Inflammasomes mediate inflammatory and cell death responses to pathogens and cellular stress signals via activation of procaspases-1 and -8. During inflammasome assembly, activated receptors of the NLR or PYHIN family recruit the adaptor protein ASC and initiate polymerization of its pyrin domain (PYD) into filaments. We show that ASC filaments in turn activate receptors of the NLR or PYHIN family. During inflammasome assembly, ASC filaments recruit and activate the inflammatory caspase-1, which processes proinflammatory cytokines, including interleukin-1β (IL-1β) and IL-18, into their active forms (4, 5). Caspase-1 activation also leads to an inflammatory type of cell death termed pyroptosis (6). Recent studies have shown that procaspase-8 is also recruited to inflammasomes (7, 8). The role of caspase-8 as an initiator of the apoptotic cascade when recruited to death receptor signaling complexes is well documented (9), and recruitment of procaspase-8 to inflammasomes can also induce apoptotic cell death (7, 8). However, the mechanism by which procaspase-8 is recruited to inflammasomes to allow cross-talk between inflammatory and apoptotic signaling pathways is unclear.

Inflammasome assembly depends on protein-protein interactions mediated by members of the death fold superfamily. The death fold consists of six α-helices in an anti-parallel arrangement, and the four recognized subfamilies are the pyrin domain (PYD), caspase recruitment domain (CARD), death domain (DD), and death effector domain (DED). Homotypic PYD-PYD and CARD-CARD interactions mediate conventional inflammasome formation. Inflammasome responses are initiated when certain pattern recognition receptors, such as pyrin (familial Mediterranean fever protein), and members of the death fold superfamily activate the inflammasome. We show that ASC filaments in turn activated receptors of the NLR or PYHIN family recruit the adaptor procaspases-1 and -8. During inflammasome assembly, adaptive responses to pathogens and cellular stress signals via activation of procaspase-8 interactions within and/or between filaments may be similar. Interestingly, we observed condensation of procaspase-8 filaments containing the catalytic domain, suggesting that procaspase-8 interactions within and/or between filaments may be involved in caspase-8 activation. Procaspase-8 filaments may also be relevant to apoptosis induced by death receptors.

Inflammasomes are multiprotein complexes that mediate inflammatory and cell death responses to pathogens and cellular stress signals (1–3). Deregulated inflammasome activation is associated with several common diseases, which include type II diabetes, obesity, atherosclerosis, Alzheimer disease, and multiple sclerosis (3). Thus, inflammasomes are regarded as potential therapeutic targets for many diseases. Canonical inflammasomes recruit and activate the inflammatory caspase, caspase-1, which processes proinflammatory cytokines, including IL-1β and IL-18, into their active forms (4, 5). Inflammasomes can also induce apoptotic cell death (7, 8). However, the mechanism by which procaspase-8 is recruited to inflammasomes is unclear.

Significance: The data give insights into cross-talk between apoptotic and inflammatory pathways and procaspase-8 activation.

**Background:** ASC mediates inflammasome assembly, recruiting procaspase-1 and procaspase-8 to initiate inflammation and cell death.

**Results:** ASC pyrin domain (PYD) surfaces that mediate filament assembly bind procaspase-8 death effector domains (DEDs) and induce filaments.

**Conclusion:** Procaspase-8 DED filaments are initiated from ASC PYD filaments.

**Significance:** The data give insights into cross-talk between apoptotic and inflammatory pathways and procaspase-8 activation.

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**Figure:**

Inflammasomes are multiprotein complexes that mediate inflammatory and cell death responses to pathogens and cellular stress signals (1–3). Deregulated inflammasome activation is associated with several common diseases, which include type II diabetes, obesity, atherosclerosis, Alzheimer disease, and multiple sclerosis (3). Thus, inflammasomes are regarded as potential therapeutic targets for many diseases. Canonical inflammasomes recruit and activate the inflammatory caspase, caspase-1, which processes proinflammatory cytokines, including IL-1β and IL-18, into their active forms (4, 5). Caspase-1 activation also leads to an inflammatory type of cell death termed pyroptosis (6). Recent studies have shown that procaspase-8 is also recruited to inflammasomes (7, 8). The role of caspase-8 as an initiator of the apoptotic cascade when recruited to death receptor signaling complexes is well documented (9), and recruitment of procaspase-8 to inflammasomes can also induce apoptotic cell death (7, 8). However, the mechanism by which procaspase-8 is recruited to inflammasomes to allow cross-talk between inflammatory and apoptotic signaling pathways is unclear.

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the pryn and HIN domain-containing (PYHIN) or nucleotide-binding domain and leucine-rich repeat-containing (NLR) families are activated by appropriate pathogen-associated stimuli or cellular stress signals (1, 3, 11). AIM2 (absent in melanoma 2) and NLRP3 (NLR family, pryn domain-containing 3) are well characterized members of the PYHIN and NLR families, respectively (12–15). AIM2 uses its HIN domain to bind and oligomerize along the length of cytosolic DNA (12, 13, 16), whereas NLRP3 contains a nucleotide-binding oligomerization domain and is activated by a wide range of stimuli (17). Both AIM2 and NLRP3 have an N-terminal PYD, and their activation and oligomerization promote recruitment of the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), which consists of a PYD and a CARD (12, 13, 15, 18). Once ASC clustering is initiated, it proceeds in a rapid fashion likened to prion polymerization (19), accumulating all of the ASC in the cell into a single “speck.” The speck appears to be initiated by polymerization of the ASC PYD to form a filament (20), with further condensation into a speck structure involving CARD-CARD interactions (21, 22). Although the resulting inflammasome-induced ASC oligomer is visible in cells as a speck by low resolution microscopy (23–25), the filamentous nature of ASC at the inflammasome has been demonstrated by high resolution microscopy (20, 26). The ASC CARD, which is exposed on the outside of the filament, can recruit procaspase-1 via a homotypic CARD interaction (27), and electron microscopy (EM) of complexes formed in vitro shows that oligomerized ASC induces polymerization of caspase-1 CARD into similar filaments (20). In contrast, the recruitment of procaspase-8 to inflammasomes appears to be mediated by the ASC PYD (8). However, procaspase-8 contains tandem DEDs rather than a PYD (28); hence, an atypical PYD/DED interaction may mediate initial procaspase-8 recruitment.

A striking feature of the death fold family, first identified in the death domain subfamily, is the ability to use up to six surfaces to mediate three types of interactions, which are designated type I, II, and III (29, 30). EM-based structural analysis of an ASC PYD filament revealed that it forms a triple helix, involving the same type I, II, and III interactions (20). The type I interactions are lateral, between ASC PYD subunits in a helix, and type II and III interactions occur between the three strands that comprise the triple helix (20). Short regions of oligomerized AIM2 or NLRP3 PYD were observed to promote formation of ASC filaments in vitro (20). Other work has shown that mutagenesis of ASC PYD self-association sites disrupts its interactions with the PYDs of NLRP3 and pyrin (31, 32). Furthermore, mutations of type I, II, or III interaction sites on AIM2, NLRP3, and pyrin PYDs disrupt their interaction with ASC PYD (20, 32). Together, this work suggests that all PYDs interact using conserved death fold domain binding modes.

Here we investigate the protein-protein interactions involved in recruitment of procaspase-8 by ASC and characterize the interaction between ASC PYD and the tandem DEDs of procaspase-8. The interaction modes of DEDs are less well characterized than PYD. Homotypic interactions between the procaspase-8 tandem DEDs and the single DED of the adaptor FADD are critical for assembly of the death receptor signaling complexes (28, 33), and mutagenesis studies implicate a type I-like interaction for self-associations of both FADD DED and procaspase-8 DEDs (34–38). However, it is unclear how the tandem DEDs of procaspase-8 are recruited to death-inducing complexes by either FADD or ASC. Our results suggest a common mode of interaction for ASC PYD in self-association and in association with both AIM2 PYD and procaspase-8 DEDs. Furthermore, we show that ASC induces procaspase-8 DED filaments in vitro and that procaspase-8 DEDs form striking filaments upon recruitment to inflammasomes in vivo. Interestingly, full-length procaspase-8 is more condensed at inflammasomes than the DEDs alone, indicating that the catalytic domain may mediate condensation.

Experimental Procedures

Plasmids—For in vitro translation, procaspase-8 DED1 (residues 1–94), DED2 (residues 95–216), or DED1-DED2 (residues 1–216) and AIM2 PYD (residues 1–107) were individually cloned into pET28b. Plasmids expressing GST-ASC PYD (wild type and mutants) have been described previously (31). For mammalian cell expression, full-length procaspase-8 (C360S mutant) was cloned into pEF6/V5-His TOPO or pcDNA 3.1 with either a C-terminal Myc or HA tag, respectively, whereas procaspase-8 DED1-DED2 (residues 1–216) was cloned into pcDNA 3.1 with a C-terminal V5 tag. pcDNA3.1-AIM2 was from Ricky Johnstone (Peter MacCallum Cancer Centre, Melbourne, Australia). The mCherry tag with a stop codon was cloned into pEF6-V5-His vector upstream of the V5 and His tags to generate pEF6-mCherry. Full-length AIM2 was cloned into pEF6-mCherry to generate pEF6-AIM2-mCherry. The linker and HIN domain of AIM2 (98–343/ end) was cloned into pEF6-mCherry to generate pEF6-AIM2-mCherry. The procaspase-8 DEDs (residues 1–216), ASC PYD (residues 1–96), and NLRP3 PYD (residues 1–100) were separately cloned into pEF6-HIN1; ASC-HIN2; ASC-HIN3; and pEF6-PYD, the cDNA of monomeric GFP (residues 1–230) which has been described previously (31, 39). For fluorescence polarization assays and/or electron microscopy studies, ASC PYD (residues 1–106), ASC CARD (residues 107–195), and full-length ASC (residues 1–195) was cloned into pDB.His.MBP for expression with an N-terminal His6-MBP tag that was cleavable with TEV protease. Mutations were introduced into the His6-MBP tagged full-length ASC construct by QuickChange mutagenesis (Agilent Technologies). To generate His6-GFP-tagged ASC PYD, the cDNA of monomeric GFP (residues 1–230) which has an A206K mutation (40), was first cloned into pET28a (to generate pET28a–GFP–A206K), and ASC PYD (residues 1–106) was inserted downstream of GFP. For sortase labeling, the sequence of SUMO was first cloned into the pDB.His.MBP backbone. The procaspase-8 DEDs (residues 1–186) was subsequently inserted to allow expression with an N-terminal His6-MBP and a C-terminal SUMO tag. To reduce self-association of the DEDs, a Y8A mutation was introduced into procaspase-8 DED1 using QuickChange mutagenesis, and the sequence encoding the peptide LPETGG, which is compatible for sortase labeling, was introduced downstream of the SUMO sequence.

Procaspase-8 Filaments at Inflammasomes

Plasmids—For in vitro translation, procaspase-8 DED1 (residues 1–94), DED2 (residues 95–216), or DED1-DED2 (residues 1–216) and AIM2 PYD (residues 1–107) were individually cloned into pET28b. Plasmids expressing GST-ASC PYD (wild type and mutants) have been described previously (31). For mammalian cell expression, full-length procaspase-8 (C360S mutant) was cloned into pEF6/V5-His TOPO or pcDNA 3.1 with either a C-terminal Myc or HA tag, respectively, whereas procaspase-8 DED1-DED2 (residues 1–216) was cloned into pcDNA 3.1 with a C-terminal V5 tag. pcDNA3.1-AIM2 was from Ricky Johnstone (Peter MacCallum Cancer Centre, Melbourne, Australia). The mCherry tag with a stop codon was cloned into pEF6-V5-His vector upstream of the V5 and His tags to generate pEF6-mCherry. Full-length AIM2 was cloned into pEF6-mCherry to generate pEF6-AIM2-mCherry. The linker and HIN domain of AIM2 (98–343/end) was cloned into pEF6-mCherry to generate pEF6-AIM2-mCherry. The procaspase-8 DEDs (residues 1–216), ASC PYD (residues 1–96), and NLRP3 PYD (residues 1–100) were separately cloned into pEF6-HIN1; ASC-HIN2; ASC-HIN3; and pEF6-PYD, the cDNA of monomeric GFP (residues 1–230) which has been described previously (31, 39). For fluorescence polarization assays and/or electron microscopy studies, ASC PYD (residues 1–106), ASC CARD (residues 107–195), and full-length ASC (residues 1–195) was cloned into pDB.His.MBP for expression with an N-terminal His6-MBP tag that was cleavable with TEV protease. Mutations were introduced into the His6-MBP tagged full-length ASC construct by QuickChange mutagenesis (Agilent Technologies). To generate His6-GFP-tagged ASC PYD, the cDNA of monomeric GFP (residues 1–230) which has an A206K mutation (40), was first cloned into pET28a (to generate pET28a–GFP–A206K), and ASC PYD (residues 1–106) was inserted downstream of GFP. For sortase labeling, the sequence of SUMO was first cloned into the pDB.His.MBP backbone. The procaspase-8 DEDs (residues 1–186) was subsequently inserted to allow expression with an N-terminal His6-MBP and a C-terminal SUMO tag. To reduce self-association of the DEDs, a Y8A mutation was introduced into procaspase-8 DED1 using QuickChange mutagenesis, and the sequence encoding the peptide LPETGG, which is compatible for sortase labeling, was introduced downstream of the SUMO sequence.
For preparation of biotinylated proteins, the sequence encoding a biotin acceptor peptide was inserted into the pDB. His.MBP plasmid. The procaspase-8 DEDs (residues 1–186)-Y8A mutant was cloned into this plasmid to allow expression with both an N-terminal His6-MBP tag and C-terminal biotin acceptor peptide.

**Protein Expression and Purification**—Expression and purification of wild-type and mutant GST-ASC PYD for GST pull-down assays were performed as described previously (31). Proteins for fluorescence polarization and/or EM studies were overexpressed in BL21(DE3) cells grown at 37 °C to an A600 of ~0.6 and induced with 0.4 mM isopropyl-β-D-galactopyranoside overnight at 18 °C. To express biotinylated protein, a plasmid expressing procaspase-8 DEDs-Y8A mutant with N-terminal His6-MBP tags and a C-terminal biotin acceptor peptide was co-transformed into BL21(DE3) cells with the pDW363 vector expressing the BirA biotin ligase (41). BirA allowed in vivo biotinylation of the expressed protein by supplementing cultures with 50 μM biotin during overnight induction. After overnight induction, cells were harvested and lysed by sonication in a buffer containing 20 mM HEPES, pH 8, 200 mM sodium chloride, 10% glycerol, 5 mM imidazole, and 5 mM β-ME. For purification of monomeric constructs (His6,-MBP-tagged ASC constructs and His6-MBP-procaspase-8 DEDs-Y8A-SUMO-LPETGG), the cell lysate was centrifuged at 40,000 × g for 40 min. For purification of His6-GFP-tagged ASC PYD, the cell lysate was centrifuged at 30,000 × g for 30 min. The supernatant was incubated with nickel-nitriotriacetic acid resin for 1 h and washed with lysis buffer containing 20 mM imidazole. Bound proteins were eluted by lysis buffer containing 300 mM imidazole and subjected to size exclusion chromatography on a Superdex 200 column.

**In Vitro GST Pull-down Assays**—GST pull-down assays were performed as described previously (31), except that binding was carried out overnight at 4 °C. In addition, when full-length AIM2 was in vitro translated for binding assays, it was diluted 1:4 with binding buffer and filtered through a low protein binding Millex-GP 0.22-μm membrane (Millipore) to remove precipitated protein.

**Cell Culture**—HEK293 cells were maintained in DMEM with 4.5 g/liter glucose, 110 mg/liter sodium pyruvate supplemented with Glutamax-1 (Life Technologies), 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin. For transfections, cells were plated in medium without antibiotics and transfected using a 2.5:1 (μl/μg) ratio of Lipofectamine 2000 (Life Technologies) to DNA as per the manufacturer’s instructions.

**Microscopy**—HEK293 cells (100,000 cells) were seeded in 24-well dishes on coverslips coated with poly-L-lysine (Sigma). For transfection, 200 ng of each plasmid was combined to a final concentration of 400 and 600 ng. After incubation overnight (~18 h), cells were fixed for 15 min with 4% paraformaldehyde prepared in PBS/CaCl2/MgCl2 and then permeabilized and blocked in PBS containing 10% FCS, 0.3% saponin, 0.1% sodium azide for 45 min. ASC was detected using rabbit polyclonal anti-ASC (N-15) (Santa Cruz Biotechnology, Inc.) overnight at 4 °C followed by goat anti-rabbit Alexa Fluor-488 (Life Technologies) for 1 h at room temperature. AIM2 was detected using the anti-AIM2 mAb 3B10 (42) followed by goat anti-mouse Alexa Fluor-555 (Life Technologies). Procaspe-8 (C360S)-V5-His was detected using mouse monoclonal antibody (clone SV5-Pk1) to the V5 tag (AbD Serotec) followed by goat anti-mouse-Alexa Fluor-555. All antibodies were diluted in antibody dilution buffer (1× PBS containing 2% FCS, 0.06% saponin), and unbound antibodies were washed away using PBS containing 0.04 % Tween 20. 4′,6-diamidino-2-phenylindole (DAPI) was added to the secondary antibody at a final concentration of 2 μg/ml. Coverslips were mounted on slides with fluorescent mounting medium (Dako). Microscopy was performed using a Zeiss Axioplan 2 microscope using a Plan-NEOFLUAR ×20 objective and captured using an AxioCam MRm imaging camera with AxioVision LE software. All images were collected using identical settings. Brightness and contrast were adjusted identically unless stated otherwise using Adobe Photoshop CS6 software. For higher resolution imaging of ASC and procaspase-8 full-length or DEDs alone, cells were co-transfected as above with plasmids expressing ASC, AIM2, and either V5-tagged procaspase-8 DEDs or Myc-tagged procaspase-8 (C360S) and stained as above with appropriate antibodies. Myc-tagged procaspase-8 was detected using a c-Myc monoclonal antibody harvested from the 9E10.2 hybridoma cell line followed by goat anti-mouse-Alexa Fluor-555. Cells were viewed on a Personal DeltaVision Olympus IX71 inverted wide field deconvolution microscope (Applied Biosystems) using a plan-apochromat ×60/1.42 oil objective. Multicolor Z stacks were captured at 0.2 μm using a Roper Scientific CoolSNAP HQ2 monochrome camera with softWoRx image acquisition software, deconvoluted using standard settings (10 iterations), and then merged using Fiji image software (ImageJ 1.45b, National Institutes of Health) where maximum projection images were generated.

**Electron Microscopy of Inflammasomes Reconstituted in HEK293 Cells**—HEK293 cells were co-transfected with plasmids expressing ASC, AIM2, and HA-tagged procaspase-8 (C360S) and incubated overnight. Cells were then fixed in 4% paraformaldehyde, embedded in gelatin, and infused with polyvinylpyrrolidone/sucrose overnight and then frozen in liquid nitrogen. Ultrathin cryosections were collected onto Formvar-coated grids and immunolabeled using standard protocols using a polyclonal HA antibody (Sigma) followed by 10-nm protein A-gold (Abacus ALS). Sections were viewed on a JEOL 1011 electron microscope (JEOL Australasia) at 80 kV, and images were captured using the iTEM analysis program (Soft Imaging System, Olympus). Brightness and contrast of images were adjusted using Adobe Photoshop CS6 software.

**Quantification of ASC Speck-containing Cells by Flow Cytometry**—Assays were performed as described previously (39) with some modifications. To quantify the ability of AIM2 to induce speck formation by ASC WT/mutants, HEK293 cells (200,000 cells) were plated in 12-well plates and grown overnight. Transfections for each well contained empty vector alone or 50 ng of plasmid expressing ASC WT/mutant and 50 ng of plasmid expressing AIM2 or empty vector. Empty vector was added to each sample so that the final amount of DNA was 800 ng. DNA-Lipofectamine complexes were added to cells, followed by centrifugation of the plates at 700 × g for 10 min. Cells
were harvested after ~16 h and fixed for 15 min with 75% ethanol. Cells were immunostained using the rabbit polyclonal anti-ASC (N-15) (Santa Cruz Biotechnology) at a 1:1500 dilution for 90 min and goat anti-rabbit Alexa Fluor-488 (Life Technologies) at a 1:10,000 dilution for 60 min. The immunostained cells were analyzed on a BD Biosciences Accuri C6 flow cytometer.

Procaspase-8 Filaments at Inflammasomes

To quantify the ability of the various mCherry-tagged AIM2 fusion proteins to induce speck formation by ASC-GFP, HEK293 cells (100,000 cells) were plated in 24-well plates and grown overnight. Varying amounts (1, 3, 10, 30, or 100 ng) of plasmid expressing the different mCherry-tagged AIM2 fusion proteins or mCherry-tagged AIM2 or AIM2 alone were co-transfected with 100 ng of plasmid expressing ASC-GFP. Empty vector (pEF6 plasmid) was added to each sample so that the final amount of DNA was 400 ng. Control samples with empty vector alone or ASC-GFP alone (100 ng) were also prepared. DNA was transfected as above. Cells were harvested 16 h post-transfection and analyzed directly on the Accuri C6 flow cytometer.

In both cases, ASC- or GFP-positive cells were gated and used for further analysis of speck formation using height to area analysis. The percentage of speck-forming cells was determined in a window of moderate ASC expression, below the level at which ASC spontaneously forms specks (39).

Procaspase-8 Activation Assay—HEK293 cells (100,000 cells) were seeded in 24-well plates, grown overnight, and then transfected with 200 ng of pcDNA-ASC WT/mutant and 200 ng of pcDNA-AIM2 or empty vector (pcDNA3.1+). Cells were incubated overnight and then lysed using 200 μl of lysis buffer (66 mM Tris, pH 7.5, 2% SDS). The cell lysates were precipitated with 4 volumes of ice-cold acetone, and the entire pellet was resuspended in SDS-PAGE sample buffer and analyzed by Western blotting. Blots were probed using the mouse monoclonal C15 antibody (Enzo Life Sciences), which detects the p18 subunit of caspase-8, the rabbit polyclonal antibody (AL177) to ASC (AdipoGen), the monoclonal antibody (3B10) to AIM2 (42), and the rabbit monoclonal antibody (5G10) to the S6 ribosomal protein (Cell Signaling Technology) and developed using enhanced chemiluminescence.

Fluorescence Polarization Assay—Labeling was by sortase reaction (43) using 5 μM calcium-independent sortase, 30 μM substrate (His6-MBP-procaspase-8 DEDs (residues 1–186)-Y8A-SUMO-LPETGG), and 500 μM nucleophile (GGG-TAMRA). The mixture was incubated on ice overnight and passed through a Superdex 200 column to remove excess nucleophile. The monomeric fraction of TAMRA-labeled protein after size exclusion chromatography was diluted to a suitable concentration for the assay. A detailed methodology has been published (20). Briefly, TEV protease was added to monomeric TAMRA-labeled His6-MBP-procaspase-8 DEDs (residues 1–186)-Y8A-SUMO-LPETGG to cleave the solubilizing His6-MBP tag and allow the protein to spontaneously polymerize into filaments at a slow rate. The addition of nucleotides to the reaction accelerated filament formation. Fluorescence polarization readings were taken on the SpectraMax M5e (Molecular Devices) using excitation/emission at 561 nm/585 nm. Three readings for each data point were averaged for analysis.

Results

Procaspase-8 Interaction with ASC Requires Both DEDs—Our previous study indicated that procaspase-8 interacts with ASC PYD but not ASC CARD, but the regions of procaspase-8 involved are unknown (8). Here we tested whether the individual DEDs or both DEDs of procaspase-8 were required for the interaction. The individual or tandem DEDs were in vitro translated with [35S]methionine and used for binding assays with GST-ASC PYD or GST alone. Each of the individual DEDs interacted with GST-ASC PYD, although binding of DED1 was very low (Fig. 1). The tandem DEDs (DED1-DED2) resulted in the highest binding, with 5% of the input bound, whereas only 1 or 3% of the input of DED1 and DED2, respectively, bound to GST–ASC PYD. An estimate of the percentage bound was made because the individual DED1 and DED2 constructs contained 4 and 5 labeled methionines, respectively, whereas the tandem DED construct contained 8 methionine residues. Tandem DEDs were also tested at half-concentration to approximate the total 35S signal seen with the individual DEDs and allow direct comparison of the signal strength. This confirmed that both DEDs together mediated optimal interaction of procaspase-8 with ASC PYD.

Procaspase-8 Interaction with ASC PYD in Vitro Is Disrupted by Mutations to Residues That Are Also Important for ASC Self-association and Interaction with AIM2—Prior work indicated that ASC PYD interaction surfaces for NLRP3 and pyrin PYDs overlap with its self-association surfaces (31, 32). Here we investigated the binding surfaces for both procaspase-8 and AIM2 on ASC PYD using a panel of individual point mutations that span the surface of ASC PYD. The key mutations previously reported to disrupt ASC PYD self-association without altering structure (E13A, K21A, R41A, D48A, and D51A) (31) also disrupted its interactions with procaspase-8 and AIM2 (Fig. 2, A and B), although E13A had less effect on procaspase-8 than AIM2 recruitment. The structure of the ASC PYD filament shows six interaction surfaces referred to as type Ia, Ib, Ila, IIb, IIIa, and IIIb (20) (Fig. 2C). The key mutations that disrupted ASC-ASC, ASC-AIM2, and ASC-procaspase-8 interactions are located on the type Ia (D48A and D51A), Ib (K21A), and IIIb (E13A) surfaces and boundary of the Ib/IIa surfaces (R41A) of ASC PYD (Fig. 2C).

Mutations of some residues in self-association surfaces in the ASC PYD filament (20) diminished interaction with pro-
Procaspase-8 Filaments at Inflammasomes

All of the mutations that diminished interactions of ASC PYD with procaspase-8 and AIM2 PYD are present in ASC PYD self-association surfaces defined in the filament structure (20), and/or they diminished ASC PYD self-association (31). The fact that mutations R3A and L25A, which map to ASC PYD self-association surfaces, have a greater effect on procaspase-8 or AIM2 interaction, respectively, than on ASC PYD self-association indicates that the effect of these mutations is a direct effect on binding of procaspase-8 and AIM2 rather than an indirect consequence of disrupting ASC PYD self-association. Thus, the most likely explanation for the results is that ASC PYD uses the same six surfaces to interact with AIM2 PYD and procaspase-8 DEDs via conserved binding modes.

Procaspase-8 and AIM2 Are Recruited to Speck-like Structures Formed by ASC Mutants That Partially Self-associate—To ascertain whether key residues important for in vitro interactions of ASC PYD were also important for in vivo interactions at the AIM2 inflammasome, we reconstituted the AIM2 inflammasome in HEK293 cells. HEK293 cells do not express AIM2, ASC, or procaspase-1 but do express procaspase-8. Cells transfected with a plasmid expressing AIM2 alone showed diffuse localization of AIM2 without any evidence for speck formation (Fig. 3A). However, co-expressed AIM2 and ASC perfectly co-localized to inflammasome specks (Fig. 3A). Although ASC can form specks spontaneously when expressed alone above a certain threshold, speck formation is greatly increased in the presence of AIM2 (39). We surmise that AIM2 clustering nucleates the formation of an ASC speck, and reciprocally, the interaction with ASC stabilizes AIM2 self-association so that it remains part of a stable speck.

Expressed ASC mutants E13A and K21A condensed to form less compact speck-like structures than WT ASC, with a large fraction of AIM2 forming a concentrated focus within the loose ASC speck (Fig. 3A). Similar results were obtained with the ASC D48A and D51A mutants (not shown). Because these full-length ASC mutants evidently maintain some interaction with AIM2 in vivo, it seems that the in vitro binding assays using ASC PYD alone (Fig. 2) are a more stringent test of interaction probably due to the lack of ASC CARD for stabilization of oligomerization. The ASC R41A mutant was strikingly different because it formed elongated filaments (Fig. 3A), similar to those formed by ASC CARD alone (44). We surmise that ASC mutants E13A, K21A, D48A, and D51A partially retain the ability to self-associate via the PYD to form speck-like structures, whereas the ASC R41A mutant has lost the ability to self-associate via the PYD. Our data are in general agreement with a recent study showing that D48A and R41A mutations in GFP-ASC disrupt formation of a compact speck (22). However, in that study, these two mutations caused a similar degree of defect. The difference in results could reflect the ASC-GFP fusion used in the other study. In cells expressing the ASC R41A mutant, AIM2 remained diffuse and did not co-localize with the ASC R41A filaments.

To quantify the ability of AIM2 to promote formation of specks/speck-like structures by WT and mutant ASC, respec-
or presence of AIM2 (Fig. 3) had a very low number of speck-containing cells in the absence of WT ASC or ASC mutants E13A, K21A, D48A, and D51A. Using this method, the percentage of ASC speck-containing cells was determined. Co-expression with AIM2 markedly increased the number of speck-containing cells with expression of the mutant ASC molecules that form less compact specks. Procaspase-8 Filaments at Inflammasomes

We subsequently tested the effect of ASC PYD mutations on procaspase-8 recruitment to specks formed by WT or mutant ASC in the presence of AIM2. Procaspase-8 localization was diffuse when expressed alone but localized to specks formed by WT ASC (Fig. 4). In specks containing AIM2 and ASC mutant E13A or K21A, procaspase-8 was concentrated at the center of the looser speck-like structures (Fig. 4A). Similar results were observed in cells expressing ASC mutants D48A and D51A (not shown). However, procaspase-8 remained diffuse in cells expressing the ASC R41A mutant (Fig. 4A). We conclude that the individual point mutants E13A, K21A, D48A, and D51A that partially retain the ability to self-associate in vivo via the PYD also retained some ability to interact with AIM2 and procaspase-8 in vivo, whereas the R41A mutant, which is unable to self-associate via the PYD, is unable to interact with AIM2 and procaspase-8.
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Procaspase-8 Processing Is Reduced in Cells Expressing ASC PYD Mutants That Form Loose Specks—Because procaspase-8 was recruited to the speck-like structures formed by ASC mutants E13A, K21A, D48A, and D51A, endogenous procaspase-8 processing was assessed in cells expressing ASC WT or mutants with or without AIM2. Normal processing of procaspase-8 occurs in two steps; one cleavage between the two catalytic fragments releases p43 (DEDs linked to the large catalytic subunit) and p10 (small catalytic subunit). A second cleavage removes the DEDs to release the p18 large catalytic subunit (33). Cells expressing WT-ASC showed AIM2-dependent processing of procaspase-8 to the p43 and p18 fragments (Fig. 4B). ASC-R41A, which does not form specks (Fig. 3A) or interact with procaspase-8 in vitro (Fig. 2A), did not show any processing of procaspase-8. In general, there was reduced procaspase-8 processing with ASC mutants K21A, D48A, and D51A, which all generated looser specks. Interestingly, although the E13A mutant formed loose ASC specks, procaspase-8 often formed tight foci within these (Fig. 4B), and E13A was able to induce processing of procaspase-8 almost as well as WT-ASC. Notably, E13A retained partial interaction with procaspase-8 in vitro (Fig. 2A) unlike mutants K21A, D48A, and D51A. Curiously, the E67A mutant that showed enhanced interaction with procaspase-8 showed only modest processing of procaspase-8 to the p18 form.

Procaspase-8 DEDs Can Induce ASC Speck Formation—Because in vitro binding studies with ASC mutants indicated that the interaction modes of ASC PYD with AIM2 PYD and procaspase-8 are similar and overlap with ASC PYD self-association modes, we conjectured that if the procaspase-8 DEDs (DEDs<sup>PC8</sup>) or the ASC PYD (PYD<sup>ASC</sup>) were fused to the AIM2 HIN domain (HIN<sup>AIM2</sup>), then the resulting fusion proteins (DEDs<sup>PC8-HINAIM2</sup> and PYD<sup>ASC-HINAIM2</sup>, respectively; Fig. 5A) should be able to induce ASC specks. In agreement with this hypothesis, when DEDs<sup>PC8-HINAIM2</sup> was expressed in vitro with GFP-tagged ASC (ASC-GFP), it induced ASC-GFP to form specks, as detected by the flow cytometry-based assay (Fig. 5B). However, it was not as efficient as AIM2 at inducing specks. As expected, the AIM2 HIN domain alone had a negligible effect on speck induction (Fig. 5B). Surprisingly, PYD<sup>ASC-HINAIM2</sup> was less efficient than DEDs<sup>PC8-HINAIM2</sup> and AIM2 at inducing ASC-GFP specks (Fig. 5B). In addition, when the NLRP3 PYD, which is normally linked to a nucleotide binding and oligomerization domain, was fused to the AIM2 HIN domain, the resulting protein (PYD<sup>NLRP3-HINAIM2</sup>) induced ASC-GFP specks as efficiently as the procaspase-8 DEDs construct (DEDs<sup>PC8-HINAIM2</sup>). Assessment of the morphology of the specks induced by microscopy showed that all of the fusion proteins simult...
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FIGURE 4. Procaspase-8 is recruited to the AIM2-induced speck-like structures formed by ASC mutants and is weakly activated in cells expressing these mutants but not in cells expressing the R41A mutant that forms filaments. A, HEK293 cells were transfected with plasmids expressing WT ASC alone, V5-tagged procaspase-8 C360S inactive mutant (PC8) alone, or AIM2 with PC8 and either WT or mutant ASC. Cells were immunostained with anti-ASC (green) or anti-V5 (red), and the nuclei were counterstained with DAPI (blue). The brightness of cells expressing ASC-WT alone or procaspase-8 alone or ASC-R41A mutant with AIM2 and procaspase-8 was increased to improve visibility. B, plasminogen rich plasma expressing WT or mutant ASC were co-transfected either with a plasminogen rich plasma expressing AIM2 or with empty vector into HEK293 cells. After 24 h, processing of endogenous procaspase-8 to the p43 and p18 forms was detected by Western blotting. A band below the p43 form marked with an asterisk is constitutively present and has not been identified. The level of S6 ribosomal protein was used as a loading control. The data are representative of two independent experiments.

FIGURE 5. Procaspase-8 DEDs can substitute for AIM2 PYD to induce ASC specks with lower efficiency. A, procaspase-8 DEDs or PYDs of ASC and NLRP3 were fused to the AIM2 HIN domain and mCherry-tagged. B, HEK293 cells were transfected with plasmids expressing ASC-GFP and increasing amounts of plasminogen expressing either mCherry-tagged AIM2 or one of the mCherry-tagged AIM2 HIN fusion proteins. At 16 h post-transfection, cells were analyzed by flow cytometry to quantify the number of cells containing GFP-ASC specks. For each sample, the percentage of speck-containing cells in the same expression window of ASC-GFP expression is shown and is plotted against the expression levels of the mCherry-tagged proteins, which were also determined by flow cytometry and are expressed as mean fluorescence intensity (MFI). Data from two replicates are plotted separately. The data are representative of two independent experiments. Notably, some mCherry-tagged proteins were expressed at higher levels with equivalent amounts of plasmin sank transfected.

ASC-induced Polymerization of Procaspase-8 DEDs Is Dependent on ASC PYD—Formation of filaments is a characteristic of PYDs and CARDs (19, 20). Furthermore, procaspase-8 has been proposed to form polymers at death receptor signaling complexes (37, 45), and upon overexpression, procaspase-8 DEDs alone are known to form filaments (46). Thus, we hypothesized that ASC may induce procaspase-8 polymerization into a filament. A fluorescence polarization assay was used to quantitatively investigate the ability of ASC to induce procaspase-8 polymerization in vitro. The assay is based on the fact that a polymer moves more slowly in solution compared with its constituent monomers. Thus, when excited with polarized light, the emitted fluorescence from a labeled polymer that moves slowly remains highly polarized, whereas the emitted fluorescence from labeled monomers that tumble rapidly is less polarized. Because purified procaspase-8 DEDs lacking the catalytic domain spontaneously form oligomers, we expressed a Y8A mutant that reduced self-association and allowed purification of monomers when fused to an N-terminal His6-MBP tag and a C-terminal SUMO tag. The fusion protein also contained a C-terminal sortase recognition motif for labeling with a fluorescent probe (43). The fluorescently labeled His6-MBP-procaspase-8 DEDs-Y8A-SUMO fusion protein was incubated either alone or with two different concentrations of ASC PYD, ASC CARD, or full-length ASC, also prepared as monomers by

Similarly recruited ASC to a compact speck (Fig. 6). Overall, the data support similar interaction modes between homotypic PYD interactions and the procaspase-8 DEDs/ASC PYD interaction.
caspase-8 DEDs-Y8A but were not as effective as full-length ASC PYD filaments increased the rate of polymerization of procaspase-8 DEDs-Y8A. The GFP-ASC PYD fragments used in this study were incapable of nucleating DED filaments faster than they form spontaneously. Indeed, analogous recent work examining the formation of ASC PYD filaments showed a lack of spontaneous polymerization (20). Because GFP-tagged ASC PYD had a tendency to form oligomers upon purification, we investigated whether these aggregates would be able to induce polymerization of procaspase-8 DEDs-Y8A. The GFP-ASC PYD filaments increased the rate of polymerization of procaspase-8 DEDs-Y8A but were not as effective as full-length ASC (Fig. 7C). Thus, we surmise that ASC PYD has to be in an oligomerized state to be able to promote polymerization of procaspase-8 DEDs.

EM Analysis of Procaspe-8 DED-ASC Complexes Indicates Formation of Procaspe-8 DED Filaments with Unidirectional End Labeling of ASC—As previously stated, the procaspase-8 tandem DEDs form filaments spontaneously. Thus, to visualize complexes that are assembled in the presence of monomeric forms of both proteins by EM, MBP-tagged procaspase-8 DEDs-Y8A monomers were cleaved with TEV protease alone or in the presence of MBP-tagged full-length ASC monomers. Procaspase-8 DEDs-Y8A monomers alone assembled into multiple short filaments, whereas procaspase-8 DEDs-Y8A monomers incubated in the presence of ASC yielded fewer longer filaments (Fig. 8). Labeling of the ASC/procaspase-8 DEDs-Y8A filaments indicated that procaspase-8 DEDs-Y8A was labeled all along the length of the filament, whereas ASC labeling was only detected at one end of the filaments (Fig. 8). We infer that ASC can induce assembly of procaspase-8 DEDs into...
filaments and that the two proteins appear to segregate rather than forming an intermingled filament.

**Inflammasome-associated Procaspase-8 Is Filamentous**—Formation of procaspase-8 filaments in vivo upon recruitment to AIM2 inflammasomes reconstituted in HEK293 cells was investigated by high-resolution fluorescence microscopy. ASC and procaspase-8 both appeared filamentous on the basis of projections from the periphery of the speck (Fig. 9). However, procaspase-8 was more concentrated in the center of the speck than ASC, in agreement with a recent study (47). In contrast, procaspase-8 DEDs without the catalytic domain were recruited to inflammasomes in a more peripheral location and were strikingly filamentous with long projections extending from some specks. Interactions between catalytic domains in separate filaments are a likely explanation for the compaction of the full-length procaspase-8 filaments. Together, these data indicate that procaspase-8 is induced to form filaments upon recruitment to inflammasomes and that the catalytic domain plays a role in compaction of procaspase-8 at the inflammasome. Consistent with a previous study (46), the procaspase-8 DEDs spontaneously formed filaments when expressed alone, but these were extremely long and were not initiated from a central structure, whereas full-length procaspase-8 was diffuse.

To further examine procaspase-8 within the inflammasome, immunoelectron microscopy was used on HEK293 cells containing reconstituted AIM2 inflammasomes with HA-tagged procaspase-8 (Fig. 9). A representative image shows anti-HA gold labeling decorating a dense mass consistent with the presence of procaspase-8 at inflammasomes and resembles immunogold EM of ASC at inflammasomes (20). Gold labeling (arrows) can be seen on smaller filamentous projections at the periphery of the inflammasome, with detection limited to those found within the plane of the section (Fig. 9). Thus, both fluorescence microscopy and EM provide evidence for procaspase-8 filaments associated with inflammasomes.

**Discussion**

Recent studies on filament formation by PYD and CARD domains of inflammasome components have given key insights into inflammasome structure and suggested a role for procaspase-1 filaments in caspase-1 activation (19, 20, 48, 49). Here we show that procaspase-8 DEDs also form filaments upon recruitment to inflammasomes. Procaspase-8 DED filaments were induced in vitro by ASC clusters, suggesting that an ASC PYD filament nucleates the DED filament. In vitro binding studies showed that both DEDs were required for optimal binding to ASC PYD and that the DED-PYD interaction utilized conserved binding modes also seen in homotypic PYD interactions. Recruitment of procaspase-8 DEDs to ASC speck in vivo was strikingly filamentous, but interestingly, full-length procaspase-8 was more condensed in reconstituted inflammasomes, suggesting interactions between the catalytic domains. We speculate that filament formation and interaction between procaspase-8 catalytic domains on separate filaments are important for activation.

The interaction between ASC PYD and procaspase-8 DEDs is unusual because the death fold domains generally form homotypic interactions within a subfamily. However, in phylogenetic analyses, the DEDs and PYDs are the most closely related death fold domains (10, 50), which may explain this interaction. Homotypic PYD interactions are mediated by type I, II, and III interaction modes (20, 31, 32, 50), and our data are consistent with the same interaction modes being used between procaspase-8 DEDs and ASC PYD. Published data (35, 38, 51) show a role for a type I-like interaction between the tandem DEDs of procaspase-8 and the viral inhibitory protein MC159. This is mediated by a typical type Ib interaction site involving helices 1 and 4 of DED2 but involves helices 2 and 5 of DED1 instead of a typical 1a site, which comprises helices 2 and 3. A phenylalanine residue (Phe-30) from the interface of MC159 is conserved in FADD DED and procaspase-8 DEDs (34, 36, 37). This conserved phenylalanine in procaspase-8 DED2 (Phe-122) is important for interaction with FADD DED (35), and mutation of this residue and the nearby Ile-128 facilitated purification of soluble procaspase-8 DEDs in a recent crystal structure determination (38). These data suggest that the type I interaction is conserved in these other DEDs.

Our data indicate that ASC PYD filaments nucleate procaspase-8 DED filaments. Immunogold labeling showed a terminal location for ASC and suggested that the two different
domains do not intermingle along the length of the filament. Because only GFP-ASC PYD aggregates or full-length ASC, which has a high tendency to aggregate, can induce procaspase-8 DED filaments, ASC PYD must be self-associated in a filament before it can induce procaspase-8 DED filaments. This is in agreement with the events thought to take place in vivo when an activated pattern recognition receptor, such as AIM2 or NLRP3, recruits ASC via its PYD and induces ASC filaments that condense into a speck (12, 13, 15, 20–23, 25). A proposed model for nucleation of a procaspase-8 DED filament by the triple helical ASC PYD filament is presented in Fig. 10. In an ASC PYD filament, type I interaction surfaces mediate interactions between subunits in a helix, whereas type II and III interaction surfaces mediate interactions between the helices (20).

Thus, the type II and III interaction surfaces exposed at the end of an ASC PYD filament are likely to play a major role in forming a platform for recruitment of procaspase-8 via complementary sites. Homotypic type I interactions between adjacent DEDs may then be favored, driving the filament to become a pure DED oligomer. The structure of the DED filament is yet to be described.

AIM2 PYD filament structures have recently been determined (16, 52). Models developed for inflammasome formation suggest that oligomerization of AIM2 along DNA permits the clustering of a small number of AIM2 PYD into very short filaments that then nucleate ASC oligomerization. This is consistent with the observation of AIM2 capping one end of ASC filaments formed in vitro (20). Similarly, here ASC initiated
Procaspase-8 filaments and capped one end. In both these cases, the AIM2 or ASC cap formed only a short terminal section of the filament. Together, these data are consistent with filaments having a polarity that allows growth in one direction only. Thus, we assume that during inflammasome formation, a filament initiated by AIM2 PYD is continued by ASC PYD and then procaspase-8 DEDs in a linear fashion. Here we found that AIM2 with procaspase-8 DEDs in place of the normal AIM2 PYD still promoted formation of ASC specks. However, the relatively low efficiency observed may be accounted for by this DED-PYD interaction being of opposite polarity to the physiological mode because ASC would normally recruit procaspase-8 in the growing filament. Nevertheless, a degree of functional substitution emphasizes the general conserved nature of DED and PYD interactions.

Our finding that procaspase-8 filaments are induced at inflammasomes, which have been shown to induce apoptosis (8), suggests a role for filament formation in procaspase-8 activation and induction of apoptosis. The importance of procaspase-8 dimerization for activation of its proteolytic activity has been established, and roles for intradimer and interdimer cleavage for procaspase-8 activation have been proposed (53, 54), but it is not clear how procaspase-8 is recruited and activated at apoptosis-inducing signaling complexes. Procaspase-8 recruitment and activation have mostly been studied at the death receptor-mediated death-inducing signaling complex (DISC). One model proposed is of discrete DISC complexes with intra-DISC and inter-DISC interactions important for procaspase-8 activation and processing, whereas an alternate model is a network linked together by ligand binding and/or FADD self-association to form a honeycomb structure to which procaspase-8 is recruited (35, 54–57). However, recent co-immunoprecipitation studies of DISC complexes have revealed a high stoichiometric ratio of procaspase-8 to the other components of the DISC, leading to the proposal that procaspase-8 forms polymers at the DISC (37, 45). However, the nature of the polymers was unclear. Furthermore, although previous studies have shown that overexpression of procaspase-8 DEDs results in the formation of filaments that recruit endogenous procaspase-8 and induce apoptosis (46), the biological relevance of these filaments to natural death-inducing complexes was not clear. Our data demonstrate polymerization of procaspase-8 into filaments at an apoptosis-inducing complex and rationalize the relevance of both of these earlier findings. Furthermore, because full-length procaspase-8 was more condensed at the inflammasome compared with the DEDs alone, interfilament interactions may be mediated by the catalytic domains. It is yet to be ascertained whether interactions between catalytic domains within a filament or between filaments mediate dimerization and interdimer processing.

The effect of the cellular inhibitor of procaspase-8, cFLIPL (cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein long form) (58), on procaspase-8 activation at the inflammasome is yet to be tested. cFLIPL is also a tandem DED-containing protein but has a catalytically inactive caspase-like domain (59) and has recently been shown to promote activation of caspase-1 by the AIM2 and NLRP3 inflammasomes (60). Its function was attributed to interaction of the caspase-like domain of cFLIPL with NLRP3 and AIM2. Given the interaction of procaspase-8 DEDs with ASC, testing the interaction of cFLIP DEDs with ASC may give additional insights into the mode of cFLIP action at inflammasomes. Furthermore, because the tandem DEDs of cFLIP are known to interact with procaspase-8 DEDs (61), cFLIP may form a mixed filament with procaspase-8 and could modulate activation.

In addition to its role in apoptosis, in some circumstances, caspase-8 can mediate pro-IL-1β processing and is important for NLRP3 inflammasome priming via TLR4 (Toll-like receptor 4) signaling (62–64). Intriguingly, caspase-8, together with cFLIP, has a role in mediating cell survival via inhibition of necroptosis, which is important for lymphocyte survival and expansion (9, 65). Finally, caspase-8 is proposed to mediate NF-κB activation in response to viral infections (66, 67). Thus, caspase-8 is important for immune function and homeostasis. The contribution of filament formation to these different functions is yet to be elucidated. Inflammasome-mediated procaspase-8 oligomerization and activation raise the possibility that filament formation may be a requirement for caspase-8 activation in other apoptotic signaling platforms, such as the DISC.

**FIGURE 10. Model to illustrate interaction of ASC PYDs and procaspase-8 DEDs.** Shown is a flattened view of a proposed ASC PYD-procaspase-8 DED filament to show the different types of death domain interactions. ASC PYDs belonging to each strand of the triple helix are colored yellow, light green, or dark green, whereas procaspase-8 tandem DEDs are colored pink. Our data indicate that procaspase-8 DEDs are recruited to ASC PYD via the same type I, II, and III interactions that mediate assembly of the ASC PYD triple helix, and we hypothesize that the same interactions mediate assembly of the DED filament. Type I, II, and III interactions are indicated on domains at/near the interface as thick black, blue, and orange lines, respectively. The model shows how both DEDs of procaspase-8 can interact with ASC PYDs at the interface between the filaments.
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