhibition to activation upon ligand engagement. With clearly identified ligands such as flagellin (such as *Salmonella typhimurium* FliC or *Legionella pneumophila* FlaA) and rod proteins from the Type III secretion system (T3SS) (such as PrgJ), the NAIP-NLRC4 inflammasomes have been ideal targets for structural and mechanistic dissection [28]. Reconstituted PrgJ-NAIP2-NLRC4 inflammasome using CARD-deleted NLRC4 ($NLRC4_{\Delta CARD}$) revealed disk-like structures with 11 or 12 subunits (Figure 2a) [29••,30••]. Biochemical characterization and gold labeling surprisingly showed that only one subunit in each disk belongs to the PrgJ-NAIP2 complex, while the remaining subunits are $NLRC4_{\Delta CARD}$ [29••,30••]. Because NAIP2 also has a similar domain organization to NLRC4, cryo-EM reconstruction was performed by applying the 11-fold or 12-fold symmetry assuming that all subunits are $NLRC4_{\Delta CARD}$ and uncovered the active conformation of $NLRC4_{\Delta CARD}$ (Figure 2b) [29••,30••].

In comparison with the crystal structure of $NLRC4_{\Delta CARD}$ in complex with ADP in a closed, auto-inhibited conformation [31•], the active conformation featured a large domain rotation (~90°) at the junction between the NBD-helical domain 1 (HD1) module and the winged helix domain (WHD)-helical domain 2 (HD2)-LRR module, leading to an open conformation of $NLRC4_{\Delta CARD}$ that is likely facilitated by nucleotide exchange (Figure 2b). Recent cryo-EM structures of the FlaA-NAIP5-$NLRC4_{\Delta CARD}$ inflammasome resolved a NAIP from the $NLRC4_{\Delta CARD}$ subunits [32••,33•] (Figure 2c). Here, the active NAIP5 is in complex with FlaA, revealing the molecular basis for ligand recognition by NAIP5. Through ligand–receptor binding analysis, the authors proposed multi-surface innate immune recognition as an efficient way to overcome single-point mutation-induced pathogen invasion.

The fact that only one subunit belongs to the ligand–sensor complex in these inflammasome structures suggests an elegant mechanism of ligand-induced inflammasome activation (Figure 2d). Upon ligand engagement, NAIP undergoes conformational changes to bind to an $NLRC4_{\Delta CARD}$ protomer, and induces conformational changes of $NLRC4_{\Delta CARD}$ to expose the oligomerization or 'catalytic' interface for the recruitment of another $NLRC4_{\Delta CARD}$ molecule. The self-propagation proceeds until a whole disk-like inflammasome is assembled (Figure 2d). The conformational change from auto-inhibited to active $NLRC4_{\Delta CARD}$ releases the positively charged 'catalytic' surface to recruit the negatively charged NBD surface of another molecule to initiate and propagate $NLRC4_{\Delta CARD}$ polymerization [29••,30••].

These studies provide important insights and raised two intriguing questions that require further structural studies of activated inflammasomes: 1. Do other NLRs form homo-oligomers or hetero-oligomers with the help of other NLRs, just like the NAIP-NLRC4 inflammasome?

Figure 2



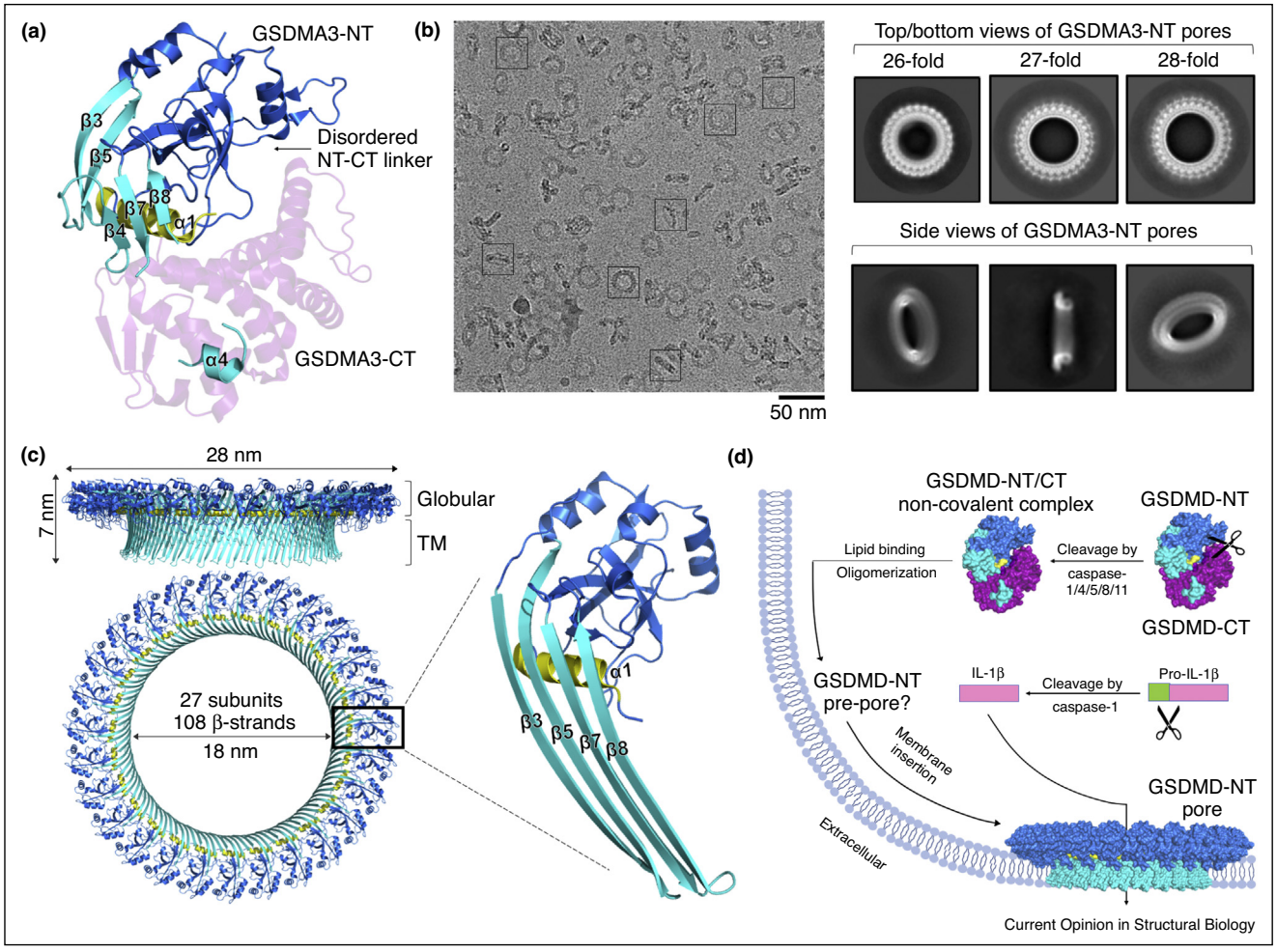
Nucleated polymerization of the NAIP-NLRC4 inflammasome.
(a) A cryo-EM micrograph showing boxed disk-like PrgJ-NAIP2-NLRC4$_{\Delta CARD}$ inflammasome particles, 11-mer 2D class averages, and 12-mer 2D class averages [30••]. **(b)** Auto-inhibited (PDB ID: 4KXF) and active conformations of NLRC4$_{\Delta CARD}$. The latter is a single subunit from the cryo-EM structure of the disk-like PrgJ-NAIP2-NLRC4$_{\Delta CARD}$ inflammasome complex (PDB ID: 6B4B, 3JBL). **(c)** Mechanism of FlaA recognition within a partial FlaA-NAIP5-NLRC4$_{\Delta CARD}$ inflammasome disk (PDB ID: 6B5B). **(d)** The whole process of NLRC4$_{\Delta CARD}$ inflammasome activation from a single FlaA-bound NAIP5 to a final 11-subunit disk (PDB ID: 6B4B, 3JBL, 6B5B). **(e)** Cryo-ET map of the FliC-D0$_L$-NAIP5-NLRC4 inflammasome (EMDB ID: 2901), showing a spiral architecture with a central CARD filament.

2. How do the scaffold domains of NLRs – namely CARD and PYD – integrate into the disk-like inflammasome structure? Clues to the second question come from cryo-electron tomography (cryo-ET), which revealed that CARD may form the central helical filament of the NAIP-NLRC4 inflammasome surrounded by the disk-like structure in a spiral manner [34•] (Figure 2e). Indeed, the dimensions and architecture of the NLRC4^{CARD} filament support its integration into the core region [21]. Taken together, progress in NAIP-NLRC4 inflammasome research through the development of cryo-EM and cryo-ET methods provides valuable guidance for further studies of NLR inflammasomes.

Mechanistic elucidation of gasdermin pore formation by cryo-EM

Both canonical and non-canonical inflammasomes can induce inflammatory and lytic cell death commonly referred to as pyroptosis. Two independent genetic screens identified GSDMD, which belongs to the

Figure 3



Molecular mechanism of GSDM pore formation.
(a) Crystal structure of mouse GSDMA3 (PDB ID: 5B5R) with key secondary structure elements labeled, GSDMA3-NT colored in blue, cyan, and yellow, and GSDMA3-CT colored in magenta. **(b)** A cryo-EM micrograph of detergent-solubilized GSDMA3 pores and 2D class averages of the pores generated in RELION [40••,46]. **(c)** Cryo-EM structure of the 27-fold symmetric GSDMA3 membrane pore (PDB ID: 6CB8) with dimensions indicated and a magnified view of a pore-form GSDMA3-NT subunit. Color schemes follow those of the crystal structure in (a). **(d)** A model for pore formation by the GSDM family, where GSDM-NTs might oligomerize into a membrane-associated pre-pore before insertion of the β-barrel to form a transmembrane pore that allows the passage of cytoplasmic contents such as IL-1β.

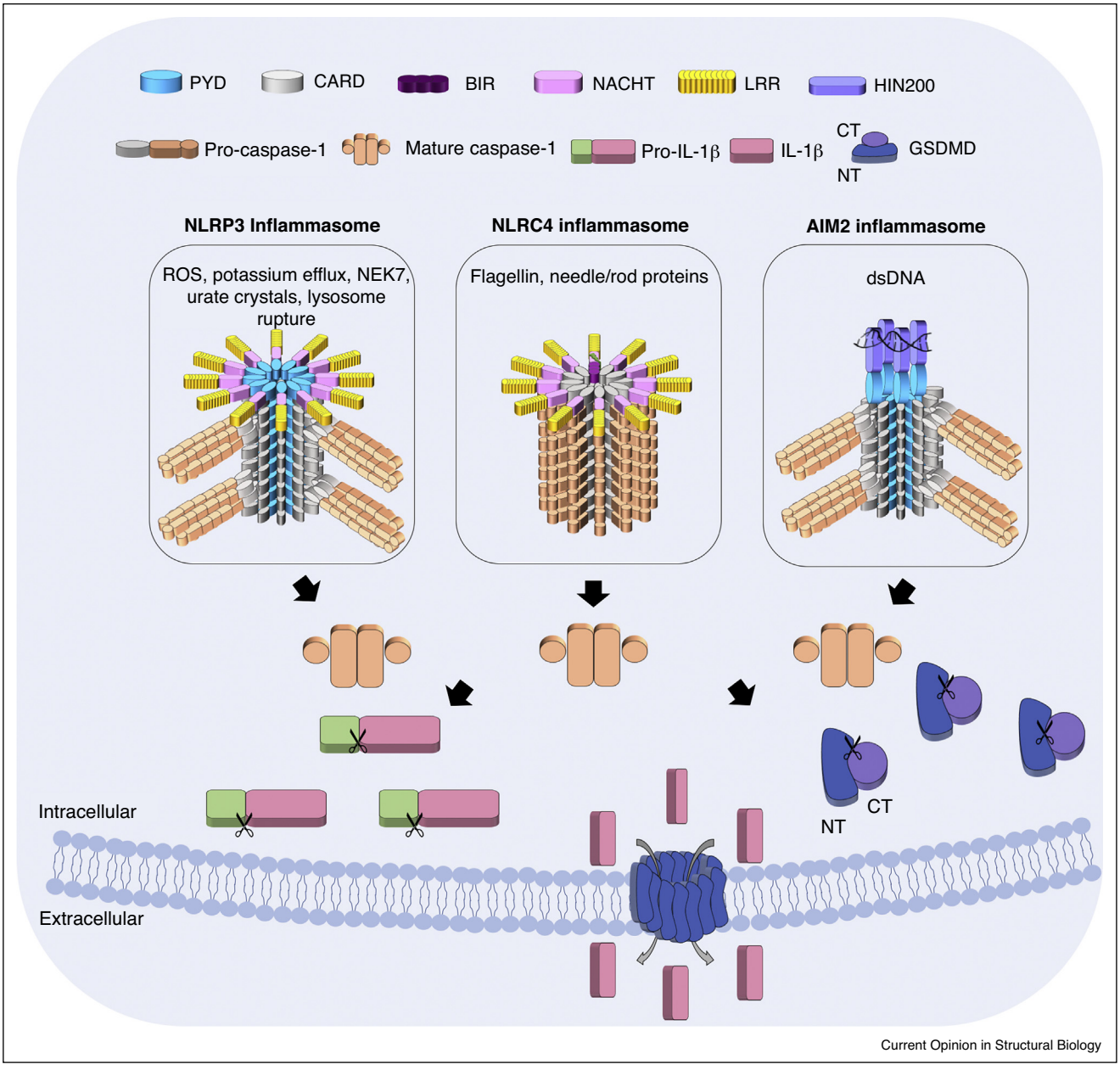
GSDM family, as the executioner of pyroptosis downstream of caspase-1 and murine caspase-11 (human homologs caspase-4 and caspase-5) [11,12]. These inflammasome-activated caspases, as well as a caspase-8-dependent pathway during *Yersinia* infection [35,36], proteolytically activate GSDMD. GSDMD-NTs promote pyroptosis by directly binding membrane lipids and forming oligomeric membrane pores, while GSDMD-CT has an auto-inhibitory function that is removed by caspase cleavage [37•,13•]. Adding to the complex signaling network involving GSDMD, recent studies have highlighted a non-pyroptotic role of GSDMD and placed GSDMD upstream of the NLRP3 inflammasome [38,35,39].

The X-ray crystal structure of mouse GSDMA3 provided valuable visualization of the auto-inhibited, inactive conformation of GSDM family proteins (Figure 3a) [37•]. However, a thorough structural understanding of GSDMs is hindered by the instability and heterogeneity of GSDM pores that render crystallization unfeasible. By testing GSDMs of different species, Ruan *et al.* reconstituted GSDMA3 pores on cardiolipin-containing liposomes and used cholate to solubilize the pores for cryo-EM structural determination [40••]. RELION 2D classification showed 26-fold, 27-fold, and 28-fold symmetric top views with 27-fold as the major class, suggesting oligomerization heterogeneity of GSDM pores (Figure 3b). Top-view 27-fold 2D classes, and all side-view classes of which the symmetry is

unclear, were selected for 3D classification without assumption of any symmetry, upon which the predominant 3D class was refined in RELION with C27 symmetry.

The final pore structure at 3.8 Å resolution features a 108-stranded antiparallel β-barrel as the transmembrane region, capped by a soluble rim formed by the globular domain of each of the 27 subunits (Figure 3c). The inner diameter of the pore is approximately 18 nm, large enough for the passage of IL-1β but not all cytosolic components, suggesting a potential size-exclusion mechanism. In comparison with the auto-inhibited crystal structure, GSDMA3-NT undergoes large conformational changes, particularly at the β3–β4 and the β7–α4–β8

Figure 4



Structural biology of inflammasome signaling.
Overview of the inflammasome activation processes for NLRP3, NLRC4, and AIM2, with domains and proteins indicated above, and the stimuli and cartoon models of NLRP3, NLRC4, and AIM2 inflammasomes shown in boxes. NLRP3 and AIM2 require the adaptor protein ASC for proximity-induced activation of caspase-1. By contrast, NLRC4 can directly recruit and activate caspase-1. Active caspase-1 cleaves and releases the auto-inhibition of GSDMD. GSDMD-NT then forms membrane pores to induce pyroptosis. Active caspase-1 also cleaves pro-IL-1β into its mature form, which is released through GSDMD pores.

regions, for membrane insertion (Figure 3a,c). Adjacent to the basic α1 helix, a cryo-EM density is visible and likely represents the negatively charged head group of the acidic lipid cardiolipin, which indicates the crucial role of lipid binding in membrane pore formation. Interestingly, lining the membrane-inserted pore is a soluble ring without extended β-strands, which could be an intermediate pre-pore conformation of GSDMs [40••]. It merits further study whether GSDM pore formation is a smooth continuum of structural transitions or comprises discrete steps from the auto-inhibited conformation, to a non-covalent complex of GSDM-NT and GSDM-CT [37•], to an oligomerized but not membrane-inserted pre-pore, and finally to a membrane pore (Figure 3d).

Outlook and challenges

Cryo-EM has offered invaluable mechanistic insights into inflammasome signaling (Figure 4). Especially regarding the NLRC4 inflammasome, high-resolution structures have revealed molecular details of ligand-induced conformational change, release from auto-inhibition, self-propagation through NBD and LRR interactions, and nucleation-induced polymerization of ASC and caspase-1 by DD-fold assembly. These structures also shed light on the activation mechanisms of other NLRs given their highly conserved domain architecture.

One future direction toward the understanding of inflammasome activation lies in ligand–receptor interactions. NEK7 kinase and lipoteichoic acid (LTA) from Gram-positive bacteria have been identified as activators of NLRP3 and NLRP6, respectively [41,42], but the structural basis for the recognition of these ligands remain elusive. Likewise, the interaction between LPS and caspase-11 in the non-canonical inflammasome pathway remains an intriguing topic [43,9,8]. In addition to ligand–receptor interactions, the cooperativity of sensor molecules in inflammasome signaling merits further study. For example, NLRP3 and NLRC4 may together orchestrate inflammasome activation in macrophages [44], which suggests that the structural scaffolds of inflammasomes may be co-formed by different NLRs. A thorough depiction of inflammasome signaling by cryo-EM, from a translational perspective, will aid structure-guided drug development against inflammatory diseases.

Despite these interesting directions, structural study of ligand–receptor complexes and higher-order protein assemblies faces many challenges. Not only are inflammasome components difficult to express and purify, instability, heterogeneity in size and shape, and orientational preference also deter the structural determination of these molecules and their complexes by cryo-EM. For example, the effector protein GSDMD employs a higher-order assembly strategy to rupture cell membranes for cytokine release and pyroptosis (Figure 4). A previous attempt at reconstituting homogeneous human GSDMD pores *in vitro* was unsuccessful due to their aggregation and deformation after being detergent-solubilized from liposomes [40••]. In addition, the cryo-EM densities at the globular domain of the GSDMA3 pore are only at a modest resolution, likely due to conformational dynamics. The field is awaiting a method to validate sequence registration into cryo-EM maps with relatively poor densities, possibly by selective labeling of reactive amino acid residues.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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