


REVIEW ARTICLE

Evaluation of ASC as a therapeutic target for Alzheimer's disease

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Funding information

National Institute on Aging; National Institutes of Health, Grant/Award Number: U54AG065181

Abstract

Neuroinflammation is increasingly recognized as a central contributor to the pathogenesis and progression of Alzheimer's disease (AD). The apoptosis-associated speck-like protein containing a CARD (ASC), encoded by the *PYCARD* gene, plays a critical role in the formation of multiple inflammasomes, including NLRP3, a key mediator of inflammation signaling. Beyond its role in inflammasome formation, extracellular ASC specks have been shown to promote amyloid- β aggregation, showing a potential link between inflammation and plaque formation. In this review, we examine the role of ASC in AD pathology and highlight emerging tools to study ASC biology and strategies for ASC targeted drug discovery.

KEYWORDS

Alzheimer's disease, ASC, cell assays, drug discovery, *PYCARD*

Highlights

- Apoptosis-associated speck-like protein containing a CARD (ASC) is a critical inflammasome scaffolding protein that promotes both neuroinflammation and A β aggregation in Alzheimer's disease (AD).
- ASC's tendency to self-aggregate complicates biophysical assays, but robust live-cell assays enable functional evaluation.
- Reported small molecule inhibitors lack specificity and have a high probability of being false positives, highlighting the need for future drug discovery efforts.

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1 | BACKGROUND

The gene *PYCARD* codes for the protein ASC which functions as a molecular scaffolding protein component of various inflammasomes. Inflammasomes are innate immune protein complexes that evolved to assemble in response to specific environmental or cellular stimuli especially those associated with cellular damage or infections.¹ Several inflammasomes require the adaptor protein ASC for full assembly and caspase-1 activation, including Absent in Melanoma 2 (AIM2), members of the NOD-like receptor (NLR) family such as NLRP1, NLRP3, and NLRC4 (NLR family CARD domain-containing protein 4, also known as IPAF), and the Pyrin inflammasome, although some of these can still function even in the absence of ASC.^{2–6} Many of these inflammasomes have been connected to Alzheimer's disease (AD),^{7–9} but NLRP3 remains the most often studied with its pathology, placing it at the core of AD related inflammasome research.^{10–13}

The assembly of the NLRP3 inflammasome occurs in two distinctive phases, priming and activation (Figure 1).^{14–18} Activation of Toll-like receptors (TLRs) or interleukin-1 receptors (IL-1Rs) recruits adaptor myeloid differentiation primary response 88 (MyD88), initiating a signaling cascade through IL-1 receptor-associated kinases (IRAK1 and IRAK4)¹⁹ and the E3 ubiquitin ligase tumor necrosis factor receptor-associated factor 6 (TRAF6).²⁰ TRAF6, together with the E2 conjugating a complex of Ubiquitin-conjugating enzymes Ubc13/Uev1A, cat-

alyzes K63 linked polyubiquitination of itself and transforming growth factor β -activated kinase 1 (TAK1), creating a platform for the recruitment of TAK1-binding proteins TAB1 and TAB2/3 complexes. TAK1 then phosphorylates and activates two major downstream pathways.²¹ First, the I κ B kinase (IKK) complex (IKK α , IKK β , and NEMO/IKK γ) leading to the degradation of inhibitor of NF- κ B (I κ B) and subsequent release and nuclear translocation of nuclear factor kappa-B (NF- κ B). Also, the mitogen-activated protein kinase (MAPK) pathway, activating c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 kinases that converge on transcription factor Activator protein-1 (AP-1). Together, NF- κ B and AP-1 drive the expression of inflammasome related genes, including *NLRP3*, *PYCARD* (ASC), pro-caspase-1, pro-IL-18, and pro-IL-1 β .^{22,23}

Following the priming phase, a secondary activation signal is required to trigger the formation of the inflammasome complex. The depictions in Figure 1 are intentionally simplified with each step shown representing an intricate series of molecular events that converge on the assembly of NLRP3, ASC, and pro-caspase-1. For example, under resting conditions, the ASC created in the priming stage is not freely distributed in the cytosol but collects within the nucleus,²⁴ where it forms a complex with IKK α ²⁵ until triggering stimuli such as ATP or nigericin cause ASC to be released and exported from the nucleus to the cytoplasm by chromosome region maintenance 1 (CRM1) also known as exportin 1 (XPO1).²⁶ Several such stimuli can

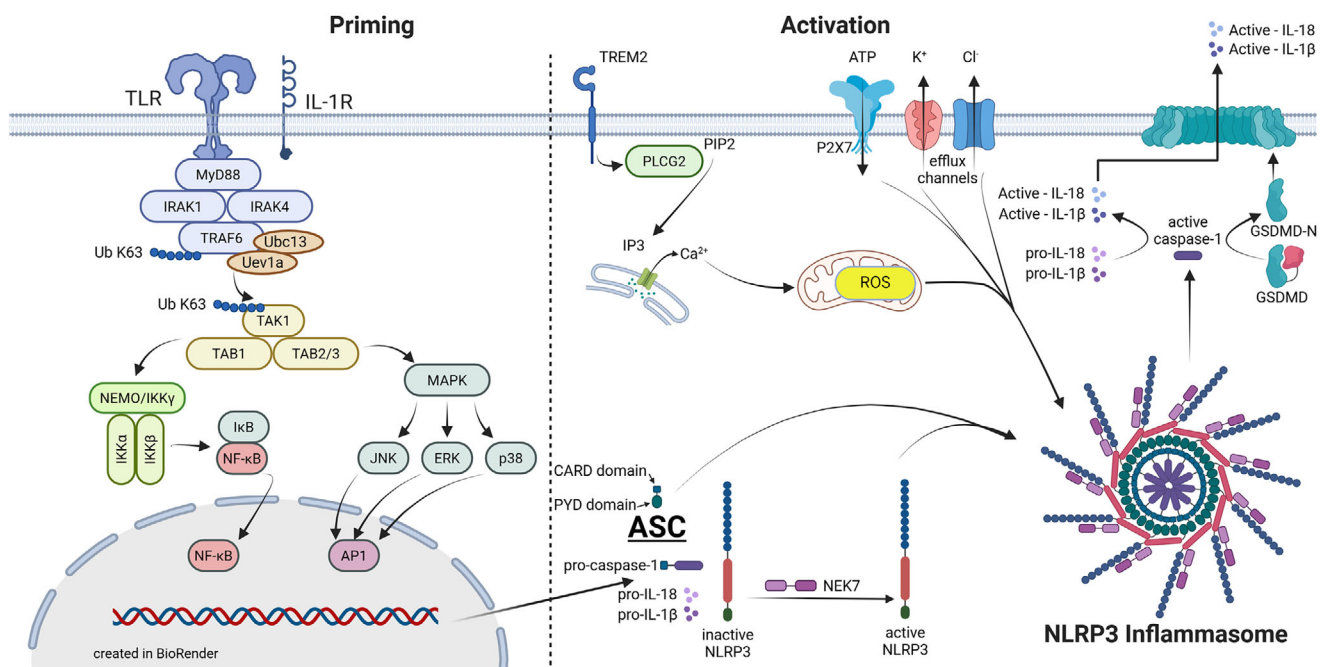


FIGURE 1 The NLRP3 inflammasome signaling pathway. Left: *Priming*. TLR/IL-1R signaling activates NF- κ B/AP-1, inducing NLRP3, ASC (PYCARD), pro-IL-1 β , and pro-IL-18 expression. Engagement of TLR/IL-1R recruits MyD88 and assembles the myddosome (MyD88/IRAK4/IRAK1). IRAK1 activates TRAF6, which undergoes K63-linked ubiquitination (with Ubc13/Uev1A) to activate the TAK1/TAB2/3 complex. TAK1 then signals through two pathways: phosphorylation of the IKK complex (IKK β /IKK α with scaffold NEMO/IKK γ) to release NF- κ B, and activation MAPKs (JNK, p38, ERK) to form AP-1. Right: *Activation*. Various inputs, including ATP, K⁺ efflux, Cl⁻ flux, mitochondrial ROS, and Ca²⁺ mobilization (e.g., PLCG2 activation) can trigger the assembly of the NLRP3 inflammasome. NEK7 activates NLRP3 allowing aggregation with ASC via PYD-PYD interactions. Pro-caspase-1 is recruited via CARD-CARD domains leading to the formation of active caspase-1, maturation of IL-1 β /IL-18, and cleavage of GSDMD. GSDMD-N pores are formed allowing the release of active IL-1 β /IL-18.

provide this initiate this activation stage. ATP binding to the purinergic receptor P2X7 induces potassium efflux,²⁷ while nigericin acts as a K⁺/H⁺ ionophore.²⁸ Similarly, activation of phospholipase C gamma 2 (PLC γ 2) promotes ER calcium release through inositol triphosphate (IP $_3$) mediated channels,²⁹ which secondarily increases mitochondrial Ca²⁺ uptake and reactive oxygen species (ROS) production.³⁰ The activation of NLRP3 is regulated by E3 ligases,^{31,32} and NLRP3 must also undergo a conformational change mediated by NEK7^{14,33,34} before oligomerization and complexation with ASC can commence.

The formation of inflammasome complexes is initiated by the binding of sensor proteins, such as NLRP3 or AIM2, to their respective ligands. Binding of an inflammasome sensor protein to its ligand initiates the inflammasome assembly process by recruiting the scaffolding protein ASC through its PYD domain to nucleate into fibril structures.³⁵ ASC protein fibrils recruit protein caspases through caspase recruitment domains (CARD) leading to caspase-1 activation. This protein assembly and recruitment process results in the formation of the large paranuclear ASC aggregate or speck. Super resolution imaging of ASC proteins reveals a striking redistribution of diffuse ASC proteins to dense speck structures upon stimulation in THP-1 cells.³⁶ The activated caspase enzymes in the inflammasome complex proteolytically mature cytokines to their active forms including IL-1 β and IL-18. This process of inflammasome dependent cytokine maturation is impaired in PYCARD knockout mice, which are viable but have impaired processing of IL-1 β and IL-18 while other cytokines including tumor necrosis factor alpha (TNF- α) are not affected.³⁷ This caspase and cytokine activation mechanism can be blocked in a cell free system using ASC targeting antibodies.³⁸ The inflammasome can also initiate pyroptosis or inflammatory cell death in which pores of gasdermin D (GSDMD) protein form in the cellular membrane.³⁹ Membrane rupture leads to the release of the aforementioned cytokines and the ASC specks into the extracellular space where they can continue to exert biological activity and may serve to perpetuate inflammation where they act as a mechanism of immune signal propagation.⁴⁰

Neuroinflammation is an established feature of AD pathophysiology with several immune genes having been identified as genetic risk factors for AD.⁴¹ Increased levels of cleaved IL-1 β and NLRP3 inflammasome activation have been detected in AD patient brains.⁴²⁻⁴⁴ In other studies, NLRP1 and caspase-6 have been found to be elevated in AD cortexes and cerebellums compared to healthy controls.^{45,46} In transgenic mouse models of AD, deletion of the NLRP3 reduces plaque burden and improves cognition.⁴⁷ It has been shown that NLRP3 itself can be activated by aggregated amyloid beta, which may serve as one mechanism of inflammasome activation in the AD brain.^{48,49} However, it should be noted that one study found no effect upon NLRP3 disruption in transgenic AD rodent models, suggesting that NLRP3 inflammasome specific activity may be dispensable for A β pathology in this context as other inflammasomes may be able to compensate.⁵⁰

ASC specks may also be of importance in AD by acting as molecular seeds of amyloid plaques. ASC specks have been shown to enhance A β aggregation and intrahippocampal injection of ASC specks into an AD mouse model increased the number and area of amyloid plaques.⁵¹ Moreover, fluorescent imaging revealed the presence of ASC protein

in AD patient amyloid plaque deposits. Total ASC protein levels in AD patient serum are higher than in control patients.⁵² Interestingly, ASC specks may have some value as a biomarker of neurodegeneration because ASC specks are more abundant in the serum and cerebrospinal fluid (CSF) of AD and Parkinson's disease patients compared to healthy controls.⁵³ Furthermore, ASC fibrils have been shown to enhance the toxicity of A β and promote pyroptosis of primary microglial cells, potentially contributing to toxic inflammation.⁵⁴

ASC dependent inflammasome activity has been directly linked to tauopathy. Aggregated tau species can act as both priming and activation signals for the NLRP3 inflammasome. Aggregated PHF6 (paired helical filament 6), the tau derived hexapeptide VQIVYK (Val₃₀₆-Lys₃₁₁) that models the aggregation prone core of pathogenic tau, caused a short lived increase of PYCARD gene expression and ASC protein levels in HMC3 cells accompanied by increases in pro- and mature IL-1 β .⁵⁵ In lipopolysaccharide (LPS) primed primary mouse microglia, treatment with tau seeds triggered an increase in IL-1 β in a similar, though attenuated, fashion to nigericin.⁵⁶ As expected, this increase was abolished when microglia taken from ASC^{-/-} mice were used. Tauopathy mouse models similarly show progressive inflammasome engagement. Tau22 mice exhibit age dependent increases in ASC and cleaved caspase-1, while also showing higher levels of ASC specks compared to wild-type (WT) controls. ASC knockout Tau22 mice displayed markedly lower tau hyperphosphorylation and aggregation.⁵⁷ Similar results were seen with PS19 mice,⁵⁶ supporting a loop in which tau aggregates activate the inflammasome, and inflammasome signaling in turn amplifies tau pathology. The NLRP3 inflammasome regulation of pTau occurs, at least in part, through control of kinases and phosphatases. The ASC knockout Tau22 mice had less inactive protein phosphatase 2A (PP2A) and less of its natural inhibitor protein phosphatase methylesterase-1 (PME-1). These changes together with less Ca²⁺/calmodulin-dependent protein kinase II- α (CaMKII- α), shift the balance away from hyperphosphorylation.⁵⁷ Finally, hippocampal injection of A β -rich brain homogenates from APP/PS1 mice robustly increased tau hyperphosphorylation in Tau22 mice, but not the ASC knockout. These results show that the NLRP3 inflammasome is one of the critical mechanisms by which amyloid pathology can be translated into tauopathy.

An increase of ASC expression and the presence of specks has been observed along the continuum from cognitively normal controls to early AD and to more advanced stages in both serum and CSF.⁵³ In one study, patients labeled as having intermediate AD, characterized by higher Thal A scores (wider A β spread), CERAD C scores (greater neuritic plaque density), and more advanced tau pathology, had significantly more ASC positive cells across multiple hippocampal and cortical regions than low AD cases.⁵⁸ Using two ASC antibodies, they reported that ASC immunoreactivity increased in neurons (IC100 antibody) and in microglia like cells (mouse antiASC), while NLRP1 localized predominantly to neurons and NLRP3 primarily to microglia.

Importantly, ASC specks alone are not the most informative biomarker. In one study, serum ASC specks distinguished early AD from controls with only modest accuracy with an area under the curve (AUC) in the receiver operating characteristic (ROC) of 64%. When

ASC specks were evaluated relative to other aggregates, performance improved substantially with the ASC/A β ratio reaching an AUC of about 78%, and the composite (ASC + p-tau-AT8)/A β achieving 92% for early AD versus controls, with similar trends and higher values reported for more advanced disease stages.⁵³ Further analysis showed that small round ASC specks, a morphology associated with recent inflammasome activation and pyroptotic release, were enriched in AD (and Parkinson's) samples. Together, these studies support a stage linked increase in inflammasome activation, with ASC present in both neurons and microglia in the progression of AD.

Overall, these findings indicate that ASC is a central amplifying link between amyloid pathology, tauopathy, and chronic inflammasome activation. Targeting ASC by preventing its recruitment into inflammasomes, and thus limiting IL-1 β /IL-18 maturation and extracellular ASC speck formation, may therefore offer a strong strategy to modify, slow, or prevent the disease.

2 | EXPRESSION OF PYCARD MRNA AND ASC PROTEIN

The *PYCARD* gene is conserved across species, including both mice and humans. In both species, *PYCARD* is predominantly expressed in immune related tissues, such as lymphoid tissues and bone marrow, underscoring its involvement in immune responses.^{59–63} Notably, in the healthy brain, *PYCARD* expression is relatively low in both humans and mice, suggesting a limited role in central nervous system functions under normal, noninflammatory conditions.

In mice, a single nucleotide polymorphism (SNP) in the 3' untranslated region (UTR) of the *PYCARD* gene has been shown to affect mRNA stability and protein expression levels, thereby influencing inflammasome activity.⁶⁴ This SNP leads to increased *PYCARD* mRNA stability and higher ASC protein levels, resulting in enhanced inflammasome activation.

ASC protein in humans has three alternative isoforms resulting from differential splicing, referenced in UniProt as Q9ULZ3, as Q9ULZ3-1, Q9ULZ3-2, and Q9ULZ3-3. Notably, one splice variant lacks exon 2, which is observed under specific conditions, such as certain inflammatory responses.⁶⁵ Additionally, *PYCARD* has 236 genetic variants listed in UniProt, which can be evaluated for somatic occurrence and impact levels. However, the clinical significance of these variants and mutations remains largely unknown.

PYCARD exhibits varying expression levels across tissues as reported on the Human Protein Atlas website (Figure 2). Notably, *PYCARD* expression is highest in tissues such as spleen and bone marrow, emphasizing its tissue enhanced specificity in immune related organs and its potential role in regulating inflammatory responses and immune signaling pathways. This distinct pattern of expression underscores *PYCARD*'s dominant role in immune related functions, while hinting at its specialized, context dependent activity in other tissue systems.

The Human Protein Atlas reports that ASC protein levels vary across cell types across various tissues (Figure 3). High expression lev-

els are predominantly observed in immune cells, including Hofbauer,⁶⁶ Kupffer,⁶⁷ and Langerhans⁶⁸ cells where NLRP3 inflammasome activity has been investigated. In healthy brain tissue, moderate protein expression is seen in microglia cells, but nearly absent in neurons and astrocytes in a noninflammatory state.

PYCARD has also been evaluated on the Agora AD Knowledge Portal. Driven in large part by a Multi-omic Score of 1.96 out of 2, the overall Target Risk Score is 3.28 out of 5 placing this gene in the top 20% of all analyzed and making *PYCARD* an attractive AD target.

3 | PROTEIN STRUCTURE

ASC is ~24 kDa cytosolic protein comprised of mainly two domains: N-terminal pyrin domain (PYD) and C-terminal CARD joined by 23 amino acid linker (Figure 4A). This long flexible linker allows for independent movement of the two domains, facilitating the binding of each to their respective partners via PYD-PYD (e.g. NLRP3) and CARD-CARD (e.g. pro-caspase-1) interactions (Figure 1). The full-length nuclear magnetic resonance (NMR) structure of ASC shows six helix bundle of each PYD and CARD domain.⁶⁹ The AlphaFold3 model of full-length ASC indicates a high confidence score for both PYD and CARD domains, but less confident score (pIDDT < 50) for the linker region. Structural alignment of the reported solution structure with the AlphaFold3 model shows almost complete overlap with PYD domain but not the CARD domain (RMSD = 1.031 Å). These differences are a consequence of the highly flexible loop connecting the two domains (Figure 4B).

On comparing the solution structure of ASC PYD domain with the cryo-EM structure³⁵ (Figure 4C), there is a slight difference in the positioning of helix4 (RMSD = 1.343 Å) and in case of CARD domain,⁷⁰ there are differences in helix 1,4,5, and 6 (RMSD = 1.309 Å). We also compared both domains with the other proteins containing PYD and CARD domains. The PYD domain of NALP1⁷¹ contains a long-disordered loop instead of the helix 4 (RMSD = 1.191 Å) (Figure 4D). Similarly, there are differences in the helices 1, 4, and 6 compared to the NLRP1 CARD domain⁷² (RMSD = 1.152). These differences between solution and cryo-EM structures of ASC domains along with interprotein differences suggest the possibility that these regions may play an important role in filament formation.⁷²

There are four isoforms of ASC due to alternative splicing of the *PYCARD* gene: ASC, ASCb, ASCc, and ASCd (Figure 5). ASC, the canonical isoform, is the full-length protein (~22 kDa) containing both of the PYD and CARD domains. ASCb is similar to ASC, containing both domains, but the linker is only three amino acid long. ASCc and ASCd lack the full PYD or CARD domains. ASCc contains the CARD domain and a partial PYD domain, while the CARD domain is absent in ASCd. It has been shown that canonical ASC oligomerizes faster than ASCb.⁷³ ASC oligomerizes into filamentous disks whereas ASCb self assembles in linear filaments. Different cell types showed different levels of expression for these ASC isoforms. One study showed that ASC and ASCb are abundantly expressed in THP-1 cells while ASC and ASCc isoforms were expressed in J774A1 macrophages.⁷⁴ These isoforms when coexpressed form different kinds of aggregates. In HEK-293

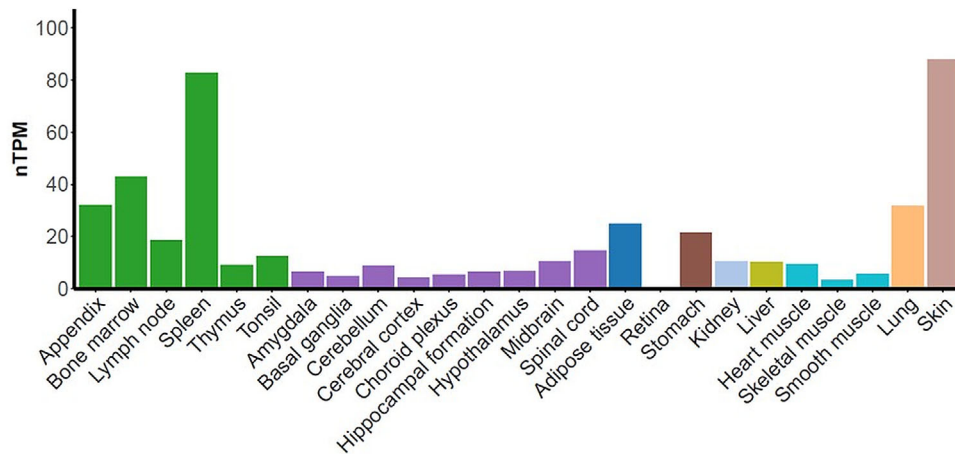


FIGURE 2 Gene expression. RNA expression of *PYCARD* in multiple tissues quantified in normalized transcripts per million (nTPM), integrating RNA-seq data from the Human Protein Atlas (HPA) and the Genotype-Tissue Expression (GTEx) project. Tissues are grouped by system (e.g., immune/lymphoid in green, central nervous system in purple, etc.). Expression is highest in immune-related organs (spleen and bone marrow) and skin with and lower levels in most CNS regions.

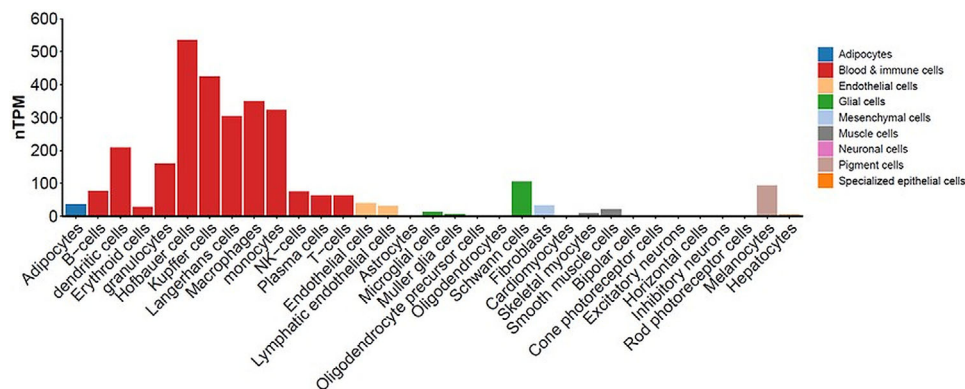


FIGURE 3 Protein levels. Relative ASC protein levels across cell types quantified in normalized transcripts per million (nTPM), as reported from Human Protein Atlas (HPA). Cell types are colored by class (e.g., blood and immune in red, endothelial in peach, glial in green, etc.). Highest expression is observed in immune cells, while glial cells are low or undetectable.

cells, the coexpression of ASCb and ASC results in irregular perinuclear aggregates, while the coexpression of ASC and ASCc showed the formation of circular perinuclear aggregates.

Despite structural similarity and 100% sequence identity between ASC and ASCb, the NMR and dynamic light scattering (DLS) studies showed differences in the self-assembly of ASC and ASCb isoforms. In case of ASC, the PYD-PYD interactions initiate the self-association and then the CARD domains interact. In contrast, the PYD and CARD domains of ASCb interacts equally to self-assemble. These differences in oligomerization indicate that the linker plays a crucial role in reorientation of the PYD and CARD domain of ASC.^{35,73} In addition to differences in the domain positioning, the kinetics of oligomerization also vary. Real-time NMR (RT-NMR) studies on both these isoforms indicate that ASC undergoes a fast-kinetic phase where the PYD-PYD interactions initiate the assembly followed by the CARD-CARD interactions, converting monomeric ASC to oligomers and then in the second slower kinetic phase both PYD and CARD comes together

to form the filaments. In ASCb the PYD and CARD domains interact equally to form filaments. ASC oligomerizes faster and in more uniform fashion whereas ASCb self-associates more slowly and forms disordered filaments.⁷³

Crystallizing full-length ASC or even the individual domains remains challenging because of the self-oligomerization properties of the protein. As a result, few x-ray crystal structures of ASC domains are available. In a recently published study, a crystal structure of human ASC CARD with the Maltose-binding protein (MBP) tag was shown. The MBP tag helps the protein to solubilize and prevents it from aggregation.⁷⁵ A cryo-EM study of ASC revealed a 3.8 Å structure of the ASC PYD filament, demonstrating a unified assembly mechanism for inflammasomes through nucleation-induced filament formation. The PYD domain of sensor proteins like AIM2 and NLRP3 interacts with the ASC PYD, nucleating the helical cluster. Subsequently, the CARD domain of ASC nucleates caspase-1 by initiating CARD-CARD interactions. Additionally, it has been shown that the CARD domains of

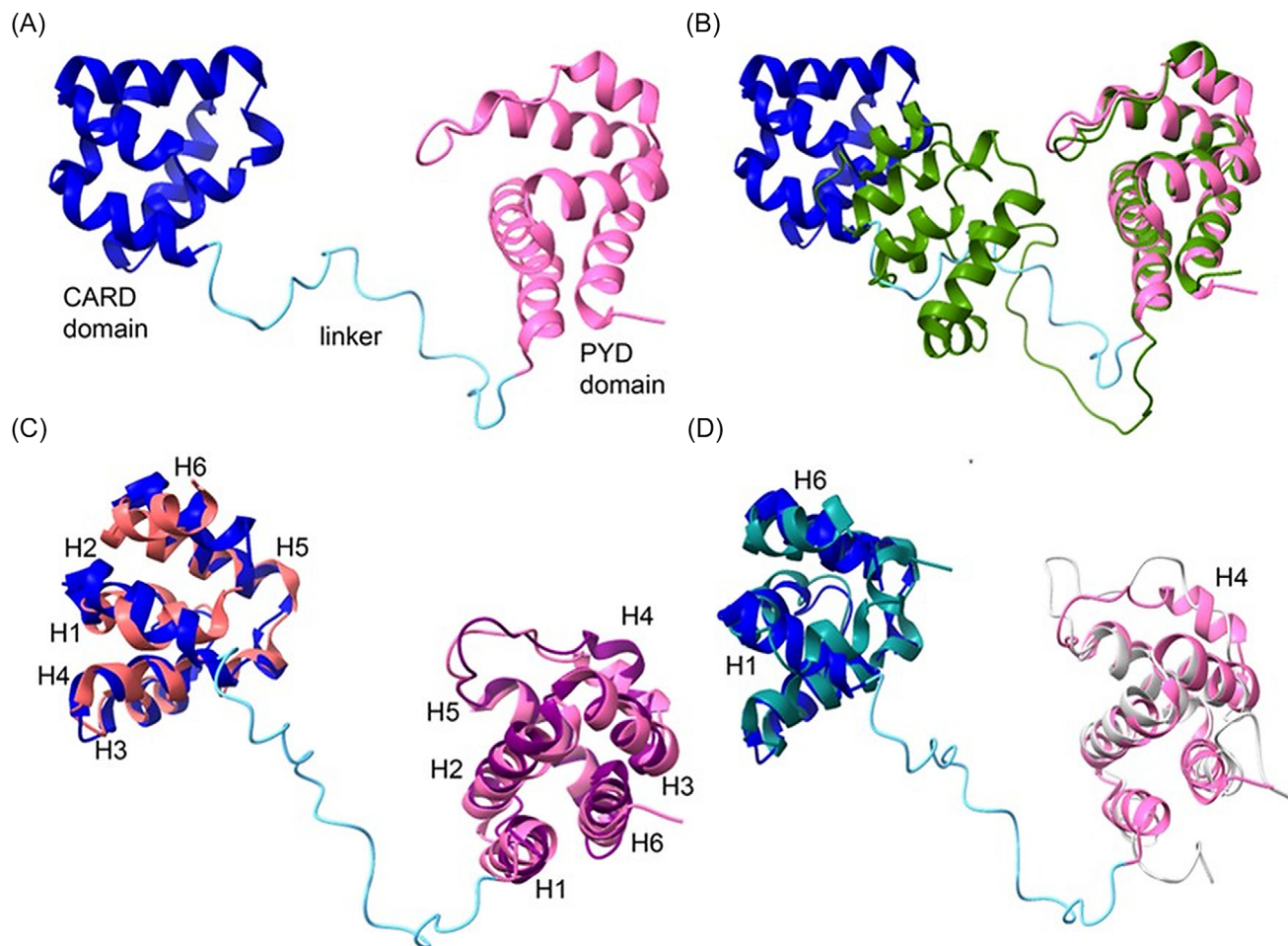


FIGURE 4 Structure of full-length ASC protein and its structural comparison. (A) Solution structure of full-length ASC, pink shows the PYD domain, blue shows the CARD domain and linker region is shown in cyan color (PDB: 2KN6). (B) Solution structure of ASC superimposed with AlphaFold3 model of ASC (green). (C) Solution structure of ASC superimposed with the individual cryo-EM structure of PYD (PDB: 3J63; dark pink) and CARD (PDB: 6N1H; orange) domains. (D) Solution structure of ASC superimposed with the PYD domain of NALP1 in gray (PDB: 1PN5) and the CARD domain of NLRP1 in teal (PDB: 6K7V).

NLRs can also form filamentous structures by interacting directly with the CARD domain of caspase-1, independent of ASC.³⁵ Another cryo-EM study shows the 3.17 Å filament structure of ASC CARD domain with NLRC4 CARD domain suggesting that both ASC and NLRC4 initiate caspase-1 assembly and activation using a unified filament-based nucleation mechanism.⁷⁰

There are three interaction interfaces that are known to be involved in ASC oligomerization. Type 1 interface are interactions between the helices of PYD-PYD domain, Type2 and Type3 interface involve the helices-loop interactions.⁷⁶ Different ASC mutations have been reported that have shown disruption in speck formation. Type I mutation includes K21A, K26A, D48N, and D51R, Type II mutations are M76A, Y59A, Q79E and E80R, and Type III mutation involves L15A, E13R, and R41E. Some mutations like L25A, D48A, and R160A are suggested to compact the ASC speck assembly, while others (Q79E and E80R) have been reported to disrupt the speck assembly. Some residues in the CARD domain play a specific role in ASC speck formation and inflammasome assembly. CARD domain mutants like D128R,

D132R, and E144R have also been reported to disrupt ASC oligomerization thus resulting in nonfunctional inflammasome assembly. Similar to PYD interface, three interfaces (Type I, II, and III) have been identified in CARD-CARD domain interactions.⁷⁰ The ASC CARD filament structure suggested that in Type I interface residues (R119, E130, D134, and R160) involves the electrostatic interactions between the helices of the CARD domain (helix 2 of one molecule and helix 1 and 4 of other). Type II interface residues (W169 and Y187) involve hydrophobic interactions and Type III is same as Type I showing charge-charge interactions between R160 and D143 or E144.

A nanobody (variable heavy chain [VHH]) has been reported to interact with the CARD domain of ASC, demonstrating that VHH binds to the ASC CARD interface, which is essential for ASC self-oligomerization and the recruitment of pro-caspase-1 through CARD-CARD interactions. To generate a monomeric ASC CARD, two residues (N128 and E130), predicted to impair CARD-CARD interactions, were mutated (N128A/E130R). This mutant was then used to crystallize the VHH-ASC CARD complex. The study revealed a 4.2 Å



FIGURE 5 Isoforms of ASC. (A) Sequence alignment of ASC, ASCb, ASCc, and ASCd. (B) Domain representation of ASC and its isoforms. Full length ASC (195 amino acid) is composed of N-terminal PYD (pink) and CARD (blue) domain connected by 23 amino acid long linker (cyan). ASCb contains an in-frame deletion in the linker region (residues 93-111), ASCc lacks 60 amino acids (residues 26-85) in the PYD domain and in ASCd, deletion of nucleotides 107-134 induces a frameshift that replaces residues 36-195 with a 69 amino acid novel domain.

resolution structure of the VHH-ASC CARD complex, showing that VHH blocks the Type II interface of ASC, thereby restricting the recruitment of the caspase-1 CARD domain and ultimately preventing inflammasome activation.⁷⁷ In a recent study, ASC PYD domain was crystallized with a protein binder specific to the PYD domain. This protein binder called “repebody” (rB7) contains leucine-rich-repeat (LRR) modules and ~30 kDa in molecular weight. The rB7 showed high affinity and specificity for the ASC PYD domain. To crystallize the rB7-ASC PYD complex, ten residues were removed from the C-terminus of ASC PYD domain. This study showed that rB7 disrupts the ASC oligomerization, but this is accompanied by an increase of caspase-1 activity. It was proposed that the ASC CARD domains from disassembled specks resulted in an enhanced interaction with caspase-1 CARD.^{78,79}

ASC can form both homo and hetero oligomeric assemblies, but the PYD and CARD domains do not interact with each other.⁷⁶ For the inflammasome assembly, the ASC acts as molecular glue, where the CARD domain interacts with the CARD domain of the procaspase-1 and PYD domain interacts with the PYD domain of NLR sensor proteins.⁷⁶

4 | TOOL MOLECULES





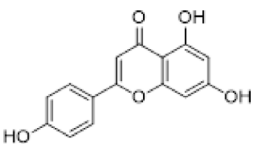
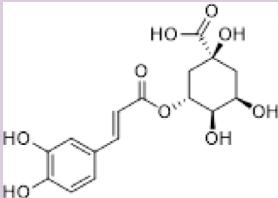
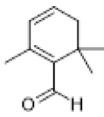
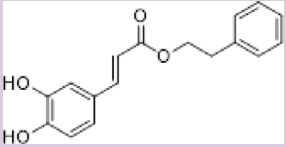
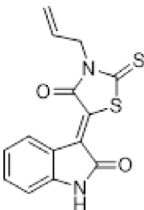
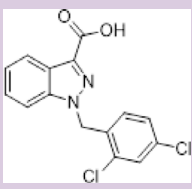
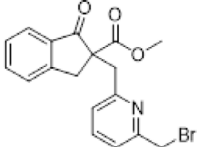
When selecting or developing tool molecules to modulate a target, it is critical to recognize that such modulation can occur through various mechanistically distinct routes. For the purposes of this work, we will group these routes into three broad classes, acknowledging that individual cases may be exceptions or blur these boundaries. **Class I: Direct modulation.** In this case, the molecule physically engages the

target protein itself and either inhibits or enhances its activity through orthosteric or allosteric binding. Classical small molecule inhibitors and many blocking or agonist antibodies fall into this category. **Class II: Post-translational modulation.** Here, the molecule alters function by changing the target protein’s post-translational state (e.g., phosphorylation, ubiquitination, or cleavage) in ways that regulate activation, stability, localization, or oligomerization. A Class II agent may bind the target directly (e.g., shielding a phosphorylation site) or bind to the protein (kinase, phosphatase, or E3 ligase) that installs or removes the relevant modification. **Class III: Upstream pathway modulation.** In this case, the molecule does not act directly on the target protein itself, but instead perturbs signaling earlier in the pathway to indirectly alter the target’s abundance or activity. An example is illustrated in Figure 1, where the inhibition of IRAK4 suppresses NF- κ B activity and thereby limits ASC expression. Gene-silencing approaches such as siRNA also belong in this category.

All three strategies have been explored as ways to block ASC’s contribution to inflammasome assembly (Table 1), caspase-1 activation, downstream cytokine maturation, and even amyloid plaque seeding. Given that ASC does not have a natural ligand and is primarily involved in protein–protein interactions (PPIs), the primary strategy of Class I inhibitors would be to inhibit ASC’s interactions with other PYD or CARD domain containing proteins in the inflammasome pathway, such as NLRP3, or to interfere with ASC’s ability to self-oligomerize to form filaments and specks.


Of all the reported strategies to target ASC’s role in inflammasome formation, the most advanced to date are the efforts to develop ASC antibodies. This Class I strategy is being actively pursued in the private sector, and once they are made public may serve as useful comparators

TABLE 1 Tool molecules.

Structure	Name	Probable MOA	Observed effects	Ref.
	IC100 by ZyVersa	Class I	Reduced IL-1 β and ASC specks	80, 81
	ACI-6635 by AC Immune	Class I	Increased phagocytosis of ASC specks. Reduced Iba1 and GFAP	ADPD 2024
	VHH _{ASC}	Class I	Reduced IL-1 β	77
	rB7 (repebody)	Class I	Disassembled extracellular ASC specks	79
	Apigenin, Contained in <i>artemisia princeps</i> extracts	Class II PAINS	Reduced IL-1 β , IL-6, and TNF- α mRNA, IL-6, and TNF- α protein, and ASC specks	84–86
	Chlorogenic acid, Contained in <i>artemisia princeps</i> extracts	Class II PAINS	Reduced IL-1 β and ASC specks	88
	Safranal	Class III	Reduced NLRP3 expression, IL-1 β and ASC specks	89
	Caffeic acid phenethyl ester (CAPE)	Class I PAINS	Reduced IL-1 β , active caspase-1, and ASC specks	91
	MM01	Class I PAINS	Reduced ASC protein levels (but not PYCARD mRNA), IL-1 β , active caspase-1, and pyroptosis	109–110
	Lonidamine (LND)	Class I	Reduced IL-1 β , IL-18, active GSDMD, and ASC specks	117
	8A	Class I	Reduced IL-1 β and ASC specks	125

(Continues)

TABLE 1 (Continued)

Structure	Name	Probable MOA	Observed effects	Ref.
	siRNA	Class III	Reduced ASC, NLRP3, NF- κ B, and α -synuclein protein levels, and active IL-1 β , IL-18, GSDMD, and caspase-1	126-128

Note: Various types of tool molecules have been reported, including (1) monoclonal antibodies, antibody fragments, and proteins; (2) small molecules; (3) siRNA. These tool molecules exert their effects via three broad mechanisms: Class I, direct contact; Class II, modulation of post-translational modifications; and Class III, interruption of upstream signaling. The observed phenotypic effects seen in cells and in vivo are summarized.

for novel small molecule alternatives. IC100, a fully humanized monoclonal antibody (mAb) developed by ZyVersa Therapeutics,^{80,81} has demonstrated the ability to penetrate the brain and even enter cells via the FcRn-mediated antibody recycling pathway. Once inside cells, IC100 interferes with ASC polymerization, preventing the assembly of ASC specks, which are essential for inflammasome activation. This inhibition leads to a significant reduction in IL-1 β release in human whole blood, an important marker of inflammasome activity and systemic inflammation.

AC Immune presented their work on ACI-6635 at the International Conference on Alzheimer's and Parkinson's Diseases (ADPD) in 2024. This mAb has shown cross-reactivity in mouse and human. The reported mechanism of action involves exploiting its IgG effector function to increase the phagocytic uptake of ASC aggregates, thus lowering the seeding potential of ASC specks to form amyloid beta plaques. Further, ACI-6635 reduced Iba1 and GFAP in AD mouse models.

The alpaca derived single domain antibody VHH_{ASC} was expressed via inducible expression in THP-1 cells and bound to the CARD domain of human ASC, sterically occluding the interface to block CARD-CARD interactions to prevent ASC speck formation.⁷⁷ This inhibition was found to lead to a significant reduction in NLRP3, AIM2, and NLRC4 inflammasome mediated IL-1 β release.

Another method to target ASC disruption is the use of a binding protein such as rB7.⁷⁹ This 30 kD protein was identified using a phage display against the ASC PYD domain. rB7 is capable of disassembling already formed ASC specks into smaller oligomers. Because rB7 cannot penetrate into cells, it acts solely on extracellular ASC specks. Surprisingly, these smaller ASC oligomers had high caspase-1 activity, potentially suggesting a role for specific ASC aggregation states in regulating the activity of caspase-1, possibly through improved recruitment of caspase-1 through the exposure of CARD domains.

Small molecules also provide viable approaches in targeting ASC protein using the strategies of all three classes. The majority of the reported small molecules are either natural products or repurposing molecules designed for other targets. Apigenin is a flavonoid polyphenol natural product found in fruits, vegetables, and other plants and has been implicated in multiple diseases (amnesia, anxiety, cancer, depression, diabetes, insomnia, neuroinflammation, osteoarthritis, etc.) through a wide variety of pathways (ERK/JNK, PI3K/Akt, MAPK, etc.).^{82,83} Apigenin has been reported to lower mRNA levels of IL-1 β , IL-6, and TNF- α , protein levels of IL-6, and TNF- α , and ASC specks in LPS induced THP-1 derived macrophages.⁸⁴ It inhibits ASC oligomer-

ization indirectly in Class II fashion by blocking the phosphorylation of key residues, such as mTyr144 (equivalent to hTyr146), by Syk or Pyk2 kinases, preventing the activation of ASC and the formation of NLRP3 and AIM2 inflammasomes.⁸⁵ Apigenin was also found to inhibit the formation of AIM2 inflammasomes, but not NLRC4.⁸⁶

Artemisia princeps is an herb used in far-eastern traditional medicine for its antioxidant, anti-inflammation, and analgesic properties.⁸⁷ The extracts contain a variety of small molecules including apigenin, diosmetin, eriodicytol, eupafolin, kaempferol, vitex, jaceosidine, quercetins, and chlorogenic acid.^{87,88} These extracts inhibit the formation of IL-1 β and ASC oligomers by blocking mTyr144 phosphorylation in LPS treated bone marrow derived macrophages (BDBMs). Again, both the NLRP3 and AIM2 inflammasomes were affected but not NLRC4.

The small molecule safranal in the spice saffron from crocus flowers has also been shown to disrupt ASC oligomerization and speck formation and ultimately prevent IL-1 β maturation.⁸⁹ Selectivity was observed with only the NLRP3 inflammasome being affected and not AIM2. These effects are most likely due to upstream events reducing NLRP3 expression, but interestingly not PYCARD expression. Thus, at best safranal should be considered a Class III inhibitor of the phenotype, but not of ASC itself.

Another small molecule from folk medicine, caffeic acid phenethyl ester (CAPE) derived from honey bee propolis,⁹⁰ shows more direct effects on ASC.⁹¹ CAPE binds to ASC as seen by surface plasmon resonance (SPR) analysis, presumably at the PYD domain. Additionally, CAPE had no impact the mRNA levels of PYCARD, indicating that the mechanism of action is solely associated with the protein itself (i.e. Class I). Treatment of primary mouse macrophages resulted in suppressed monosodium urate (MSU) crystals-induced caspase-1 activation and IL-1 β production with similar results observed in in vivo mouse models. The formation of ASC specks was also diminished.

It is important to note that polyphenols like apigenin and CAPE are known for their non-selectivity.⁹² In general, flavonoids like apigenin bind various proteins, acting as inhibitors, activators, or modulators. Although this polypharmacology can drive anti-inflammatory effects in cellular models and even in vivo, it also poses challenges. Targeting multiple pathways complicates the precise mechanistic studies required for target enablement and risks undesired pharmacology. For example, apigenin is also reportedly involved in many other cellular pathways including inhibition of monoamine oxidase-A and B,⁹³ elevation of NAD⁺ levels by the inhibition of CD38,⁹⁴ downregulation of cyclin-dependent kinase 1 (CDK1) expression via ribosomal protein S9 (RPS9) inhibition,⁹⁵ and a wide variety of cancer and

inflammation related pathways though the exact cell targets remain unclear.^{96,97} Chlorogenic acid has been shown to bind to cyclooxygenase-2 (COX2)⁹⁸ and lipoxygenases,⁹⁹ and is implicated in modulating the activity of AKR1B10,¹⁰⁰ AMPK,¹⁰¹ and tyrosinase¹⁰² enzymes. Unfortunately, the use of CAPE as a molecular probe is also complicated by polypharmacology and poor drug-like properties. Although CAPE did show direct binding to ASC, it has also been reported to impact other inflammation related targets such as NF- κ B¹⁰³ and nuclear factor erythroid 2-related factor 2 (Nrf2).^{104,105}

Many of the compounds described above are not only nonselective, they also contain functional groups commonly associated with pan-assay interference compounds (PAINS).¹⁰⁶⁻¹⁰⁸ Functional groups such as polyphenols, Michael acceptors, quinones, hydrazones, and rhodanines are well known to generate recurrent false positives in high throughput screens through mechanisms like redox cycling, aggregation, covalent modification of proteins, metal chelation, and other assay dependent artifacts. The presence of a PAINS motif is not proof that a molecule is a false positive, but it does substantially increase the likelihood of it being a false positive. As a result, such compounds must be treated with particular caution, and rigorous validation must be performed before advancing the molecule.

MM01, discovered through screening for pro-caspase-1 activation, inhibits ASC participation by preventing oligomerization presumably by direct binding to the PYD domain.^{109,110} Although there is no reported biophysical evidence (e.g., SPR, ITC, etc.), MM01 did inhibit ASC mediated pro-caspase-1 reconstitution in vitro using recombinant ASC with no upstream inhibitory pathways available. Activated THP-1 cells treated with MM01 had impaired IL-1 β release and reduced cell death compared to controls, demonstrating MM01 is sufficient to reduce inflammasome activation of pyroptosis in vitro. Interestingly, MM01 also leads to a decrease in ASC protein levels, although it does not affect PYCARD mRNA levels, suggesting that it may trigger ASC degradation. Despite its promising activity, MM01 contains a rhodamine ring, a notorious PAINS moiety, likely making it a poor starting point for a drug discovery campaign. Furthermore, MM01 shares a core structure with ClpB inhibitor antimicrobials¹¹¹ which raises selectivity concerns.

The reported hexokinase 2 (HK2)/glycolysis^{112,113} inhibitor Lonidamine (LND) has also been evaluated as an ASC inhibitor after reports of this compound's impact on cancer,¹¹⁴ spermatogenesis,¹¹⁵ rheumatoid arthritis,¹¹⁶ and ischemic brain injury. In LPS-primed BMDM's treated with ATP, LND lowered levels of IL-1 β , IL-18, GSDMD, and ASC specks, while pro-IL-1 β were unaffected.¹¹⁷ Similar results were seen in vivo. This could be viewed as simply the downstream effects of inhibiting HK2 as it is reported to regulate the NLRP3 inflammasome,¹¹⁸ but Chen et al.¹¹⁷ found strong evidence that LND binds directly to ASC using surface plasmon resonance (SPR) and cellular drug affinity responsive target stability (DARTS) assays suggesting Lonidamine may be a Class I inhibitor. However, the likelihood that LND has polypharmacology is high given the numerous reports of other possible protein targets.¹¹⁹⁻¹²³

Spirodalsole is a complex polyketide isolated from the fungus *Dalmanella eschscholzii* which showed a dose dependent decrease of IL-1 β

levels in LPS-primed BMDM's treated with ATP.¹²⁴ Simpler analogs such as 8A have been found to have similar effects. Like other reported ASC inhibitors, 8A has been shown to lower cellular IL-1 β and ASC speck levels while TNF- α is unaffected.¹²⁵ When dosed in LPS treated mice, 8A decreased both protein and relative mRNA expression of IL-1 β , IL-6, and TNF- α . Target engagement with ASC was shown using microscale thermophoresis (MST) and the cellular thermal shift assay (CETSA). Although this molecule with its alkyl bromide may be a covalent modifier, neither 8A nor Lonidamine contain functional groups typically seen in PAINS compounds, greatly increasing the possibility that they are verifiable ASC binders.

Reducing the levels of the ASC protein is another viable strategy to limit inflammasome activity. This concept is seen by inhibition of the NF- κ B pathway in the priming stage which reduces the expression of NLRP3.¹⁸ Lower PYCARD expression was also observed in the treatment of MM01.¹¹⁰ Another powerful method to achieve a reduction in protein levels would be the utilization of siRNA or antisense oligonucleotides. Intrathecal administration of the siRNA could be particularly effective in avoiding immune suppression in the periphery. Conveniently, PYCARD siRNA and related materials are already commercially available (Santa Cruz Biotechnology, sc-37282). The use of PYCARD siRNA has already been shown to reduce secretion of IL-1 β .^{126,127} PYCARD knockdown in BV2 cells caused significantly reduced NLRP3 and NF- κ B levels and inhibited the cleavage of GSDMD, and release of caspase-1, IL-1 β , and IL-18.¹²⁸ Furthermore, treatment of cocultured SH-SY5Y cells with PYCARD siRNA transfected BV2 cells resulted in lower α -synuclein levels.¹²⁸

5 | IN VITRO ASSAYS AND ANIMAL MODELS

As noted above, ASC specks exhibit high affinity for A β peptides, driving the formation of large protein aggregates that contribute to the development and propagation of amyloid plaques through a cross-seeding mechanism.⁵¹ Therefore, disrupting ASC speck formation and the associated release of proinflammatory cytokines (IL-1 β and IL-18, which are matured by caspase-1), may offer a promising strategy to mitigate neuroinflammation and potentially reduce AD pathology. This approach is based on the mechanistic hypothesis that preventing ASC assembly will dampen inflammasome activation, thereby limiting the inflammatory cascade that contributes to neurodegeneration. Nonetheless, in certain pathological conditions inflammasome activation is necessary for the clearance of pathogens and damaged cells promoting neuroprotection and repair.¹²⁹ Investigating the contribution of ASC specks to AD pathology may support a precision medicine approach, in which an alternative strategy could be selectively disrupt the interaction of ASC with amyloid or diffuse plaques, with the goal of reducing the formation of dense neuritic plaques without impairing neuroprotective microglial responses.

To inform a drug discovery campaign targeting ASC, it is essential to establish and evaluate robust pharmacological assays that accurately measure ASC aggregation and inflammasome associated biological readouts. Tool molecules may be optimized to prevent ASC

aggregation to in turn disrupt theoretical ASC mediated seeding of amyloid plaques, or instead optimized for their ability to dampen ASC dependent inflammasome activation and cytokine release. Assays must be designed, optimized, and validated to inform structure-activity relationship (SAR) studies, enabling the iterative optimization of lead compounds based on their biochemical and cellular effects. Furthermore, for the *in vitro* assays should inform on the performance of the compounds *in vivo*, first in preclinical AD models, and ultimately in human clinical studies. This will ensure that ASC targeting therapeutics not only demonstrate efficacy in preclinical studies but also allow us to test our mechanistic hypothesis in clinical trials.

5.1 | Biophysical assays

It is challenging to study the interactions between ligand and ASC full length protein, because even as truncated isoforms, PYD and CARD domains oligomerize and can form aggregates, thereby creating a major hindrance through protein insolubility. The only full-length structure of ASC determined using NMR involved denaturing condition (5 M GdHCl) and subsequent refolding.⁶⁹ However, NMR is still a challenging technique to study ligand-ASC interactions because of the possibility of the refolding artifacts and of non-native conformers generated through the process. One study showed the binding of CAPE with ASC using an antibody-based affinity method of SPR.⁹¹ Conventional SPR maybe challenging due to heterogenous orientations and MST maybe unreliable due to the multiple binding sites.⁷³ There are some studies reported to understand the oligomerization of ASC and with its partners using DLS and fluorescence polarization assays,^{35,70} but the use of these techniques to understand how a potential ligand can disrupt the oligomerization of ASC are yet to be explored. Another alternative would be to screen crystallization conditions of ligand-ASC complex using any monomeric mutant of the ASC domain or using cryo-EM to understand the ASC filament-ligand interactions.

5.2 | Inflammasome priming and activation

For each of the *in vitro* experimental approaches described below it is important to carefully consider the mechanism by which ASC aggregation and inflammasome activation are initiated as some cell culture models may be particularly sensitive or resistant to specific triggering stimuli. Although these models may not fully recapitulate the complex environment of the AD brain, they can provide a tractable and reproducible system for evaluating inflammasome activation and enabling SAR studies. A priming step (see Figure 1) of immune cell activation is often required to achieve full activation and assembly of the inflammasome complex.¹³⁰ Experimentally, this priming step is often achieved through the treatment of cells with bacterial-derived TLR4 agonist LPS.¹³¹ Immune priming precedes the secondary inflammasome activation stimulus to promote upregulation of inflammasome associated

genes as well as post-translational processing of inflammasome proteins. Specific inflammasomes are then activated using a second stimulus, such as the NLRP3 activator nigericin or ATP.¹³² Inflammasome activation initiates a cascade of events which can be measured experimentally. These events include ASC protein oligomerization, caspase-1 cleavage and enzyme activation, cleavage and bioactivation of IL-1 β and IL-18 cytokines, and cleavage of GSDMD resulting in membrane pore formation and ultimately release of activated cytokines and cell death.

In the context of AD pathology, early studies provided evidence linking senile amyloid plaque with inflammasome activation. Elevated expression of IL-1 β was observed in the brains of AD patients,¹³³ and this increase was also detected in CSF.¹³⁴ Subsequent research revealed higher levels of cleaved caspase-1, active IL-1 β , cleaved GSDMD, and ASC in postmortem brain tissue from AD patients compared to age-matched, nondemented controls.^{47,51,135}

In vitro studies further support this link by showing that pattern recognition receptors (PRRs), such as TLRs, can recognize A β oligomers and protofibrils.^{49,136} For example, both protofibrils and low molecular weight A β peptides have been shown to significantly increase IL-1 β release and caspase-1 activation, independently of high molecular weight A β aggregates.⁴⁹ Additionally, it has been proposed that lysosomal disruption in AD may directly trigger inflammasome activation by releasing A β peptides intracellularly, serving as a secondary signal for ASC speck formation.^{12,48} These findings highlight the role ASC specks formation in AD pathology and the ability of A β species to act as both priming and activating signals in the inflammasome activation.

5.3 | ASC aggregation and imaging assays

When cells are appropriately stimulated to trigger an inflammasome, ASC protein reorganizes from a broad cellular distribution to organized intracellular aggregates or specks. The aggregation of ASC is directly related to its biological function as a scaffolding protein in the inflammasome and can be measured as an upstream readout of inflammasome activation.¹³⁷ These ASC aggregates can be quantified using immunofluorescent imaging.³⁶ The ASC protein can also be genetically tagged with a fluorescent protein, or a fluorescently tagged ASC can be overexpressed to visualize specks.¹³⁷ A transgenic mouse with a fluorescently labeled ASC protein allows for visualization and quantification of ASC specks in tissues.¹³⁸ Fluorescent ASC aggregation assays may be particularly amenable to high-content imaging experiments. ASC aggregates can be immunolabeled in paraformaldehyde fixed cells and imaged, or a modified fluorescently tagged ASC cell lines utilized for live-cell imaging assays. Screening of ASC aggregation can be conducted by inducing inflammasome activation by priming and activation stimuli and then imaging and counting cells for ASC specks. Alternatively, recent reports detail the use of AMNIS-based ImageStream imaging flow cytometry¹³⁹ and a dSTORM enabled SimPull assay⁵³ to measure fluorescently immunolabeled ASC specks.

5.4 | Caspase 1 cleavage and activity assays

The inflammasome has been described as a caspase-1 activation platform.¹⁴⁰ Inflammasome formation results in caspase-1 recruitment and autocatalytic activation of the caspase zymogen. Western blots of caspase cleavage serve as a useful indicator of inflammasome activation or inhibition by measuring the molecular size of caspase proteins. Practically, this can be accomplished by utilizing assays such as the Caspase-Glo 1 Inflammasome Assay in which caspase-1 proteolytic activity results in the generation of luciferase substrates for luminescent readouts.¹³⁵

5.5 | IL-1 β and IL-18 assays

The cleavage and release of IL-1 β and IL-18 is mediated by the inflammasome and can be measured in the conditioned media of stimulated cells. The release of these activated cytokines is enhanced by the formation of GSDMD pores.¹⁴¹ IL-1 β and IL-18 levels in conditioned media or other biological samples can be measured by enzyme-linked immunosorbent assay (ELISA). Standard western blotting can also be used to assess the levels of both immature IL-1 β and its cleaved active forms.

5.6 | Pyroptosis assays

Inflammatory cell death or pyroptosis can occur as a result of inflammasome activation and membrane pore formation.³⁹ Screening for cell death is performed by imaging using reagents such as fluorescent dyes or Hoechst nuclei imaging. Cell death screening can also be multiplexed with other assays by using fluorescent cytotoxicity assay reagents. The secreted contents of pyroptotic cells, such as lactate dehydrogenase (LDH), can also be measured in conditioned media.¹⁴² In addition to these readouts, cleavage of GSDMD, the executioner protein of pyroptosis, serves as a definitive molecular marker, as the N-terminal fragment generated by caspase-mediated processing drives pore formation and subsequent cell lysis.¹⁴³ Beyond traditional immunoblot detection, GSDMD activation can also be monitored using engineered reporter constructs in which proteolytic separation of fluorescent proteins enables real-time visualization of GSDMD cleavage through live-cell imaging technologies.¹⁴⁴ Pyroptotic process can be further supported by the detection of mature IL-1 β and IL-18, which are processed by caspase-1 and released upon membrane rupture; these cytokines are routinely quantified in conditioned media using ELISA or other immunoassay platforms.^{143,145}

5.7 | Microglia and myeloid cell models

Most inflammasome and ASC research in the context of the brain has been performed in microglia and other myeloid cells and mod-

els. Myeloid cell lines that can be used in the assays described above include THP-1 monocytes, and the BV2 and SIM-A9 microglial lines.^{38,146-148} Primary mouse microglia or iPSC derived microglia and can serve as a valuable second screening tool to validate results obtained with tool molecules against ASC in cell lines. Assay development for ASC using these myeloid cell lines and iPSC microglia can first be assessed for the levels of ASC protein under control and primed activation states. Inflammasome activation should also be confirmed using different stimuli including nigericin and ATP and measuring IL-1 β release and cell death to confirm that tool molecules which disrupt ASC driven inflammasome assembly are not restricted to LPS initiated inflammasomes.

Phenotypic screening for ASC targeting molecules in cell lines can be performed initially by measuring cell populations containing induced ASC specks through high content fluorescent imaging. Tool molecules which bind ASC may disrupt ASC aggregation and speck formation leading to reduced numbers of ASC speck positive cells in screens. Tool molecules may also interfere with ASC mediated caspase-1 recruitment and activation by binding to the ASC CARD domain. Caspase-1 activity can be screened by measuring IL-1 β and IL-18 in conditioned media. It is possible that different in vitro phenotypic assays may identify varying half-maximal inhibitory concentration (IC50) concentrations are required to efficiently inhibit each of these inflammasome readouts described above.

5.8 | Animal models

Pharmacokinetic and pharmacodynamic (PK/PD) studies involve oral administration or intraperitoneal injection of compounds into rodent models to determine compound levels in plasma and tissue compartments. In particular, for use in models of AD, the brain levels of compounds require careful consideration as they will need to reach sufficient concentration in the brain to disrupt ASC inflammasomes.

Fluorescent ASC reporter mice may be of value in assessing the ability of ASC tool compounds to change ASC protein levels or interfere with ASC speck formation. These mice enable the visualization of ASC speck formation, which can be detected by flow cytometry allowing examination by cell-type. Additionally, primary cell cultures of microglia and other brain cells from this animal model may also be of value for development of ASC speck assays in non-immortalized cells.

NLRP3 Inflammasomes can be activated in vivo by administering an intraperitoneal (IP) injection of LPS for inflammasome priming followed by an IP injection of ATP resulting in a potent release of IL-1 β into blood and tissues. This particular strategy has been utilized in previous research to provide in vivo validation of NLRP3 inhibitors.¹⁴⁹ Similar approaches will be of value in screening for ASC inhibitors as IL-1 β release is a downstream effect of various inflammasomes and is a consequence of caspase-1 activation. Measurements of IL-18 levels may provide additional value as expression of this cytokine is less dependent on cellular priming signals. In contrast to these acute animal experiments, studies in aging transgenic AD mouse models will likely

require longer administration of compounds to manifest in detectable phenotypes.

6 | TARGET RECOMMENDATIONS

ASC is an attractive target for developing new tool molecules for the modulation of neuroinflammation because it serves a central role that organizes multiple inflammasomes, largely independent of the initial triggering receptor. Direct ASC modulators exert their biological effects by binding to the PYD or CARD domains on the ASC protein and preventing ASC recruitment, aggregation, and ultimate formation of ASC dependent inflammasomes, while allowing for ASC independent inflammatory pathways to continue unimpeded. In some instances, certain inflammasomes can function via either an ASC dependent or independent manner, however its presence typically enhances activity.¹⁵⁰⁻¹⁵³ This will reduce the recruitment and activation of caspase-1, prevent the formation of ASC specks, and inhibit both IL-1 β and IL-18 cytokine activation and release from cells. Moreover, this may prevent the formation of membrane pores induced by GSDMD, which have recently been shown to have the ability to propagate cell death.¹⁵⁴

However, there may be unanticipated consequences for disrupting ASC aggregate assembly *in vivo*. PYCARD knockout mice are viable and appear similar to WT at baseline,¹⁵⁵⁻¹⁵⁷ but under challenges the ASC^{-/-} mice demonstrate hampered immune defenses¹⁵⁸ with dramatic increases in bacterial load and faster mortality.¹⁵⁹ This is likely due to decreased ASC dependent, inflammasome driven conversion of pro-IL-1 β and pro-IL-18 to their active counterparts,^{155,159} yet inflammation is not abolished,¹⁵⁷ because alternative proteases and parallel pathways can still generate active cytokines and sustain inflammatory responses.¹⁶⁰ This is in perfect agreement with the observed benefits of NLRP3 inflammasomes against infection^{161,162} while acknowledging the presence of other, nonASC dependent inflammasomes. Developmental considerations may apply, given evidence that the AIM2/ASC axis participates in brain homeostasis by sensing damaged neuronal DNA for clearance.¹⁶³ Additionally, beyond classical inflammasome signaling, ASC deficiency shows cell type specific, opposing effects on tumorigenesis (e.g., tumor-promoting in myeloid compartments vs. tumor-suppressive in certain epithelia).¹⁵⁶ In practical terms, pharmacologic ASC inhibition may increase susceptibility to infection requiring careful patient selection and monitoring.

Given the progress already made by ZyVera on the IC100 antibody, we propose that future academic advancements should be focused on the exploration of siRNA in AD models and the development of small molecule inhibitors. The commercially available siRNA could be quickly validated for PYCARD knock-down, modified for self-delivery, followed by *in vivo* studies in AD mouse models looking specifically at IL-1 β levels.

Attempts could be made to improve the existing small molecules, but given the apparent lack of selectivity and the similarity to PAINS substructures, the discovery of novel scaffolds will most likely be necessary. Spontaneous self-oligomerization of ASC would present

challenges to conducting biophysical high throughput screens, though careful experimental design could allow for certain biophysical assays to be run on smaller scales. Alterations to the WT structure may prevent this oligomerization while maintaining small molecule site binding sites for affinity screens may be possible but would require extensive exploration and validation. Moderate throughput screening using cellular assays is reported^{164,165} but is resource intensive and would lack target specificity. The rapid advances in AI assisted techniques have made virtual screening much more reliable.^{166,167} The reported NMR, cryo-EM, and x-ray structures could aid in the creation of a homology model suitable for virtual screening after identifying binding pockets using Schrödinger's SiteMap,¹⁶⁸ Fpocket,¹⁶⁹ CASTp,¹⁷⁰ and DoGSiteScorer.¹⁷¹ The aforementioned biophysical and cellular assays would well support virtual hit validation and SAR studies needed to improve for potency and druglike properties until a suitable chemical probe can be found to test *in vivo*. Once drug like molecules are identified, they could be tested and validated *in vivo* using acute inflammation models, such as those used in prior studies of NLRP3 inhibitors,¹⁷² before conducting longer costly studies in aged AD mice.

While this review has largely focused on myeloid cells, microglia are not the only cells in the central nervous system that have been shown to exhibit inflammasome biology. Human astrocytes have been shown to express the inflammasome sensor NLRP2.¹⁷³ NLRP1, IPAF-1, and AIM2 (but not NLRP3) mRNAs were detected naive human primary cultures of neurons, and functional NLRP1 inflammasomes were also observed.⁴⁵ Inflammasome components including ASC have been detected in oligodendrocytes and may play a role in demyelinating disease.^{174,175}

The development of highly specific ASC targeting molecules will have value across several immune diseases in which inflammasomes contribute to tissue damage. Prevention of ASC speck formation would be of particular significance for AD therapies given its dual role in NLRP3 inflammasome activation as well as amyloid beta plaque seeding.

ACKNOWLEDGMENTS

This work was supported by The Indiana University School of Medicine-Purdue TREAT-AD Center funded through the National Institute on Aging of the National Institutes of Health (U54AG065181).

BioRender was used to create Figure 1.

CONFLICT OF INTEREST STATEMENT

W. Brent Clayton, Jie Zhang, Kun Huang, Andrew D. Mesecar, Jeffrey L. Dage, Bruce T. Lamb, Alan D. Palkowitz, and Timothy I. Richardson are founders and consultants for Monument Biosciences.

All funding provided to the institution and individual authors has been disclosed in the funding information and the declaration of interest section. No other authors have conflict of interests to disclose. Author disclosures are available in the [supporting information](#).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Clayton WB, Kulas JA, Liu J, et al. Evaluation of ASC as a therapeutic target for Alzheimer's disease. *Alzheimer's Dement.* 2026;22:e71104.
<https://doi.org/10.1002/alz.71104>