

# Regulatory molecules involved in inflammasome formation with special reference to a key mediator protein, ASC

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**Abstract** The recent identification of cytosolic pattern recognition receptors (PRRs) with leucine-rich repeats, which recognize pathogen-associated molecular patterns (PAMPs), has been garnering considerable attention. Activated PRRs form molecular complexes called inflammasomes, consisting of related proteins that include procaspase 1 [interleukin (IL) 1 $\beta$  converting enzyme (ICE)]. Inflammasomes have been shown to facilitate molecular proximity, stimulate activation of procaspase 1, which consequently produces inflammatory cytokines IL-1 $\beta$  and IL-18 and ultimately lead to the initiation of innate immunity. An adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC), which recruits PRRs carrying the pyrin homologous domain (PYD) and procaspase 1 through PYD and CARD, respectively, is responsible for the formation of some inflammasomes and also activation of procaspase 1. In this review, our main attention will be directed to PYD region analysis of ASC to understand the interaction between PYD-carrying PRRs and ASC. Taking into consideration the other aspects of the *ASC* gene in the proapoptotic ability and down-regulation by methylation, the biological function of ASC will be discussed in relation to the epigenetic aspects of infection, inflammation, and cancer.

**Keywords** Pattern recognition receptors (PRRs) · Leucine-rich repeats (LRRs) · Pathogen-associated molecular patterns (PAMPs) · A PRR family of cytosolic NOD-like receptors (NLRs) · Apoptosis-associated speck-like protein containing a CARD (ASC) · Pyrin homologous domain (PYD) · NACHT-, LRR- and PYD-containing proteins (NALPs) · Caspase 1 · Inflammasome · IL-1 $\beta$  · IL-18 · IL-33 · Apoptosis

## Cytosolic receptors different from TLRs in detecting pathogens-associated molecular patterns

The host has systems that respond rapidly to diverse groups of microorganisms and pathogens, including intracellular metabolites. Recognition of pathogen-associated molecular patterns (PAMPs) is achieved by membrane-type or cytosolic pattern-recognition receptors (PRRs) containing leucine-rich repeats (LRRs). Of these PRRs, toll-like receptors (TLRs) have been most intensively investigated as membrane-type receptors for the last decade. TLRs detect PAMPs, such as lipopolysaccharides (LPS) of gram-negative bacteria, flagellin, and bacterial and viral nucleic-acid motifs [1], thereby inducing various intracellular signaling cascades for the initiation of the innate immune response.

Recently, it has been established that PAMPs are also recognized by a PRR family of cytosolic leucine-rich receptors called nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). NLRs include proteins such as NOD1, NOD2 [2], NACHT (NOD)-, LRR- and Pyrin-domain-containing proteins (PYD; NALPs)-carrying PRRs, interleukin (IL)-1 $\beta$  converting enzyme (ICE)-protease activating factor (IPAF), and neuronal apoptosis inhibitor proteins (NAIPs) [3]. Cytosolic NLRs induce signaling cascades for initiation of innate immune responses as has been seen in TLRs [4]. Additionally, Inohara et al. reported

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that NOD proteins recognize peptidoglycan-derived molecules, leading to activation of NF- $\kappa$ B [5–7].

### **Cytosolic leucine-rich NOD like receptors and IL-1 $\beta$ and IL-18 production**

Another recent topic is that some NLRs recognize exogenously and/or endogenously derived pathogenic factors [8], leading to activation of 45 kD procaspase-1 (previously well known as ICE) into 10P (10 kD) and 20P (20 kD) forms through the assembly of a cytosolic protein complex called the inflammasome [9]. Inflammasomes then induce molecular proximity advantageous to activation of procaspase 1. Active caspase-1 is required for the processing and subsequent release of immature proinflammatory cytokines, such as prointerleukin 1 $\beta$  (proIL-1 $\beta$ ) and proIL-18 to IL-1 $\beta$  and IL-18. In addition to IL-1 $\beta$  and IL-18, it has been reported that IL-33, which is involved in generating a T helper 2-cell response to allergies, is also produced from immature form cleavage by caspase-1 [10].

### **Inflammasome and Caspase 1 activation**

While proIL-1 $\beta$  is induced through NF- $\kappa$ B activation transduced by TLRs and NOD proteins, much attention has been directed to determine the mechanisms that regulate maturation of proIL-1 $\beta$  to its active form, IL-1 $\beta$  and secretion. As previously mentioned, biologically active IL-1 $\beta$  and IL-18 are generated by cleavage of proforms of these cytokines by caspase-1 [11], although the entire mechanism of how IL-1 $\beta$  and IL-18 are released from cells still remains unclear. In some cases, it seems to involve release of the entire inflammasome complex [9]. Attempts to clarify the components of various types of inflammasomes, with special attention to NLRs, are currently being done.

### **Involvement of ASC with NLR, NALP3, and inflammasome formation**

Of the NLRs, considerable attention has been given to NALP3 (Cryopyrin), whose mutation is associated with several hereditary periodic syndromes including Muckle–Wells syndrome, familial cold urticaria, and chronic infantile neurological cutaneous and articular syndrome [12–14], to clarify its recognition of PAMPs and the mechanism of inflammasome formation. It has been reported that various pathogens, toxins, bacterial RNA, and gout crystals trigger NALP3 inflammasomes by inducing a conformational change in NALP3 by an

unknown mechanism, leading to exposure of the NACHT (NOD) domain and NALP3 oligomerization. Following this, NALP3 interacts through homotypic protein–protein interactions of pyrin domains (PYDs) with apoptosis-associated speck-like protein containing a CARD (ASC), a central adaptor within the inflammasome [15]. In addition to a PYD, ASC also contains a caspase recruitment domain (CARD), which is a key domain in recruiting procaspase-1.

### **Second signal of caspase 1 activation responsible for maturation of IL-1 $\beta$ and IL-18**

According to *in vitro* studies, the second signal involved in caspase-1 activation and IL-1 $\beta$  release can be derived from the activation of purinergic receptors of the P2X<sub>7</sub> subtype by the ligand adenosine triphosphate (ATP). P2X<sub>7</sub>-receptor-deficient mice have been shown to be completely defective in IL-1 $\beta$  release in response to exogenous ATP [16]. Although the physiological relevance of ATP-mediated IL-1 $\beta$  release remains unknown, it was reported that cells under certain stressful conditions secrete ATP into the extra-cellular space, and activated P2X<sub>7</sub> receptor [16] induces potassium efflux and plasma-membrane depolarization. It has also been suggested that the depletion of intracellular potassium results in activation of caspase-1 [17, 18]. For the secretion of activated IL-1 $\beta$ , other molecules that cause membrane blebbing and pores also appear to be required [19]. For instance, the activation of calcium-independent phospholipase A2 in association with potassium depletion was reported to be a necessary factor for IL-1 $\beta$  secretion [20].

### **The adaptor protein ASC is responsible for inflammasome formation and caspase 1 activation: discovery and characteristics of ASC**

As one of the critical factors in the formation of inflammasomes, the adapter protein ASC functions as a mediator to recruit NLPs and procaspase 1. The group of Tschopp [9] first reported that ASC is indispensable for the activation of procaspase 1. In THP1 cells, they identified a caspase-activating complex called an inflammasome, comprised of caspase-1, caspase-5, ASC, and NALP1 (DEAF-CAP), a PYD-containing protein sharing structural homology with NODs. By using a cell-free system, they showed that proinflammatory caspase 1 activation and proIL-1 $\beta$  processing is lost upon prior immunodepletion of ASC; moreover, expression of a dominant-negative form of ASC in THP-1 cells blocks proIL-1 $\beta$  maturation and activation of inflammatory caspases induced by LPS *in vitro*.

ASC, which has been recognized as an adaptor protein responsible both for forming apoptosomes and inflammasomes, was originally found and identified as a molecule, which formed a speck near the nucleus of HL-60 cells in association with apoptosis induced by various reagents during the search for molecules responsible for altering the nuclear morphology of HL-60 cells. According to its cDNA sequence, ASC has a C-terminal CARD and a PYD [21]. Pyrin is known as a causative gene for familial Mediterranean fever [22]. From its structural characteristics, we intuitively assumed that ASC must be involved in both apoptosis and inflammation. Afterwards, we then reported on PYD homotypic interactions to form filaments [23] and later determined the responsible amino acid regions for this [24].

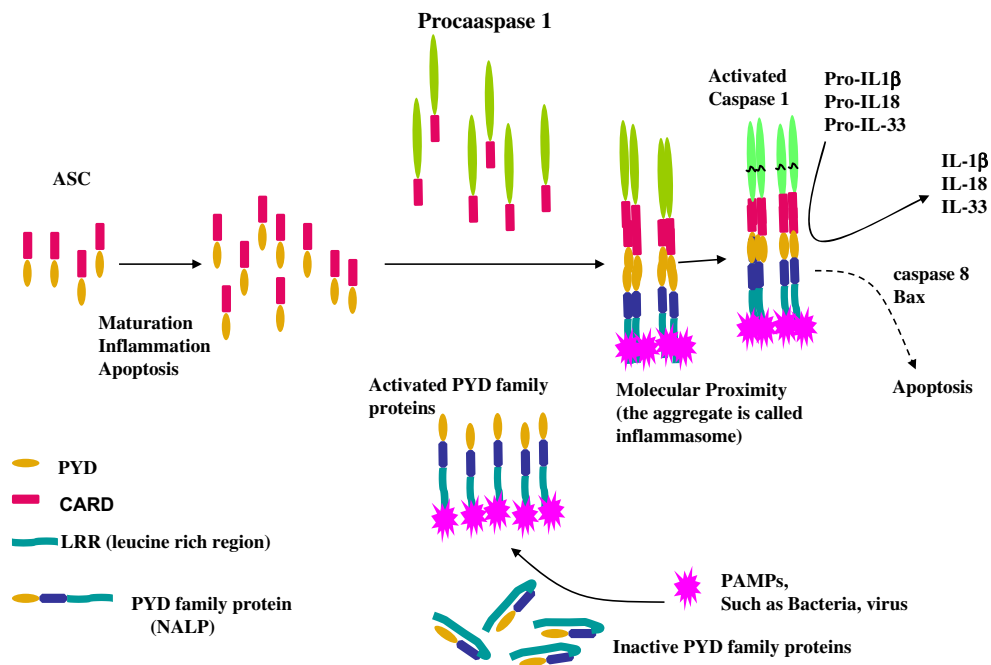
According to immunohistological experiments, ASC was not expressed in the basal layer of the epidermis, but expression became extensive towards the outer layers [25], where cells are exposed to external factors and destined to die. This was also the case in the bowel. Such an expression pattern led us to assume that ASC functions as not only a surveyor of pathogenic invaders, but also as an inducer of programmed cell death. Furthermore, ASC expression by monocytes and neutrophils was induced in a region of inflammation not usually characterized by ASC detection, indicating that ASC is involved in inflammation as well. Apoptotic inducers, such as Fas ligands, also induced the

expression of ASC [26]. Taken together, we first speculated that ASC was related both to inflammation and apoptosis (Fig. 1), which has since been established and recognized. After we reported ASC in 1999, a speedy gene hunt for the protein family containing the pyrin homologous domain and LRR was done, and the functions of some gene products in innate immunity were established.

To certify that ASC really is responsible for activating caspase 1 *in vivo* as well as *in vitro*, we generated ASC-deficient mice and analyzed their phenotypes [27]. We observed that in macrophages of the peritoneal cavity and kupffer cells of the liver, no activation of caspase 1 occurred and thereby no activation of proIL-1 $\beta$  or proIL-18. Thus, the mice were resistant to lipopolysaccharide-induced endotoxin shock [27]. Consistent results using ASC-deficient mice were reported independently by Mariathasan et al. [28].

### Endogenous inhibitors of caspase 1

Because IL-1 $\beta$  plays a crucial role in septic shock, it is rational to presume that there are numerous endogenous inhibitors of IL-1 $\beta$  activity. IL-1 $\beta$  -receptor antagonists inhibit IL-1 $\beta$  by binding the receptor without stimulating the intrinsic signal [29]. Proteinase inhibitor 9 [30],



**Fig. 1** Hypothetical functions of ASC. The NALPs (PYD-carrying PRRs) contained not only PYD, but also leucine-rich regions, which are receptors for pathogenic molecules similar to those seen in TLR. Conceptually, PYD-carrying PRRs bound to pathogenic molecules are activated to react with ASC, thus inducing molecular proximity. The aggregates formed by molecular proximity are called inflammasomes.

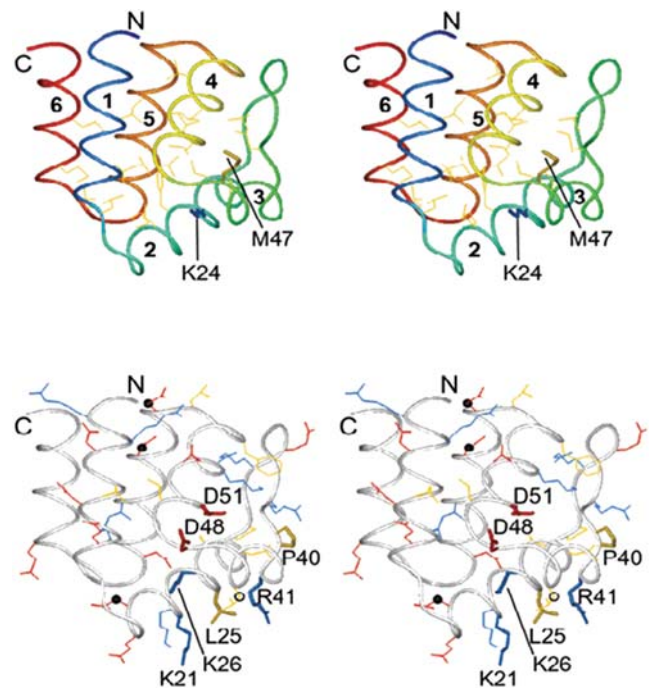
Pro-caspase 1 is also recruited to the inflammasome and activated to produce mature IL-1 $\beta$ , IL-18 and IL-33. Just afterwards, or simultaneously to, these reactions, ASC has also been suggested to induce apoptosis by recruiting caspase 8 and/or Bax. Thus, ASC functions in cells damaged by infection or other accidents to produce cytokines as a sign of danger to the host, likely leading them to programmed cell death

caspace-12 [31] and CARD 8 [32], all have a suppressive effect on caspace-1. Intact pyrin inhibits caspace-1 activation, while mutated pyrin, as identified in familial Mediterranean fever (FMF), cannot inhibit caspace-1 activation and consequent IL-1 $\beta$  release [33–35]. There seems to be two mechanisms that inhibit caspace 1 activation by *pyrin* products. One mechanism might involve direct interaction of the amino-terminal death domain (DD) of pyrin with the intracellular adaptor ASC proteins, which then inhibits caspace-1 activation. In addition, the carboxyl-terminal B30.2 domain of pyrin was recently proven to bind to the catalytic subunits(p20, p10) of caspace-1 [36]. It is intriguing that a large percentage of FMF-associated pyrin mutations reside in the B30.2 domain [36]; whereas these mutated *pyrins* might induce chronic inflammation, resistance to some infections should increase owing to an augmented innate immune response. Furthermore, several viral-negative regulators of caspace-1 activation have been described [37, 38]. In one case, the Myxoma virus encodes for a PYD-containing protein, which interacts with ASC to inhibit caspace-1 [38].

### 3D structure of the PYD of ASC showing an additional death domain resembling DD, DED, and CARD

Because the N terminal half (1–90 amino acids) of ASC is highly homologous to the N terminus of pyrin, we named it the pyrin-like domain (PYD) [21]. Thereafter, the PYD motif was found in more than 30 human and virus genes in silico and established as a new family within the DD superfamily based on a convincing combination of sequence alignment, functional association, structure prediction, and homology modeling [9, 39–42]. The DD superfamily, which is composed of six-helices, includes the DD, death effector domain (DED), and CARD families, and mediate the crucial interactions in apoptotic and inflammatory signaling pathways. Death domains function as protein–protein interaction motifs, serving as adaptors for assembling components of signaling complexes. A classic example of this is the DD of Fas, which recruits FADD through interactions with its DD and subsequently recruits procaspase 8 through interactions of its DED domain to form the death-inducing signaling complex. Death domains only associate with their own family members, forming both homo and heteromeric associations, but strictly form homotypic interactions. The structures of two DD heterodimers indicate that at least two different binding interfaces are possible, and models based on these structures suggest that both interfaces may be used simultaneously in a trimeric complex [43–45].

The three-dimensional PYD structures of ASC and NALP1 were determined by magnetic resonance imaging (MRI) spectroscopy [46, 47], revealing close structural



**Fig. 2** Three dimensional PYD structure and critical amino acids of ASC(quoted from [24]). We analyzed the three-dimensional structure of PYD in collaboration with Dr. Otting at Australia University. By including the results of mutational experiments, we could identify the responsible amino acids for PYD–PYD homotypic interaction; Basic K (lysine) and R (arginine) on helix 2 and D (aspartic acid) on helix 4 proved to form a critical region

similarity to DDs, DEDs, and CARDS. The structural alignment with other members of the DD superfamily differed from previously predicted amino acid sequence alignments, however. Although the ASC PYD region was a very close fit to the six-helix-fold model characteristic for the DD superfamily (Fig. 2), the structure of the NALP1 PYD differed from all other known DD superfamily structures in that the helix 3 was replaced by a flexibly disordered loop. As helix 3 participated in homotypic interactions with the other members of the DD superfamily, it is surprising to us and likely significant for the function of NALP 1.

The three-dimensional structures determined by MRI also suggested that two highly positively and negatively charged surfaces in ASC PYDs result in a strong electrostatic dipole moments, which are predicted to be present in related PYRIN domains as well.

### Role of charged and hydrophobic residues in the oligomerization of the PYD of ASC

There are many predictions for the mode of PYD–PYD interactions described above, but no experimental evidence with MRI has been available because PYD–PYD complexes produced biochemically form highly insoluble aggregates in physiological conditions (e.g. neutral pH),



thus making it impossible to use them for MRI spectroscopic measurement (Fig. 2).

Overexpression of ASC PYD in cells has been shown to result in filamentous structures in the cytoplasm through self-oligomerization [21]. Such cytoplasmic filament-like structures have been observed for many domains of the DD superfamily [48–51]; for example, overexpression of the DEDs of FADD and caspase-8 results in cytoplasmic filaments, and the CARDs of caspase-2 and mE10 have also been shown to form intracellular filaments through homotypic interactions. It is thought that filament formation reflects the physiological homotypic interactions of each DD fold. To assess the role of charged and hydrophobic residues in the oligomerization of ASC PYDs, a series of alanine-point mutants were made, and effects on ASC filament formation in ASC cDNA-transfected cells were examined. In addition, oligomerization-deficient alanine mutants of charged residues were further replaced with different charged or polar residues [24].

Hydrophobic residues that were critical for the formation of ASC PYD filaments were highly conserved among PYD family proteins. Most of the critical hydrophobic residues were buried inside of the PYD structures and thus, may play a crucial role in maintenance of the three-dimensional fold. The alanine-point mutants in Leu 25 (helix 2) and Pro40 (helix 3), which were partly facing the solvent outside of the PYD structure, showed deficiency in filament formation, suggesting an important role of Leu25 (helix 2), Pro40 (helix 3), and Met47 (helix 3) in interactions at the PYD–PYD interface.

Mutation experiments with charged residues revealed that the charges of Lys21 (helix2), Lys 26 (helix 2), and Arg41 (helix 3) and of Asp48 (helix 4) and Asp51 (helix 4) were critical for oligomerization of ASC PYDs. In addition, MRI analysis showed that Arg41 (helix 3) resides in proximity of Lys21 and Lys26 (helix 2). Thus, we currently propose that electrostatic forces between the positive charge cluster of helices 2 and 3 and negative charge cluster of helix 3 are critical for oligomerization of ASC PYD and possibly also for ASC interactions with the other PYD family proteins. It is also likely that the hydrophobic residues of Leu25 (helix 2), Pro40 (helix3), and Met47 (helix 3) stabilize PYD–PYD interactions formed by the charged residues. As MRI predicted the possible interactions between the positive charges of helices 2 and 3 and negative charges of helices 1 and 4, the mutational data based on the filament formation of ASC PYDs are mostly consistent with predictions from previous MRI studies [46, 24].

#### Arg42Trp pyrin mutants are associated with familial Mediterranean fever (FMF)

The positive charge of Arg41 in ASC PYDs has been predicted to be essential for PYD–PYD interactions by MRI and mutation studies [46, 24]. FMF is caused by point mutations in pyrin. One of these FMF-associated mutants, Arg42Trp, is located in the PYD of pyrin [52] and corresponds to Arg41 in ASC. In the context of the ASC

**Table 1** Activation of inflammasomes by various stimulants and ASC involvement

Inflammasome inducers (PAMPs, etc.)	Detectors (NLRs)	Involvement of ASC in IL-1 $\beta$ production and macrophage cell death
Potassium efflux-inducing agents (ATP, TLR stimulation) Staphylococcus aureus Listeria monocytogenes Bacterial RNA, Gout crystals Trinitrophenylchloride Other inducers	NALP3 (Cryopyrin) <sup>d</sup>	Indispensable for both
Francisella tularensis <sup>a</sup>	NALP?	Indispensable for both
Legionella pneumophila (cytosolic flagellin) <sup>b</sup>	IPAF+NAIP5	Probably indispensable for early reactions but dispensable later during infection or in high numbers of infectants.
Salmonella typhimurium <sup>c</sup> (SipB, cytosolic flagellin)	IPAF+NLR?	Probably indispensable for early reactions but dispensable later during infection or in high numbers of infectants

Besides the NLRs shown here, NALP 1, NALP 2, 6, 12 and CARDINAL were also reported to be involved in inflammasome activation [15, 60, 61].

<sup>a</sup> Francisella tularensis molecules in the cytosol could be detected by an unidentified NLR, which links ASC and caspase-1 activation [62, 63].

<sup>b</sup> L. pneumophila are sensed by IPAF (ICE-protease activating factor) and NAIP5 (neuronal apoptosis inhibitor protein 5)

<sup>c</sup> and perhaps S. typhimurium by IPAF and an additional NLR [64–66, 28]. ASC is important for S. typhimurium-induced caspase-1-dependent processing of IL-1 $\beta$  but less important for host-cell death.

<sup>d</sup> NALP3 recognizes various pathogens and has been intensively investigated [67–69].

PYDs, the Arg42Trp mutation was found to abolish filament formation [24]. It would be intriguing to see if the molecular mechanism behind the FMF. Arg42Trp mutation was based on the disruption of interactions between the PYDs of pyrin and ASC. This interaction is known to be of functional importance, as pyrin interacts with ASC to inhibit ASC-mediated caspase-1 activation. Notably, however, the sequence conservation between the ASC PYD and pyrin is limited. For example, of the five charged residues found to be essential for filament formation of ASC PYDs, only two of them were conserved in pyrin. Furthermore, mutation of the non-conserved residues Lys21, Asp48, and Asp51 in ASC to the oppositely charged residues Glu, Arg, and Lys, respectively, which are found in pyrin, resulted in abolishment of filament formation. Point mutations of ASC PYD were thus not sufficient to stimulate the ASC-binding function of pyrin. A more thorough mutational study of the PYD of pyrin is required to shed light on the inhibitory role of these oppositely charged pyrin residues.

### Concluding Remarks

Recently, cytosolic PRRs, containing leucine-rich repeats, have been identified and discovered to detect PAMPs. PRRs are then activated to form inflammasomes, which stimulate maturation of procaspase 1 and proIL-1 $\beta$ , proIL-18, and proIL-33 and lead to initiation of innate immunity and apoptosis of macrophage cells [53]. The inflammasome consists of cytosolic PRRs, caspase 1, and adaptor protein ASC, the latter recruiting procaspase 1 and other PRRs through PYD and CARD. It remains to be clarified which NLRs specifically recognize which PAMPs, and which NLRs does ASC specifically interact with besides NALP3 (Table 1). A year after our first report, ASC was also reported to act as a proapoptotic and tumor suppressor gene down-regulated by methylation [54]. We confirmed methylation of the ASC gene in human melanoma, colon cancer, and lung cancer, and its down-regulation correlated with the degree of methylation in CpG islands [55, 56]. They reported the dominant negative mutant for caspase 9 but not caspase 8 inhibits the apoptosis induced by over-expressing ASC in human embryonic kidney (HEK)293 cells [54]. On the other hand, a dominant negative mutant for caspase 8 but not caspase 9 inhibits the apoptosis induced by the formed oligomerization of ASC [57]. ASC was further reported to unexpectedly react with Bax and function as a promoter of apoptosis [58]. Recently, it was reported that ASC activated the cleavage of Bid by caspase 8 and induced Bax-dependent apoptosis, and that the suppression of Bid reduced the ASC-mediated apoptosis [59]. So far, although no tumorigenesis is evident in ASC-

deficient mice, chemical carcinogenesis and infection experiments are underway using ASC-deficient mice with APC- and p53-deficient mice.

Thus, taking the biological functions and the epigenetic aspects of ASC together, ASC appears to be a useful probe to understand the mutual relationships among aging, innate immunity, inflammation, and cancer.

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