



COPs and POPs Patrol Inflammasome Activation

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Abstract

Sensing and responding to pathogens and tissue damage is a core mechanism of innate immune host defense, and inflammasomes represent a central cytosolic pattern recognition receptor pathway leading to the generation of the pro-inflammatory cytokines interleukin-1 β and interleukin-18 and pyroptotic cell death that causes the subsequent release of danger signals to propagate and perpetuate inflammatory responses. While inflammasome activation is essential for host defense, deregulated inflammasome responses and excessive release of inflammatory cytokines and danger signals are linked to an increasing spectrum of inflammatory diseases. In this review, we will discuss recent developments in elucidating the role of PYRIN domain-only proteins (POPs) and the related CARD-only proteins (COPs) in regulating inflammasome responses and their impact on inflammatory disease.

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Introduction

The innate immune system is an evolutionarily conserved non-specific defense mechanism that exists to initiate an early rapid response in the event of an infection or injury. One of the hallmark responses of the innate immune system is “inflammation,” a process that potentiates a rapid influx of immune cells and the accumulation of pro-inflammatory mediators, which facilitate the clearance of infections and promote tissue repair [1]. It is well established that the recognition of molecular patterns derived from pathogens (pathogen-associated molecular patterns, or PAMPs), microbes (microbe-associated patterns or MAMPs), or host (danger-associated molecular patterns, or DAMPs, or alarmins) by the innate immune sensors initiates an inflammatory cascade [2]. These innate immune sensors are strategically located either on the surface of the cell or intracellularly in the cytoplasm or within vesicles [2]. Among the intracellular innate immune sensors, some initiate the

formation of large multimeric protein complexes called “inflammasomes,” which respond to a wide variety of ligands and exhibit unique mechanisms of activation and regulation. Inflammasomes are responsible for the activation of inflammatory caspases that promote processing of the pro-inflammatory cytokines interleukin (IL)-1 β and IL-18 and induction of pyroptotic cell death [3]. Inflammasomes can be triggered by a spectrum of different PAMPs or DAMPs, which dictate the type of inflammasome that is being assembled and the inflammatory caspase that is activated. Inflammatory caspases comprising human Caspases-1, -4, and -5 and mouse Caspases-1 and -11, are a group of phylogenetically related proteases involved in the initiation of inflammatory responses [4,5]. While Caspase-1 activation takes place in the canonical inflammasome, activation of Caspases-4/-5 and their mouse ortholog Caspase-11 occurs in the non-canonical inflammasome. Caspase-1 activation utilizes a proximity-induced dimerization mechanism [6], which initially requires the oligomerization of the

inflammasome sensor and adaptor, followed by the binding of pro-Caspase-1 to the polymerized adaptor [7,8]. The close proximity of pro-Caspase-1 molecules promotes autocatalysis and cleavage of pro-Caspase-1 into active Caspase-1, which in turn promotes pyroptosis and processes the pro-inflammatory cytokine precursors, pro-IL-1 β and pro-IL-18, thereby facilitating their maturation and secretion [6,9]. In contrast to Caspase-1, Caspase-4/-5/-11 activation occurs upon direct lipopolysaccharide (LPS) sensing by Caspases-4/-5/-11, which promotes pyroptotic cell death [10,11]. Pyroptosis is characterized by plasma membrane rupture, cell swelling, and the release of intracellular contents including the alarmins IL-1 α , polymerized ASC particles, HMGB1, and others. The pore-forming protein gasdermin D (GsdmD) is responsible for pyroptosis [12–14], but full-length GsdmD is inactive. However, cleavage of GsdmD into a 38-kDa N-terminal and 22-kDa C-terminal fragment by inflammatory caspases results in pore formation triggered by membrane insertion and polymerization of the N-terminal GsdmD fragment [15–20]. Ultimately, the pro-inflammatory cytokines and other cellular factors secreted upon inflammasome activation perpetuate the immune response by promoting the release of other inflammatory mediators, which further enhance the recruitment and activation of immune cells. Here we are focusing on the recent progress delineating the regulation of canonical inflammasomes by small endogenous inhibitory proteins containing either a PYRIN domain (PYD) or a caspase recruitment domain (CARD), which are referred to as PYD-only proteins (POPs) and CARD-only proteins (COPs), respectively.

Canonical inflammasomes were first described by Martinon *et al.* [21] in 2002 and are now recognized as a major cytosolic innate immune sensory pathway. By now, detailed mechanistic insights into the assembly and regulation of these signaling platforms have been determined [22–29]. Typically, canonical inflammasomes form a three-component signaling platform comprising a sensor, an adaptor molecule, and pro-Caspase-1. Activation of the canonical inflammasome is initiated by ligand sensing/recognition, followed by recruitment and oligomerization of the adaptor apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), which in turn recruits and promotes the polymerization of the catalytically inactive, pro-Caspase-1. The recruitment and oligomerization of these multiple proteins and the subsequent formation of this macromolecular signaling platform require efficient interaction among these proteins, which is mediated by homotypic associations between specific domains present in these proteins. Inflammasome-related proteins usually contain a CARD, a PYD, or both. The PYD and CARD belong to the death domain fold (DDF) superfamily, which also includes the death domain (DD), and death effector domain (DED) [30]. Protein domains of the DDF

superfamily are characterized by a bundle of six anti-parallel α -helices with a hydrophobic core and are well known for their participation in signaling events during apoptosis and inflammation [31–33]. Inflammasome sensors contain either a CARD or a PYD to facilitate the interaction either with pro-Caspase-1 or the adaptor protein ASC, which comprises a PYD and a CARD, and is therefore able to function as a linker between the sensors and pro-Caspase-1 to facilitate Caspase-1 activation.

Inflammasome sensors that trigger assembly and activation of canonical inflammasomes include members of the nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) family, the absent in melanoma 2 (AIM2)-like receptor (ALR) family, and Pyrin (Fig. 1). Each of these receptors recognizes specific ligands or ion fluxes to promote inflammasome assembly. NLRs exhibit a tripartite domain structure with an N-terminal PYD or CARD, a central nucleotide binding domain (NBD), and C-terminal leucine-rich repeats (LRRs). NLRs with an N-terminal PYD are classified as NLRPs, and NLRs with an N-terminal CARD are classified as NLRCs. So far, 14 NLRPs and 5 NLRCs have been identified [34]. Particularly, inflammasomes containing NLRP1 [21,35], NLRP2 [36], NLRP3 [37–41], NLRP6 [42,43], NLRP7 [44,45], NLRP12 [46], NLRC4 [47], and NLRC5 [48] have been described. Although it is still unclear whether all inflammasome sensor activation is triggered by direct ligand binding, several PAMPs have been identified that cause inflammasome activation. For instance, mouse NLRP1b senses anthrax lethal toxin [49] and NLRP7 detects acylated lipopeptides [44,45,50]. For NLRC4 inflammasome activation, PAMP recognition by NLR apoptosis-inhibitory proteins (NAIPs) triggers NLRC4 activation followed by NLRC4 self-activation and oligomerization [51]. Hence, only one NAIP molecule is incorporated into the NAIP–NLRC4 inflammasome complex [52–54]. While NAIP5 and NAIP6 trigger the formation of NAIP5/6–NLRC4 inflammasomes in response to bacterial flagellin, the NAIP2–NLRC4 inflammasome complex is formed in response to PrgJ, a bacterial type III secretion protein [55]. Extraordinarily, NLRP3 is activated by a wide range of stimuli including components from bacterial, viral, and fungal pathogens as well as endogenous molecules such as uric acid crystals and stress signals, such as extracellular ATP and ion fluxes [38,39,56–60]. Given the large diversity of agonists, it is conceivable that they converge into a common signal that triggers the NLRP3 inflammasome [61,62], and recent studies support the notion that potassium efflux is common to all NLRP3 activating signals [63]. Ligand sensors/receptors belonging to the ALR family, including AIM2 [64–67] and γ -interferon (IFN)-inducible protein 16 (IFI16) [68], have also been described to trigger the assembly and activation of a canonical inflammasome. ALRs consist of an N-terminal PYD and one or two C-terminal

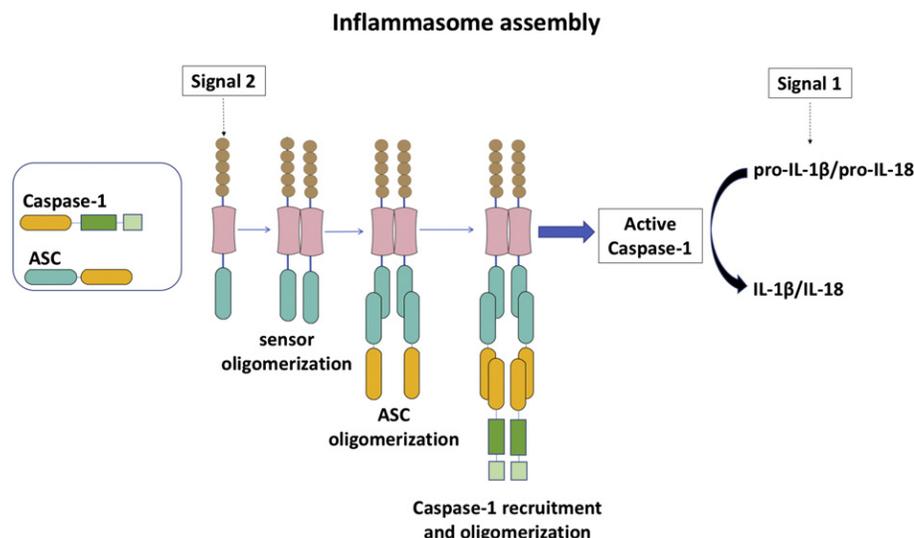


Fig. 2. A depiction of the stepwise inflammasome assembly and polymerization process.

properties, which enable the rapid formation of filamentous, self-polymerizing ASC aggregates [7,8]. While the ASC-PYD faces toward the rigid core of the ASC filament and is crucial for filament formation, the ASC-CARD is more flexible and projects toward the surface for CARD:CARD-mediated interactions with Caspase-1 to promote Caspase-1 polymerization and the assembly of ASC filaments into dense specks [92,93]. ASC specks are stable, soluble micrometer-sized aggregates that promote Caspase-1 activation through polymerization and induced proximity-mediated activation and serve as processing sites for pro-IL-1 β and pro-IL-18 (Fig. 2). Interestingly, polymerized ASC particles can be released into the extracellular space where they retain their ability to activate pro-Caspase-1 and pro-IL-1 β . In addition, ASC particles act as danger signals that can be taken up by neighboring cells *via* phagocytosis and induce Caspase-1 activation and pro-IL-1 β processing in a largely sensor-independent manner to propagate and perpetuate inflammasome responses [94,95]. Since pro-Caspase-1 encodes a CARD, ASC is quintessential for linking PYD-containing receptors/sensors to pro-Caspase-1, while CARD-containing inflammasome receptors/sensors, such as NLRC4, directly interact and activate pro-Caspase-1 [47]. Although NLRC4 is able to interact with pro-Caspase-1 independently of ASC through CARD:CARD-mediated interactions, and NLRC4-mediated pyroptosis does not require ASC, processing and secretion of IL-1 β still requires the presence of ASC [96]. ASC oligomerization and speck formation is required for efficient IL-1 β processing, but dispensable for the induction of pyroptosis, suggesting that ASC oligomerization functions as a signal amplification mechanism for Caspase-1 activation and IL-1 β processing [93].

The purpose for forming a macromolecular inflammasome complex upon ligand recognition is to ultimately provide an activation platform for Caspase-1. Subsequent to sensor and adaptor oligomerization, pro-Caspase-1 is recruited to the signaling scaffold which facilitates its activation. Inflammatory Caspases are cysteine-dependent aspartate-specific endoproteases that are produced as zymogens, which consist of an N-terminal CARD, the large p20 subunit containing the active-site cysteine within a conserved QACXG motif and a C-terminal small p10 subunit separated by a peptide linker [4]. While the active site involves both the p10 and the p20, Caspase-1 monomers have little or no activity. However, upon dimerization, a proper active site can be formed and auto-proteolytic cleavage occurs after Asp²⁹⁷ and Asp³¹⁶, removing the peptide linker between p20 and p10 and releasing the p10 subunit. Subsequently, cleavage at Asp¹¹⁹ separates the p20 subunit from the CARD [6]. The p10 and p20 subunits form a heterodimer and two heterodimers join to form the fully active mature tetrameric Caspase-1 enzyme. Pro-caspase-1 maturation is initiated in the oligomeric inflammasome complex through proximity-induced dimerization of pro-Caspase-1 molecules, which is nucleated by oligomerized ASC. This ultimately results in a locally increased concentration of pro-Caspase-1 molecules, which provides sufficient intrinsic activity to auto-proteolytically cleave pro-Caspase-1 and assembles the mature Caspase-1 tetramers that efficiently process cytokine substrates.

Since the quintessential function of the inflammasome is the conversion of zymogenic Caspase-1 into its catalytically active form, the assembly of these highly organized inflammasome complexes is the essential step. While inflammasomes are crucial for mounting an efficient host defense against pathogens [97–101],

inappropriate and uncontrolled inflammasome activation can be detrimental, as aberrant inflammasome activity is linked to an increasing spectrum of auto-inflammatory, neurodegenerative, and metabolic diseases [23,102–111]. Because the consequence of Caspase-1 activation is a potent pro-inflammatory response and ultimately cell death, the assembly of this inflammasome complex needs to be tightly regulated. First, cells employ a two-step mechanism for inflammasome activation (Fig. 3). The first step, also known as the priming step (signal 1), requires PAMP-mediated activation of Toll-like receptors (TLRs), which leads to nuclear factor (NF)- κ B-mediated transcriptional up-regulation of inflammasome components including pro-IL-1 β and NLRP3, as well as post-translational modification of NLRs, such as deubiquitination and phosphorylation [112]. In addition, in many cell types, NLRC4 and NAIPs 2/5/6 are constitutively expressed [113,114] but AIM2 and Pyrin expression can be up-regulated by IFNs [65,115,116]. The second step (signal 2) requires receptor-mediated sensing of a wide variety of endogenous and microbial ligands in the presence of accessory proteins, such as Nek7 and guanylate-binding proteins, the adaptor ASC and Caspase-1 that together facilitate inflammasome assembly and activation [22–24,26,28,117–121]. In contrast to this classical inflammasome activation, which requires pyroptosis for cytokine release, human monocytes employ a non-lytic form of alternative

NLRP3 inflammasome activation in response to LPS, which proceeds in the absence of signal 2. Here Caspase-8-mediated activation of the NLRP3 inflammasome occurs downstream of TLR4–TRIF–RIPK1–FADD–CASP8 signaling through a yet unknown mechanism [122]. Considering the pivotal role of inflammasomes in sensing threats and initiating and propagating inflammatory responses and considering the critical role that inflammasomes play in inflammatory and infectious diseases, it is plausible that a high level of control beyond the two-step activation mechanism is necessary for efficiently regulating inflammasome responses. In this regard, there are numerous reviews discussing the complex regulation of inflammasome activation and provide insights into the multiple regulatory mechanisms that directly target inflammasome components as well as upstream or downstream factors that modulate inflammasome activity [22,24,25,28,62,117,123,124]. Also, TLR-mediated signaling and the resulting inflammasome priming are highly regulated and have been reviewed elsewhere [125–128]. However, over the past several years, another simple, yet effective mechanism targeting the key step of inflammasome assembly, which is based on small endogenous decoy proteins comprising either a single PYD or CARD, has been elucidated (Fig. 3) [27,29,129]. In this review, we will discuss recent developments and the unique role of these small POPs and COPs that target inflammasome assembly

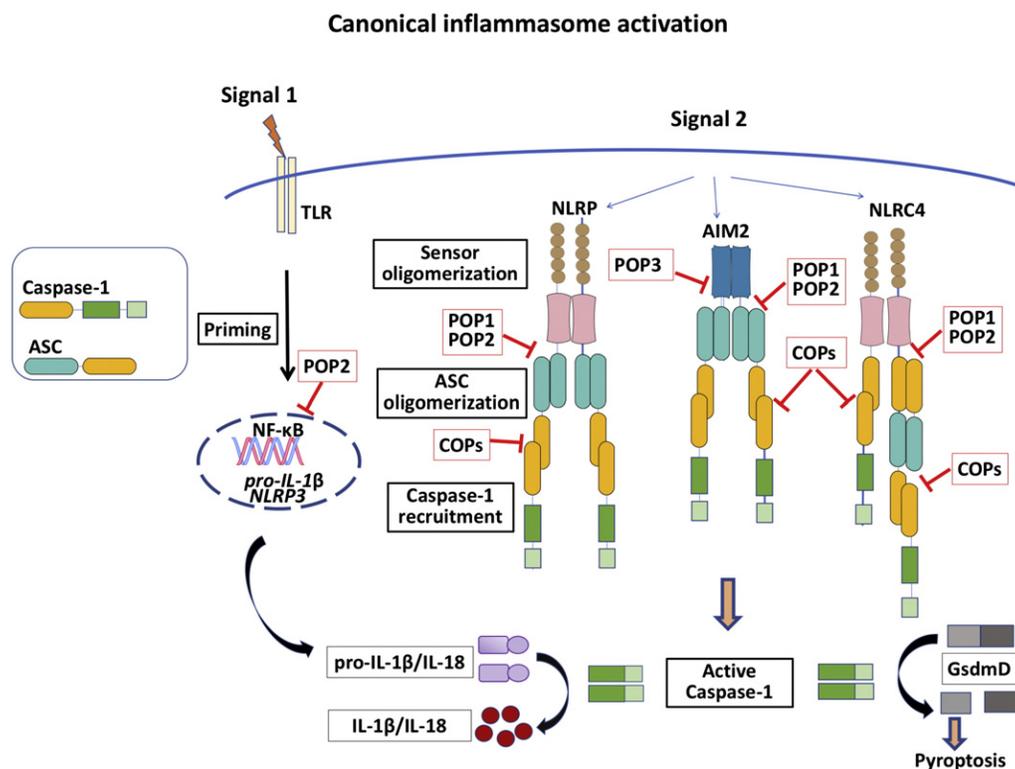


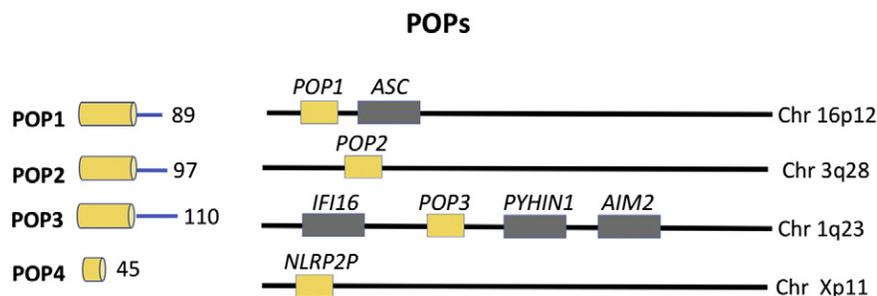
Fig. 3. Overview of canonical inflammasome signaling, indicating the steps targeted by POPs and COPs.

and thereby effectively intervene with inflammasome responses.

POPs

The PYD, which was initially referred to as PAAD (pyrin, AIM, ASC, and DD-like) or DAPIN (domain in apoptosis and IFN response), was originally discovered in the Pyrin protein (Marenostrin, *MEFV*) and is, with a few exceptions, a homotypic protein interaction domain [130–132]. There are about 23 human genes and 31 mouse genes encoding a PYD. The PYD belongs to the DDF family and typically comprises 90 aa [33,129,133,134]. Its six anti-parallel bundled α -helices assemble a hydrophobic core, but contrary to other DDF members, the residues from the α 3 helix of PYDs do not contribute to the hydrophobic core and is shorter than that of other DDF members. Thus, the length of the α 2– α 3 loop region is increased, which could influence the binding specificities of PYDs [31]. Also, the electrostatic surface charge and hydrophobicity contribute to homotypic PYD:PYD associations, enabling PYDs to function as protein–protein interaction domains. Unlike other DDF family members, the

PYD is exclusively located at the N-terminal region of proteins. While there are proteins that only encode a PYD and are therefore referred to as POPs, there are PYD encoding proteins that encode additional domains including CARD, NACHT, LRR, coiled coil, B30.2, and HIN200 domains [129,130,133,135]. POPs, also referred to as cellular POPs or PYD containing, are small proteins of about 10 to 13 kDa consisting of the PYD alone. Till date, three cellular POPs, namely, POP1, POP2, and POP3, have been identified in humans (Fig. 4) [136–139]. Recently, a truncated NLRP2P, which only consists of 45 aa and probably only forms the first two α -helices of the PYD and therefore lacks the typical six anti-parallel bundled α -helices of a PYD, has been described and termed POP4 (Fig. 4) [140]. Interestingly, mice do not have an ortholog for POPs. Instead, two murine POP genes have been annotated as *Pydc3* and *Pydc4*, which display no similarity to human POPs and are predicted to be exons of larger, multi-domain proteins. However, even the three human POPs, POP1, POP2, and POP3 are less than 20% identical to each other. Hence, they are expected to exhibit unique functionalities. Since PYDs are protein interaction domains that preferentially form homotypic interactions, POPs were predicted to interact with other



POPs	Interacts with	Functional impact
POP1	ASC-PYD	Inhibition of ASC recruitment and oligomerization
POP2	ASC-PYD ??	Inhibition of ASC recruitment and oligomerization Inhibition of NF- κ B activation
POP3	AIM2-PYD IFI16-PYD	Inhibition of ASC recruitment and oligomerization
POP4	???	Inhibition of NF- κ B activation

Fig. 4. Graphical representation of the genomic location of POPs, the length of the encoded proteins in amino acids, and their known binding partners and the functional consequence of this interaction.

PYD-containing proteins to regulate inflammasomes, which had been validated by initial biochemical over-expression studies. POPs are absent in mice, but more recently, the development of POP transgenic mice confirmed their inflammasome-inhibitory function, revealed additional activities on other innate immune pathways, and shed novel insights into their implications for inflammatory and infectious disease [138,141–143].

POP1

POP1, initially referred to as ASC2 and ASC1, was the first discovered protein consisting of only a PYD [137]. It shares 88% similarity to and 64% sequence identity with the ASC-PYD, is located in very close proximity to the ASC-encoding human chromosome locus 16p12.1, and likely arose from a gene duplication event of ASC [137] (Fig. 4). Expression of POP1 has been detected in human myeloid cells, pericytes of blood vessels, myoepithelial cells, and perineurial cells [137]. POP1 consists of 89 aa that form the characteristic six-helix bundle fold of a DD. Contrary to the other members of the DD superfamily, which often exhibit poor solubility due to self-aggregation, POP1 is monomeric and soluble [144,145]. Accordingly, microscopy studies demonstrated that POP1 is dispersed throughout the cell, and unlike ASC, it is unable to form filaments, even though residues that contribute to the filamentous structure of ASC are conserved in POP1. Nevertheless, co-expression of POP1 with ASC promotes recruitment and co-localization of POP1 and ASC in ASC specks, and the ASC-PYD has been identified to interact with POP1 [137]. Modeling studies of POP1 revealed the presence of a negative patch formed by residues from helices $\alpha 1$ and $\alpha 4$ as well as a basic patch formed by residues in helices $\alpha 2$ and $\alpha 3$. The electrostatic surface potential is identical between POP1 and ASC-PYD, and these charged surface patches contribute to the binding ability of POP1. The positive electrostatic potential surface patch (EPSP) formed by residues K20, K21, and R41 on POP1 interacts with the negative EPSP (D6, E13, D48, D54) on the ASC-PYD. In addition, the mutation of ASC-PYD residues E13 and D48 abrogates the interaction between ASC-PYD and POP1, indicating that these residues facilitate the association between these proteins [144,146]. Since the same residues (E13 and D48) along with other ASC residues (K21, R41, and D51) are required for ASC oligomerization, as well as for ASC–POP1 and ASC–NLRP3 interactions, a competitive binding mechanism, where ASC could either interact with PYD-containing sensors, such as NLRP3 or POP1, was predicted [8,147]. For a better understanding of inflammasome regulation by POP1, it is therefore crucial to determine the predominant and preferred interaction under physiological conditions and more functional insights were revealed, recently [141].

To rectify earlier results obtained by over-expression in the non-macrophage HEK293 cells, THP-1 cells and primary macrophages expressing or lacking POP1 were used in a recent study. While POP1 artificially inhibits NF- κ B as a consequence from over-expression in 293 cells, this effect was not observed in macrophages. However, POP1 was able to prevent the recruitment of ASC to the activated upstream inflammasome sensors and consequently inhibited nucleation of the ASC polymerization, which resulted in impaired Caspase-1 activation and diminished IL-1 β production in response to inflammasome activation. Conversely, siRNA-mediated silencing of POP1 in human macrophages enhanced inflammasome responses, demonstrating that POP1 has an important and unique role in regulating inflammasome assembly at the ASC polymerization step, but no effect on NF- κ B activation [141]. In addition, this study utilized transgenic mice expressing POP1 specifically in mouse monocytes, macrophages, and conventional dendritic cells (DCs). Due to the high degree of homology (80% identity) between the human and mouse ASC-PYD, the interaction of ASC-PYD and POP1 was retained in POP1 expressing mouse macrophages, and the inflammasome-inhibitory function of POP1 observed in human macrophages could be replicated in POP1 expressing mouse macrophages. Therefore, also the functional impact of POP1 during inflammatory disease could be investigated and revealed a potent anti-inflammatory activity for POP1 [141]. Even though POP1 expression was restricted to monocytes, macrophages, and DCs, POP1 abolished IL-1 β -driven neutrophil infiltration into the peritoneal cavity in response to LPS-induced peritonitis and prevented systemic inflammatory cytokine release, including IL-1 β and IL-18, and prevented hypothermia and death in response to a lethal LPS challenge. In addition to inhibiting PAMP-induced acute responses, POP1 was also able to abrogate the chronic inflammasome-dependent auto-inflammatory disease Muckle–Wells syndrome (MWS). A mouse model of MWS recapitulates human disease by expressing the disease-associated activating A350V mutation in NLRP3, resulting in uncontrolled inflammasome activation and subsequently systemic inflammation, weight loss, and death [148–151]. However, POP1 expressing mice were completely protected from this phenotype, indicating that POP1 can even protect from inflammasome responses triggered by a constitutively activated inflammasome sensor [141].

While it has been known that inflammasome components are released through pyroptosis, more recent studies demonstrated that polymerized ASC particles not only are released into the extracellular space upon inflammasome activation but also act as danger particles that are phagocytosed by neighboring cells and thereby propagate and perpetuate inflammasome responses to trigger Caspase-1 activation in a largely sensor-independent manner [94,95,152].

Importantly, ASC particles are present in the serum of auto-immune and auto-inflammatory patients and mice [94,95]. Notably, POP1 not only was able to block cytokine release but also prevented the release of ASC particles from macrophages in response to inflammasome activation, and even disabled ASC particle-mediated inflammasome responses [141]. Furthermore, while intraperitoneal injection of purified ASC particles caused IL-1 β release and neutrophil infiltration, this response was strongly ameliorated in POP1 transgenic mice [141]. Therefore, these studies demonstrated that POP1 prevents primary inflammasome activation as well as propagation and inflammasome activation in bystander cells.

Analysis of POP1 expression in macrophages revealed a highly inducible expression following TLR activation and in response to inflammatory cytokines, in particular IL-1 β and IL-18, indicating that POP1 may function in a feed forward regulatory loop. As basal POP1 expression is barely detectable, the critical early inflammasome responses during acute host defense are enabled, but once inflammatory cytokines accumulate, its NF- κ B-mediated inducible expression will raise the threshold necessary for ASC polymerization and thereby support the resolution of inflammasome responses, which is necessary to prevent excessive inflammation. A dose-dependent effect of POP1 was even detectable in transgenic mice where variable POP1 expression levels inversely correlated with serum IL-18 levels in response to intraperitoneal LPS challenge, further supporting a functional consequence of inducible POP1 expression. Remarkably, POP1 expression is reduced in cryopyrinopathy patients, including MWS, which is counterintuitive as these patients are characterized by systemic production of IL-1 β , suggesting that additional defects may prevent proper POP1 expression and may render patients more susceptible to systemic inflammatory disease. Based on this observation, a cell permeable, recombinant POP1 was validated in mice as possible therapy and successfully ameliorated inflammasome-driven disease [141].

POP2

POP2 is located on human chromosome 3q28 encoding a 97-aa protein (Fig. 4) and is only present in primates closely related to humans [136,139,153]. It shares 68% similarity to the NLRP2-PYD and 50% similarity to the NLRP7-PYD, but only 37% similarity to the ASC-PYD. POP2 interacts with the PYDs of ASC, NLRP1, NLRP2, NLRP4, and NLRP12 but not with NLRP3, NLRP10, and NLRP11 [136]. Homology modeling of POP2, based on the NLRP7-PYD, revealed that POP2 has six α -helices forming a DD fold [29]. Residues E16 and E57 from helices α 1 and α 4 of POP2 form a negative surface patch which is conserved among the PYDs of POP1, POP2, and ASC. However, the positive surface patch present in

the PYDs of ASC and POP1 is absent in POP2. Hence, it is conceivable that the negative surface patch of POP2 interacts with the positive surface patch of ASC or NLRP PYDs. Then again, NLRP1 and NLRP12, which bind to POP2, lack a strong positive surface charge. Therefore, other, non-charge-based mechanisms of PYD–PYD interactions might exist to facilitate their interactions.

Endogenous POP2 expression occurs in peripheral blood leukocytes and in human leukocyte cell lines and shows a diffuse or vesicular nuclear and cytosolic cellular localization pattern by overexpression. In the presence of ASC, POP2 and ASC co-localize [136,139]. Initial overexpression studies implicated POP2 in the inhibition of NF- κ B and inflammasomes [136,139]. Strikingly, in both instances, the α 1 helix seems to be necessary and sufficient, but while the acidic residues E6, D8, and E16 are crucial for blocking the inflammasome, they are not involved in the inhibition of NF- κ B signaling [153]. Hence, the negatively charged residues of POP2 seem to be important for PYD–PYD interactions between POP2 and ASC during inhibition of the inflammasome, but are not involved in the inhibition of NF- κ B signaling, where POP2 seems to act through a different, yet unknown, mechanism. POP2-mediated inflammasome inhibition has been determined by the lack of NLRP1, NLRP3, and NLRP12-mediated ASC speck formation in the presence of POP2 and diminished IL-1 β release from NLRP2 and NLRP3 inflammasomes, and therefore, POP2 is expected to interfere with the ASC–NLRP interactions, reminiscent of POP1. Simultaneously, two recent studies using transgenic mice expressing POP2 in monocytes, macrophages, and conventional DCs or from its own promoter provided additional insights into the role of POP2 during inflammasome activation [142,143]. Reminiscent to POP1, the highly conserved PYDs enabled human POP2 to interact with mouse ASC through their PYDs and therefore supported a comparable function as in humans. POP2 interacts with ASC and prevents recruitment to upstream PYD-containing sensors, and consequently inhibits ASC polymerization, Caspase-1 activation, IL-1 β and IL-18 secretion, and pyroptosis upon activation of NLRP3, AIM2, Pyrin, NLRC4, and NLRP1b inflammasomes by their respective agonist in mouse and human macrophages. Hence, the inflammasome-inhibitory function of POP2 is reminiscent to that of the related POP1.

However, POP2 acquired a second, distinct activity, which targets activation of NF- κ B and therefore also inflammasome priming. As the role in inflammasome assembly, inhibition of NF- κ B is conserved in humans and mice. Although the precise mechanism by which POP2 inhibits NF- κ B induction is largely elusive, Ratsimandresy *et al.* [143] mapped this activity to the non-canonical IKK ϵ and consequently to the activation of I κ B α , while TLR-induced activation of the canonical IKK α /IKK β and upstream kinases was not affected.

Earlier studies also showed that POP2 targets p65 transactivation in response to TLR activation [139].

This dual inhibitory activity is also observed in mice, as transgenic POP2 expression protects mice from LPS- and MSU-induced inflammation and from nigericin-induced acute shock, which depends on NLRP3 inflammasome activation [143]. In contrast to POP1, the POP2 reduced cytokines include IL-1 β and IL-18, which are processed by the inflammasome, as well as inflammasome-independent cytokines, including IL-6 and TNF [143]. A slightly different approach to investigate POP2 *in vivo* was employed by Periasamy *et al.* [142], who generated transgenic mice expressing POP2 from the endogenous human POP2 promoter. As expected, macrophages from POP2 expressing mice displayed reduced NF- κ B as well as NLRP3 and AIM2 inflammasome-dependent cytokine responses to inflammatory challenge and infection. Comparable to the finding by Ratsimandresy *et al.*, LPS challenge of POP2 transgenic mice resulted in reduced serum levels of inflammatory cytokines, including IL-1 β , IL-18, IL-6, and TNF, and 20% of POP2 expressing mice survived a lethal dose of LPS with an overall delay in mortality compared to littermate controls. Interestingly, despite the dampened immune response, POP2 expressing mice were protected from bacterial infections, suggesting that POP2 may balance harmful inflammatory responses, while simultaneously promoting a protective innate immune response. Indeed, inflammatory cell infiltration and tissue damage was reduced in POP2 transgenic mice, compared to littermate controls [142]. However, unexpectedly, POP2 transgenic mice showed an increased number of IFN- γ -producing macrophages and an overall increase in IFN- γ levels after bacterial infection, in spite of impaired IL-18 production. The elevated levels of IFN- γ are responsible for an increased capacity of macrophages to kill bacteria or restrain their replication [142]. Hence, while restricting inflammatory cytokine production, POP2 also enhances innate immune function in an IFN- γ -dependent manner, thereby balancing detrimental and beneficial aspects of inflammatory responses [142]. Overall, among all POPs, POP2 acquired a unique ability to interfere with inflammasome priming as well as inflammasome activation, while simultaneously promoting IL-18-independent IFN- γ production.

Similar to POP1, POP2 expression is up-regulated at late time points beyond 4-h TLR activation with LPS or treatment with inflammatory cytokines, including IL-1 β and TNF. Since continuous inflammasome inhibition could be detrimental to intestinal and metabolic health [42,154–158], a regulated expression of POP2 is critical to ensure temporary inflammasome activation before the resolution of inflammasome responses. Hence, in conjunction with other mechanisms, POP1 and POP2 seem to play a critical role in restoring homeostasis after an inflammatory response.

POP3

POP3, located on the human chromosome 1q23, is a single exon gene localized in the HIN-200 gene cluster of IFN-inducible genes between *IFI16* and *PYHIN1* (Fig. 4) [138]. Encoding 113 aa, it is the largest among the POP proteins with only 19% sequence identity to human ASC and 17% to human IFI16, but 61% identity to human AIM2, suggesting that it could have arisen from AIM2-PYD exon duplication. Unlike the other POPs that bind to the adaptor ASC, POP3 preferentially interacts with the PYD of the DNA sensors AIM2 and IFI16 [138]. Homology modeling of POP3, based on the AIM2-PYD, predicts that POP3 has five- α -helices, unlike its other DDF family counterparts that have six α -helices [29,129,138]. The interaction between POP3 and the AIM2-PYD is expected to depend on charged surface residues, but the exact amino acids involved in this interaction have not yet been mapped. Based on the high similarity of the negative EPSP within POP3 and the AIM2-PYD, one would assume that the negative EPSP, which is known to allow the AIM2-PYD to interact with the ASC-PYD, would enable POP3 interactions with ASC. Surprisingly, POP3 does not bind to the ASC-PYD, but preferentially interacts with the PYDs of AIM2 and IFI16 through a yet unknown mechanism [29,138,159].

POP3 expression is detected in monocytes and macrophages, but not in T and B cells. Interestingly, unlike the other POPs, LPS does not up-regulate the expression of *POP3*. Instead, *POP3* expression is up-regulated by IFN- β , which is consistent with its genomic localization within the type I IFN-inducible gene cluster of ALRs [138]. Co-immunoprecipitation studies show that POP3 specifically interacts with the PYD of AIM2 and IFI16, but not with the PYD of MDA and PYHIN1. In addition, in human macrophages, endogenous POP3 co-localized with AIM2 upon infection with dsDNA viruses, such as Modified Vaccinia virus Ankara and with IFI16 upon Kaposi's sarcoma-associated herpesvirus infection. In contrast to POP1 and POP2, which interact with the ASC-PYD to interrupt sensor/adaptor interactions within all ASC-dependent inflammasomes, POP3 interacts with the AIM2-PYD and specifically abrogates the AIM2:ASC complex formation upon AIM2 inflammasome activation [138]. Hence, in the presence of POP3, ASC oligomerization and Caspase-1 activation in response to AIM2 inflammasome activation are impaired, but the activation of other inflammasomes, including NLRP3 or NLRC4, remains unaffected. Furthermore, silencing of POP3 in human macrophages resulted in enhanced IL-1 β and IL-18 secretion in response to AIM2 activation. Therefore, POP3 functions as a selective inhibitor of ALR inflammasomes [138]. Since mice lack POP3 and POP3 is expressed in macrophages, Khare *et al.* [138] developed a transgenic mouse model with monocyte,

macrophage, and conventional DC-specific POP3 expression. Due to the high similarity between the PYDs of mouse and human ALRs, POP3 was able to interact with mouse AIM2 and IFI16. Consequently, POP3 inhibited ALR inflammasome activation in response to viral infection or cytosolic DNA in mouse macrophages, where the secretion of IL-1 β and IL-18, as well as ASC oligomerization and Caspase-1 activation, was greatly reduced. However, POP3 does not impact NF- κ B and MAPK signaling or the transcription of *Il1b*, *Asc*, *Aim2*, or *Ifi16* in response to ALR-agonists [138]. Importantly, mice expressing POP3 were more susceptible to MCMV infection, displaying a higher viral titer in the spleen due to impaired IL-18 and consequently IFN- γ secretion, but did not alter IL-1 β -driven neutrophil recruitment in response to NLRP3-activating MSU crystals, in a mouse peritonitis model [138]. Hence, the inhibitory effect of POP3 is specific for AIM2 and IFI16-inflammasomes only.

It is well established that viral infections promote the activation of IFN-inducible genes, and POP3, located within an IFN-inducible gene cluster, is induced upon viral infection or stimulation with type I IFN. Therefore, POP3 could be regulated by a feedback loop during viral infections. Interestingly, the absence of AIM2 elevates the AIM2-induced type I IFN response in macrophages by an unknown mechanism [71], and mice lacking AIM2 display increased serum amounts of IFN- β during bacterial infection [72]. Consistently, expression of the AIM2 inhibitor POP3 causes higher type I IFN levels as well. However, it remains to be determined how POP3 modulates the enhancement of type I IFN responses and what the functional impact of this response during type I IFN-mediated autoimmune diseases is.

POP4

The human *NLRP2P* pseudogene, located on chromosome Xp11, is thought to originate from retro transposition of an NLRP2/7-like gene, with similarities to POP2 in the first exon (Fig. 4). As pseudogene, the PYD, NBD, and LRR are not expressed; however, a recent study demonstrated that a short peptide of NLRP2P, which shows similarities to POP2, is nevertheless expressed and has been termed POP4 [140]. Like POP2, also POP4 is present only in the genomes of higher-order primates including humans, chimpanzees, orangutans, and *Rhesus macaques*. POP4 is expressed in the placenta, peripheral blood leukocytes, and bone marrow, but not in epithelial or fibroblast cell lines. The POP4 peptide consists of 45 aa, which only form two α -helices and therefore do not represent a complete PYD. However, the corresponding region responsible for NF- κ B inhibition in POP2 is retained. Hence, POP4 reduced TNF-induced NF- κ B activity but did not impact NLRP3 inflammasome-mediated

IL-1 β secretion [140]. POP4 seems to exclusively regulate NF- κ B activity by translocating to the nucleus and blocking TLR-induced RelA/p65 transactivation and secretion of NF- κ B-regulated cytokines, including TNF and IL-6 [140]. LPS stimulation induced the expression of POP4 in THP-1 cells, indicating that inflammation or infection could influence its expression similar to POP1 and POP2. As only the first two α -helices are necessary for fully functional POP2 and POP4 activities, helices 3–6 of POP2 may provide an additional level of regulation, eventually through phosphorylation, which has been observed for POP1 [137].

Viral POPs

Inflammasomes play a crucial role in the host defense against bacterial and viral infections [97,98,101,160–163], and therefore, beneficial inflammasome evasive strategies have evolved in bacteria and viruses [164–168]. Viruses employ multiple immune evasion strategies including the production of decoy cytokine receptors and Caspase-1 inhibitors to modulate inflammasome activation [165–169]. Importantly, viruses are known to hijack cellular proteins to mimic host immunomodulatory proteins that negatively regulate immune recognition and apoptosis of infected cells [169]. For instance, poxviruses, including myxoma virus and Shope fibroma virus, encode viral POPs (vPOPs) [165,166]. The 126-aa myxoma virus protein M013L is a non-secreted, early viral protein that lacks sequences for nuclear localization or secretion. M013L contains an 81-residue N-terminal PYD and similarly, the 107-aa protein gp013L of the Shope fibroma virus contains an 85-residue N-terminal PYD, which is 65% homologous to M013L [165]. Like POP1 and POP2, M013L and gp013L co-localize and interact with ASC [165] but in the absence of ASC, M013L-PYD alone localizes to discrete cytosolic bodies in a PYD-dependent manner, as deletion of the first 22-aa residues from the PYD disrupts the subcellular localization pattern. Consistent with other POPs, expression of M013L and gp013L inhibit Caspase-1 activation and secretion of IL-1 β , whereas cells infected with virus mutants lacking the vPOPs display an increase in Caspase-1 activation [165,166]. Therefore, viruses have evolved POPs to dampen the host inflammatory response to improve viral replication. While M013L seems to either inhibit [170] or induce [165] NF- κ B activation, gp013L is capable of increasing NF- κ B activation in a dose-dependent fashion as well as enhancing TNF-induced NF- κ B activation [165]. Enhanced NF- κ B activation could contribute to the tumorigenesis, which is observed as a consequence of infection with these viruses. Utilizing vPOPs to dampen host defense is conserved, as similar proteins have been identified in other pox viruses including Yaba-like disease virus (ORF18L), swinepox virus (ORF014L), and Mule deer poxvirus (gp024) [165].

COPs

The CARD, like the PYD, promotes homotypic CARD:CARD protein interactions in apoptosis and inflammation signaling pathways [31,171–174]. So far, at least 33 human genes and 24 mouse genes have been identified to encode one or two CARDS [134]. As the PYD, also the CARD is a member of the DDF superfamily and it exhibits the characteristic six antiparallel α -helices with a hydrophobic core and charged residues on the surface, which determine the protein binding specificity [32,33]. Proteins can contain only a CARD or one or two CARDS in combination with other domains including the PYD, LRR, NACHT, SH3, WD, coiled coil, PDZ, GUK, helicase, caspase, kinase, FIIND, BIR, and others [134,171]. Among the CARD-containing proteins are caspases including human Caspases-1/-4/-5 and mouse Caspases-1/-11, which contain an N-terminal CARD motif constituting their pro-domain [175–177]. Three COPs have been identified and are known as pseudo-IL-1 β converting enzyme (pseudo-ICE)/Cop (CARD16), Inca (CARD17), and Iceberg (CARD18) [178–181]. They are located on the human chromosome 11q22 in close proximity to the genes encoding the inflammatory Caspases-1, -4, -5, and Caspase-12 (Fig. 5). Since COPs display the highest similarity to the Caspase-1 CARD, COPs likely originate from a *CASP1* gene duplication event. Like POPs, all COPs are absent from the mouse and rat genome, but orthologs of Cop and Inca exist in chimpanzees and orthologs of Cop, Inca, and Iceberg exist in Rhesus monkeys. Although COPs were discovered over 15 years ago, little progress

has been made in elucidating their precise functions. So far, over-expression studies only provide limited evidence for a Caspase-1 inhibitory role of COPs through a competitive binding mechanism reminiscent of POPs.

Cop

Cop is also known as pseudo-ICE or CARD16 and exists as a long and a short variant, but only the short isoform encoding a 97-aa protein has been characterized [178,180,181]. Cop is expressed in the placenta, spleen, bone marrow, lymph node, and monocytes and its promoter region shares 83.8% sequence homology to the Caspase-1 promoter region [181,182]. In addition, it is nearly identical to the CARD of pro-Caspase-1 with 92% similarity at the nucleotide level and 97% homology at the protein level. Consequently, Cop interacts with the CARD of pro-Caspase-1 and thereby competes with Caspase-1 dimerization and activation, which results in impaired IL-1 β maturation and release [178,180,181]. However, Cop, like pro-Caspase-1, can also dimerize and even form filaments. In addition, a recent study demonstrated that Cop interacts not only with the CARD of pro-Caspase-1 but also with the CARD of ASC, which could provide an additional or alternative mechanism of inflammasome inhibition [182]. Further, Cop was suggested to induce NF- κ B [178,180]. Since all these studies utilized transient over-expression in HEK293 and HeLa cells, which is prone to artifacts, especially since Cop has a strong tendency to aggregate, further investigations are required. For a better understanding of the physiological role and mechanism of endogenous Cop-mediated

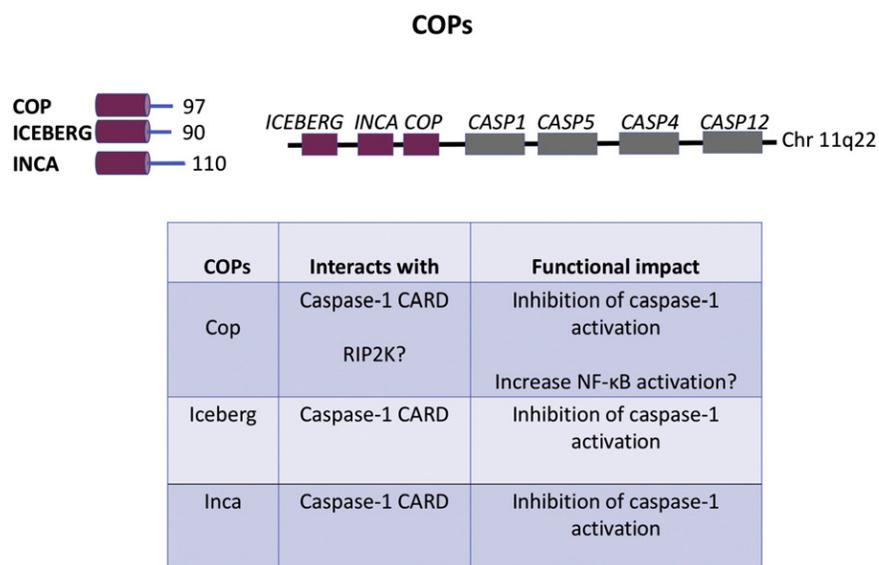


Fig. 5. Graphical representation of the genomic location of COPs, the length of the encoded proteins in amino acids, and their known binding partners and the functional consequence of this interaction.

inflammasome inhibition and NF- κ B activation, carefully designed studies in macrophages are urgently needed.

Inca

Inca, which is also known as CARD 17, is a 110-aa protein and displays 81% identity to the CARD of Caspase-1. In spite of its small size and single domain, Inca comprises four exons and three introns, where exon 1 encodes the first 2 aa, exon 3 encodes the last 18 aa, and exon 2 encodes the CARD. In addition to the similarity in the CARD, also the promoter regions upstream of the initiation codon of *CASP1* and *INCA* are 80.2% identical [182]. Inca is widely expressed in numerous tissues and its expression is up-regulated by pro-inflammatory stimuli, in particular IFN- γ , which also up-regulates pro-Caspase-1 [180]. Similar to Cop, Inca not only dimerizes but also binds to other COPs and the CARD of pro-Caspase-1 [180,183]. Its interaction with pro-Caspase-1 likely prevents Caspase-1:ASC interaction, nucleation of pro-Caspase-1 dimerization, and Caspase-1 activation, since even low concentrations of Inca are sufficient to reduce the ASC-CARD filament-induced polymerization of Caspase-1 CARD [180,183]. Inca seems to preferentially interact with the filamentous form of pro-Caspase-1 and it seems to localize exclusively to the tip of Caspase-1 CARD filaments, indicating that Inca prevents Caspase-1 CARD polymerization through a novel capping mechanism. While Lu *et al.* [183] co-purified ASC and Inca, this putative interaction had no functional impact on ASC oligomerization, which is known to be mediated through the PYD, even at stoichiometrically high amounts of Inca. Hence, Inca appears to preferentially inhibit Caspase-1 activation, also in THP-1 cells, but unlike Cop, Inca does not impact TNF-mediated NF- κ B activation [180,183].

Iceberg

Iceberg, also known as CARD18, is a 90-aa protein, which is only 52% homologous to the pro-Caspase-1 CARD and displays the least homology to the Caspase-1 CARD among the COPs. It was the first identified COP, and the only one for which the NMR structure has been solved [179]. As expected, the NMR structure of Iceberg revealed six anti-parallel α -helices with a hydrophobic core, reminiscent of other DDF members. Helices α 1, α 4, and α 6 have positively charged residues and two negative patches are formed by the α 2 and α 5 helices, as well as at the convergence point of α 4 and α 3. Similar charged patches are present on the surface of pro-Caspase-1, implicating charge-mediated interactions in the Caspase-1–Iceberg binding [179]. Like Cop and Inca, Iceberg interacts with pro-Caspase-1 and tends to self-aggregate [178,179]. By interacting with the CARD of pro-Caspase-1 through a CARD:CARD-mediated interaction, Iceberg prevents

Caspase-1 oligomerization and activation, and subsequently the release of IL-1 β [178,179,183]. However, Iceberg is less potent than Cop and Inca in preventing Caspase-1 activation [179,183], possibly due to its comparatively low homology to the pro-Caspase-1 CARD, relative to the other COPs. The tissue distribution profile is different compared to Cop and Inca, as Iceberg is predominantly expressed in the placenta and the heart, but PAMPs and pro-inflammatory cytokines, such as LPS and TNF, induce Iceberg expression in THP-1, primary monocytes, and macrophages [178,179]. A recent study identified that Iceberg is expressed in differentiating keratinocytes, where expression was elevated in psoriatic skin lesions, potentially implying that dysregulated Iceberg expression could contribute to skin inflammation [184].

Other COPs

Caspase-12

Human Caspase-12 is located in the same gene cluster as COPs, on chromosome 11q23, and is expressed as a full-length or truncated protein. While the full-length Caspase-12L has only been identified in 20% of the population, particularly in individuals of African descent, the truncated Caspase-12S, which is essentially a COP that originated from a point mutation introducing a premature stop codon, is expressed by most the population [185]. Caspase-12L confers hypo-responsiveness to LPS-stimulated cytokine production and subsequently constitutes a risk factor for developing severe sepsis and sepsis related mortality. Accordingly, mice lacking Caspase-12 are resistant to sepsis and clear systemic bacterial infections more efficiently [185,186]. Since over-expressed Caspase-12 interacts with Caspase-1 and prevents its activation, Caspase-12 seems to function as a negative regulator of Caspase-1 [186]. However, these studies have come into question recently, as studies by Walle *et al.* [11,187] demonstrated that this phenotype was not due to the lack of Caspase-12, but to the lack of Caspase-11 in the 129 mouse strain, which is defective in non-canonical, but not canonical inflammasome responses, and which was used to knock out *Casp12*. Newly generated C57BL/6 strain *Casp12*^{-/-} mice revealed intact non-canonical and canonical inflammasome responses and Caspase-1 activation. Therefore, based on recent revelations, the proposed role of Caspase-12 as a negative regulator of Caspase-1 activation is questionable and warrants further investigation.

NOD2-S splice variants

NOD2, a member of the NLR family, is composed of two tandem CARDs, a NACHT and LRRs, and it is

located on chromosome 16. NOD2 is a cytosolic sensor that recognizes muramyl dipeptide (MDP), a peptidoglycan derived from bacterial cell walls, and genetic variants have been linked to Crohn's disease [188,189]. NOD2 is also linked to inflammasome activation, in concert with NLRP1 and NLRP3 [190,191]. An alternatively spliced form of NOD2, NOD2-S, containing only one of the CARDs, has been identified and suppresses MDP responses, as well as NF- κ B activation [192]. NOD2-S interacts with NOD2 and its activating kinase RIPK2, and thereby prevents the formation of the Nodosome and the subsequent release of inflammatory cytokines. Another NOD2 splice variant, NOD2-C2, has been identified. It encodes both tandem CARDs, but unlike NOD2-S, NOD2-C2 over-expression causes likely artificial MDP-independent NF- κ B induction, but is still able to compete with MDP-mediated activation of NOD2 [193].

ASC-c splice variant

In addition to full-length ASC, three different ASC splice variants have been identified, referred to as ASC-b, ASC-c, and ASC-d. These splice variants lack different parts of ASC. ASC-c lacks the majority of the PYD, ASC-d lacks the CARD and retains only 35 residues of the PYD, and ASC-b lacks the flexible linker portion that connects the PYD and CARD [88]. Lack of these domains affects their subcellular localization and ability to act as inflammasome adaptor. ASC-b interacts with the PYD of NLRP3 and still functions as an activating inflammasome adaptor. ASC-c co-localizes with pro-Caspase-1, but prevents inflammasome activation and IL-1 β secretion *in vitro*, and functionally resembles a COP. Although ASC-d retains a part of the PYD, it is unable to interact with NLRP3 and therefore cannot function as the inflammasome adaptor, but also failed to function as a POP, as it cannot compete with ASC [88]. Based on these findings, it is feasible that differential splicing of other multi-domain CARD and PYD proteins may produce a functional COP or POP.

CARD8

CARD8 is also known as tumor-up-regulated CARD-containing antagonist of caspase nine (TUCAN) or CARD inhibitor of NF- κ B-activating ligands (Cardinal), is located on human chromosome 19q13, and is encoded by 13 exons [194]. CARD8 consists of an N-terminal function to find domain (FIIND) and a C-terminal CARD. The FIIND is also present in NLRP1, and similarity in intron and exon arrangement of the FIIND-CARD portion between CARD8 and NLRP1 suggests a common ancestral gene, but like COPs, *CARD8* is absent in rodents [194,195]. After autoproteolytic cleavage within the FIIND, only the CARD remains intact [195] and could therefore qualify

as a COP, as it is also similar to the Caspase-1 CARD. CARD8 was found to interact with Caspase-1, Cop, and Iceberg and binding of CARD8 to Caspase-1 prevents Caspase-1 activation and the release of IL-1 β [196]. Cop and Iceberg seem to compete with CARD8 for Caspase-1 binding, because the presence of Cop or Iceberg resulted in diminished interaction of CARD8 with Caspase-1. In addition, CARD8 interacts with IKK γ /NEMO to inhibit NF- κ B activation, which, however, is not mediated by the CARD [197]. At least two CARD8 protein isoforms exist [198]. Several hereditary polymorphisms of CARD8 are associated with inflammatory diseases [199], which warrants further functional studies on their impact on inflammasome activity.

Viral COPs

Similar to vPOPs, a 91-aa COP, termed GIV-CARD, has been identified in Grouper iridovirus (GIV), which shows high similarity to human Caspase-1 and COPs [200]. GIV-CARD inhibits apoptosis [200]; however, it remains to be determined whether GIV-CARD impacts inflammasome activation.

Perspectives

While substantial progress has been made in understanding the mechanism by which inflammasomes are activated and in elucidating the core principles of inflammasome signaling over the past 16 years, it also became obvious that a tight regulation is essential for ensuring a balanced response that promotes localized host defense, but also resolves in a timely manner to prevent uncontrolled and deleterious systemic inflammation. We are just beginning to understand these checkpoints that evolved to enable such a regulated inflammasome response and homeostasis. The presence of at least six small regulatory proteins that directly guard the assembly of a single-protein complex consisting of only three proteins, a sensor, ASC, and Caspase-1 is quite fascinating and, to our knowledge unprecedented, and this multi-level inflammasome regulation emphasizes the critical importance and ability of quick and efficient immune responses, and their resolution and restoration of homeostasis. While some redundant and unique functions of POPs have been elucidated, particularly their sensor selectivity and their ability to interfere with inflammasome priming, our knowledge about unique and redundant functions of COPs is still very incomplete. Besides their ability to bind to Caspase-1 and to possibly prevent its activation, there is little evidence about potential unique functions of COPs. Their monomeric or filamentous features, as well as their ability to nucleate Caspase-1 CARD filament formation, show some interesting differences, and while Inca seems to employ a novel capping mechanism to prevent the polymerization of the

Caspase-1 CARD, Iceberg co-polymerizes with the Caspase-1 CARD into filaments, but the Caspase-1 inhibitory function of Iceberg is still controversial. Will some COPs be able to modulate Caspase-1 activity, while others will completely shut it down? How and under which physiological circumstances will these differences in Caspase-1 regulation make a difference and will COPs be able to steer the fate of a cell in a certain direction that allows cytokine processing and release and/or pyroptosis? These and many more questions are currently under investigation and provide an exciting field for future research. While POPs and COPs ultimately all block Caspase-1 activation through interactions with different inflammasome components and thereby block its IL-1 β and IL-18 processing function, their role in regulating pyroptosis has not been well investigated. Only POP1 and POP2 have been shown to exhibit dampened lactate dehydrogenase release in response to canonical inflammasome activation and have thereby been implicated in regulating pyroptosis. Therefore, more detailed studies on the effects of POPs and COPs on Caspase-1-mediated pyroptosis, which is triggered by different inflammasome sensors and GsdmD cleavage, need to be carried out. However, since POPs and COPs prevent Caspase-1 activation, we presume that they block Caspase-1-mediated GsdmD cleavage and pyroptosis as well. Once we learn more details about POP and COP functions, we expect to uncover their unique cellular roles. This is particularly intriguing, as the CARD is utilized more frequently and in several distinct signaling pathways and is not only restricted to inflammasome signaling. While the inflammasome pathway is fairly conserved, COP and POP regulators evolved very recently, and it will be of interest to define these potentially more deleterious consequences of uncontrolled inflammasome responses in humans compared, for example, to mice. Research into the POP and COP rheostats is still in its nascent stages, but novel mouse models that were generated over the past several years already provided novel insights into the function of the POP family, while we are still awaiting such insights for the COP family. Although the role of POPs and COPs has been underappreciated, these mouse models compellingly demonstrated that these proteins, even at very low expression levels, can have a profound impact on the severity of inflammatory disease, which is further supported from initial findings of impaired expression of POP1 in cryopyrinopathy patients, providing the exciting prospect that with sequencing of more patients, defects in POPs and COPs may potentially be more broadly linked to inflammasomopathies. A future challenge will certainly be to provide more insights into the function of these protein rheostats, to mechanistically define their unique and common properties, to define their expression during inflammatory and infectious diseases, and to explore their usefulness as a potential therapeutic.

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Abbreviations used:

AIM2, absent in melanoma 2; ALR, AIM2-like receptor; CARD, caspase recruitment domain; COP, CARD-only protein; DAMP, damage-associated molecular pattern; EPSP, electrostatic potential surface patch; IL, interleukin; IFN, interferon; DD, death domain; DDF, death domain fold; GsdmD, gasdermin D; LPS, lipopolysaccharide; MDP, muramyl dipeptide; MWS, Muckle–Wells syndrome; NAIP, NLR apoptosis-inhibitory protein; NBD, nucleotide binding domain; NF, nuclear factor; NLR, NOD-like receptor; NOD, nucleotide-binding and oligomerization domain; PAMP, pathogen-associated molecular pattern; POP, PYD-only protein; PYD, PYRIN domain; TLR, Toll-like receptor.

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