Distinct axial and lateral interactions within homologous filaments dictate the signaling specificity and order of the AIM2-ASC inflammasome

Mariusz Matyszewski, Weili Zheng, Jacob Lueck, Zachary Mazanek, Naveen Mohideen, Albert Y. Lau, Edward H. Egelman & Jungsan Sohn

Inflammasomes are filamentous signaling platforms integral to innate immunity. Currently, little is known about how these structurally similar filaments recognize and distinguish one another. A cryo-EM structure of the AIM2PYD filament reveals that the architecture of the upstream filament is essentially identical to that of the adaptor ASCPYD filament. In silico simulations using Rosetta and molecular dynamics followed by biochemical and cellular experiments consistently demonstrate that individual filaments assemble bidirectionally. By contrast, the recognition between AIM2 and ASC requires at least one to be oligomeric and occurs in a head-to-tail manner. Using in silico mutagenesis as a guide, we also identify specific axial and lateral interfaces that dictate the recognition and distinction between AIM2 and ASC filaments. Together, the results here provide a robust framework for delineating the signaling specificity and order of inflammasomes.
Inflammasomes are filamentous signaling platforms and play key roles in the metazoan innate immune system. These supra-structures assemble upon detecting molecular signatures arising from various intracellular catastrophes, such as genomic instability, dysfunctional organelles, and pathogen invasion. Mammals have at least 15 different receptors that lead to the assembly of inflammasomes whose ultimate goal is to induce the polymerization of procaspase-1, activating the zymogen protease by proximity-induced autoproteolysis. Caspase-1 then executes two key innate immune responses: the cleavage/maturation of pro-inflammatory cytokines, such as interleukin-1β and -18, and the initiation of pyroptosis. Inflammasomes play essential roles in host defense against pathogen invasion (e.g., coronaviruses, herpesviridae, and Listeria monocytogenes). In addition, malfunctioning inflammasomes promote acute and chronic autoimmune diseases (e.g., severe COVID-19, rheumatoid arthritis, and systemic lupus erythematosus (SLE)) and metabolic disorders (type 2 diabetes) and even tumorigenesis (colon cancer, lung cancer, and oral cancer).

Inflammasome receptors contain multiple functional domains for autoinhibition, signal recognition, and oligomerization. Importantly, the N-terminal pyrin domain (PYD) acts as the primary signal transduction module in the vast majority of inflammasomes. PYDs are six-helix bundles that belong to the death-domain (DD) superfamily and can assemble into helical filaments. For instance, incoming signals such as viral nucleic acids induce the assembly of a receptor PYD filament, leading to the oligomerization of the CARD of ASC (ASC CARD) to recruit and trigger the polymerization (activation) of procaspase-1 (ASC: apoptosis-associated speck-forming protein containing caspase-recruiting domain; CARDs are also six-helix bundles that belong to the DD family).

Although the structural mechanisms by which inflammasomes assemble are increasingly better understood, little is known about the mechanisms that direct the signaling order (sequence) and specificity. For instance, all published cryo-electron microscopy (cryo-EM) structures of PYD filaments show essentially the same helical architectures (six subunits per helical turn). All CARD filaments also show the same helical architectures (four subunits per helical turn). These observations then led to a well-accepted model, in which the architectural complementarity between upstream and downstream filaments underpins the recognition. However, it raises a considerably more complex problem as to how these similar helical filaments built from homologous protomers distinguish and recognize one another within respective subfamilies. Here, we address this fundamental mechanistic issue in the cytosolic double-stranded (ds)DNA-sensing AIM2-ASC inflammasome. AIM2 (absent in melanoma 2) is a bipartite protein composed of the N-terminal PYD followed by the dsDNA-binding HIN domain (hematopoietic interferon-inducible nuclear antigen). Upon binding cytosolic dsDNA via its HIN domain, AIM2PYD assembles into filaments, inducing the polymerization of ASC. AIM2 is essential for the host defense against numerous pathogenic viruses and bacteria. AIM2 also plays vital roles in neuronal development by regulating timely cell death. However, dysregulated AIM2 leads to various maladies, such as SLE, chronic kidney diseases, and lung cancer.

We present a cryo-EM structure of the AIMA2PYD filament at 3.2 Å resolution, which reveals that its architecture is indeed identical to that of the ASCPYD filament. Using our structure, we then investigate how AIMA2PYD and ASCPYD filaments recognize, and distinguish each other by Rosetta and molecular dynamics (MD) simulations. Our in silico analyses consistently suggest that the energy landscapes that underpin the assembly of individual filaments do not impose directionality. By contrast, the energy landscape that governs the recognition between AIMA2PYD and ASCPYD is polarized in a head-to-tail manner. Multiple biochemical experiments corroborate that individual filaments assemble bidirectionally. Moreover, AIMA2PYD and ASCPYD filaments do not co-assemble, and the signal transduction from AIM2 to ASC occurs unidirectionally only when at least one is oligomeric. Using Rosetta-based in silico mutagenesis as a guide, our biochemical and cellular experiments consistently show that lateral interfaces of AIMA2PYD drive its bidirectional assembly. We also identify specific axial interfaces that mediate the recognition between AIMA2PYD and ASCPYD. Together, we demonstrate that distinct interfaces within homologous filaments direct signaling order and specificity of inflammasomes. We also set forth a broadly applicable multidisciplinary platform for delineating the signal transduction order, specificity, and directionality of filamentous assemblies.

**Results**

The cryo-EM structure of AIMA2PYD. Using EM of negatively stained samples (nSEM), we previously found that the helical symmetry of the AIMA2PYD filament is consistent with that of the ASCPYD filament, and thus proposed that architectural complementarity is important for their recognition. However, the published high-resolution cryo-EM structure of the AIMA2PYD filament displays an altered helical architecture because the N-terminal green fluorescence protein (GFP)-tag interferes with assembly. Thus, we first determined the cryo-EM structure of the AIMA2PYD filament using an untagged recombinant protein.

Cryo-EM images showed that AIMA2PYD filaments are straight helical rods. The average power spectrum of 512-pixel-long nonoverlapping filament segments showed that the AIMA2PYD filament displays a six-start, C3 helical symmetry of 54.4° rotation (~6 subunits per helical turn) and an axial rise of 14 Å. These parameters are remarkably similar to those of the ASCPYD filament, further solidifying the concept that the upstream receptors provide structural templates for downstream assemblies in inflammasomes.

We fit the crystal structure of AIMA2PYD into the EM map for initial modeling, and the refined high-resolution map allowed us to model in most bulky and aliphatic side chains. The resolution of the final model was 3.2 Å according to the gold standard method (Supplementary Fig. 1A). The diameter of the outer rim is ~94 Å and that of the inner cavity is ~25 Å. The structure of individual AIMA2PYD protomers is identical to the crystal structure of AIMA2PYD monomer (Supplementary Fig. 1B), thus indicating that, unlike the PYD of NLRP6, an AIMA2PYD monomer does not undergo any conformational changes during activation. As seen from the ASCPYD filament, each AIMA2PYD subunit contributes three unique protein–protein interaction interfaces (Fig. 1E).

The type 1a:1b interface is largely composed of side-chain interactions, while the type 2a:2b and type 3a:3b interfaces involved both side-chain and backbone interactions (Fig. 1E). We also noted several side chains previously implicated in filament assembly throughout different interfaces (e.g., L11, D19, F27, and I46; Fig. 1E). Aligning the new AIMA2PYD filament to the GFP-AIM2PYD filament demonstrates that although the lateral interactions are largely conserved, the axial positions are significantly different due to the altered helical symmetry (five subunits per turn in the GFP-tagged filament vs. six subunits per turn in the untagged filament; Supplementary Fig. 1C). On the other hand, aligning the cryo-EM structures of AIMA2PYD and ASCPYD filaments demonstrates their congruent architectures (Fig. 1F). The subtle difference in subunit assembly...
positions between AIM2\textsuperscript{PYD} and ASC\textsuperscript{PYD} filaments along the helical axis could reflect the unique side-chain interactions that mediate their respective filament assembly or the inherent flexibility of biomolecular structures (Fig. 1F). Nevertheless, the near perfect architectural complementarity between AIM2 PYD and ASCPYD filaments supports the idea that upstream filaments provide structural templates for the assembly of downstream filaments\textsuperscript{14,17,26,37}.

Deciphering the specificity and directionality of the AIM2-ASC inflammasome using Rosetta and MD. AIM2\textsuperscript{PYD} and ASC\textsuperscript{PYD} monomers are homologous and structurally highly conserved (root-mean-squared-deviation, RMSD 0.5 Å), and our new cryo-EM structure shows that they indeed assemble into essentially identical filaments (Fig. 1F). These observations raise significantly more complex questions as to whether and how these suprastructure distinguish and recognize each other. Importantly, such questions are germane to all filamentous signaling platforms employing PYDs or CARDs\textsuperscript{21,22,26,37–39}. Thus, to establish a broadly relevant method for tackling these questions, we employed a computational approach using Rosetta. First, we tested whether RosettaDock\textsuperscript{40} could recapitulate the cryo-EM structures by docking an AIM2PYD monomer into our AIM2PYD filament structure (also an ASCPYD monomer to the ASCPYD filament (PDB ID: 3J63))\textsuperscript{18}. For instance, each PYD protomer provides three unique interfaces in AIM2PYD and ASCPYD filaments (i.e., six distinct surfaces; Figs. 1E and 2A). To facilitate docking experiments, we generated a honeycomb-like side view of AIM2PYD and ASCPYD filaments, in which the center protomer
Fig. 2 | In silico studies suggest that homotypic filaments assemble bidirectionally and the recognition between AIM2 and ASC occurs unidirectionally.

A Cartoon representations of honeycombs. Each interface type is labeled in the hexagons which represent PYD monomers. B Rosetta docking strategy. Top docking indicates that a ligand monomer docks onto the top surface of the pocket, while bottom docking is the opposite. C Plots of Rosetta interface energy scores vs. RMSD for docking an ASCPYD monomer on the top or bottom of AIM2PYD pockets. The B represents simulations conducted in C. D Plots of Rosetta interface energy scores vs. RMSD for docking an ASCPYD monomer on the top or bottom of AIM2PYD pockets. E A model of the AIM2PYD filament recognition of the ASCPYD filament. F G Rosetta interface energy scores at individual filament interfaces for homotypic and heterotypic assemblies. AIM2PYD-AIM2PYD, ASCPYD-ASCPYD, ASCPYD-AIM2PYD, and AIM2PYD-ASCPYD. Each hexagon represents AIM2PYD or ASCPYD monomer. H A plot for the difference in free energy (ΔΔG) for dissociating ligand PYDs from the top or bottom pockets.

makes all six possible contacts (Fig. 2A). We then divided the honeycomb into six unique subsections consisting of one ligand docked into a pocket created by three adjacent subunits (Fig. 2B). Using the local docking method in Rosetta, we performed 5000 independent docking simulations between a ligand–pocket pair from each subsection, then compared the interface energy and RMSD from the cryo-EM structures.

For each filament, both parameters decreased concurrently, while displaying uniform energy scores from all subsections (Fig. 2C (arrow) and Supplementary Fig. 2A), indicating that RosettaDock can recapitulate the cryo-EM structures. The more favorable energy scores from the AIM2PYD filament suggest that it is more stable than the ASCPYD filament (typically −70 s for AIM2PYD complexes vs. −50 s for ASCPYD complexes). Importantly, the uniform energy scores throughout the top and bottom subsections (Fig. 2C and Supplementary Fig. 2A) suggest that individual filaments would assemble bidirectionally. Next, we docked an ASCPYD monomer (ligand) onto all six pockets of the AIM2PYD filament and vice versa (Fig. 2D and Supplementary Fig. 2B). We first noted that the interface energies are not as favorable as the AIM2PYD•AIM2PYD complexes (−60 or worse for ASCPYD•AIM2PYD complexes; Fig. 2D and Supplementary Fig. 2B). Moreover, docking ASCPYD on the top pockets of the AIM2PYD filament was significantly more favorable than docking at the bottom. (Fig. 2D orange vs. gray). Docking AIM2PYD on the ASCPYD filament also showed that AIM2PYD prefers the bottom half of the ASCPYD filament with the energy scores as favorable as the homotypic ASCPYD assembly (Supplementary Fig. 2A, B (2B orange vs. gray)). These results suggest that individual filaments assemble bidirectionally, while the recognition between AIM2PYD and ASCPYD occurs unidirectionally, where the top of the AIM2PYD filament recognizes the bottom of the ASCPYD filament (Fig. 2E).

Next, we used Rosetta InterfaceAnalyzer to evaluate the interaction energies between homotypic and heterotypic interactions at the individual interfaces of the honeycomb (Fig. 2F). The AIM2PYD complex also showed the most favorable overall interface energy scores (Fig. 2G). Moreover, for the respective homotypic assembly of AIM2PYD and ASCPYD, the type 1 interface contributed most significantly with the top and bottom halves displaying symmetric energy scores (Fig. 2F). On the other hand, the interface energy scores were consistently worse, when
ASC\textsuperscript{PYD} was placed at the center of the AIM2\textsuperscript{PYD} honeycomb, except for the one between the type 2a of ASC\textsuperscript{PYD} and 2b of AIM2\textsuperscript{PYD} (Fig. 2F vs. 2G). The overall energy scores between the top and bottom halves were again asymmetric, preferring a head-to-tail-like direction, in which the top of AIM2\textsuperscript{PYD}s favoring the bottom of ASC\textsuperscript{PYD} and vice versa (Fig. 2E, G; the small difference in energy scores between Fig. 2F, G likely stemmed from the subtle architectural differences in two filaments).

To test whether the simulation results are not biased by a particular algorithm, we then used MD to calculate the free energy required to dissociate a ligand PYD from each pocket described in Fig. 2B (i.e., stability; Supplementary Fig. 2C, D). Consistent with the results from RosettaDock, MD simulations suggested the AIM2\textsuperscript{PYD} filament complex to be most stable, followed by ASC\textsuperscript{PYD}•AIM2\textsuperscript{PYD} then ASC\textsuperscript{PYD} complexes. (Supplementary Fig. 3B; see also Supplementary Fig. 3C for images showing the dissociation of each monomer before and after the simulation). We compared the sum of energies required to dissociate a ligand PYD from the top vs. bottom halves (Fig. 2H; Supplementary Fig. 2C). Individually, AIM2\textsuperscript{PYD} and ASC\textsuperscript{PYD} complexes showed mostly uniform energy landscapes from either filament pole, with both filaments showing more stable interactions at the bottom (Fig. 2H and Supplementary Fig. 3A, B). The moderate asymmetry suggests that the bottom interfaces might be preferred for homotypic assembly, or it could also reflect the intrinsic noise from sampling multiple conformations in an all-atom MD simulation. Nevertheless, consistent with Rossetta simulations, significantly more energy was required to dissociate ASC\textsuperscript{PYD} from the top of the AIM2\textsuperscript{PYD} filament than the bottom (Fig. 2H and Supplementary Fig. 3B). Together, our in silico analyses consistently suggest that individual filaments assemble bidirectionally, AIM2\textsuperscript{PYD} strongly prefers to assemble homotypically, and the recognition between AIM2\textsuperscript{PYD} and ASC\textsuperscript{PYD} occurs via the type 2 interface.

**In vitro experiments corroborate in silico predictions.** To test our simulation results, we first tracked the assembly of fluor-labeled recombinant AIM2\textsuperscript{PYD} and ASC\textsuperscript{PYD} filaments via confocal fluorescence microscopy. When we mixed two populations of differentially labeled maltose-binding-protein-tagged (MBP)-AIM2\textsuperscript{PYD} at 1:1 ratio and triggered polymerization by cleaving MBP via Tobacco Etch Virus protease (TEVp)\textsuperscript{19}, the two colors colocalized in the same filaments (Fig. 3A, AIM2\textsuperscript{PYD}•AIM2\textsuperscript{PYD}); differentially labeled ASC\textsuperscript{PYD} populations also colocalized in the same filaments (Fig. 3A, ASC\textsuperscript{PYD}•ASC\textsuperscript{PYD}). Importantly, when we preassembled the AIM2\textsuperscript{PYD} filament labeled with one color and added AIM2\textsuperscript{PYD} monomers labeled with a different color, nascent filaments extended from both axial poles of existing filaments (Fig. 3A, (AIM2\textsuperscript{PYD} filament) + AIM2\textsuperscript{PYD}); ASC\textsuperscript{PYD} filaments also displayed random bidirectional assembly (Fig. 3A, (ASC\textsuperscript{PYD} filament) + ASC\textsuperscript{PYD}). These results corroborate that homotypic filament complexes assemble bidirectionally. Next, we mixed differentially labeled MBP-AIM2\textsuperscript{PYD} and MBP-ASC\textsuperscript{PYD} at 1:1 ratio, and monitored their filament assembly upon triggering polymerization via TEVp. Here, each protein appeared to be oligomerized separately without colocalizing on the same filament, and two distinct filaments interacted only at one specific axial pole (Fig. 3B, AIM2\textsuperscript{PYD}•ASC\textsuperscript{PYD}). Such a unidirectional interaction was even more evident when we added excess nascent proteins over pre-formed filaments (Fig. 3B, (AIM2\textsuperscript{PYD} filament) + ASC\textsuperscript{PYD} and (ASC\textsuperscript{PYD} filament) + AIM2\textsuperscript{PYD}). In addition, no significant Förster resonance energy transfer (FRET) signals were observed when we triggered the assembly of a donor-labeled AIM2\textsuperscript{PYD} and acceptor-labeled ASC\textsuperscript{PYD} (Fig. 3C, see also ref. 19), indicating that AIM2\textsuperscript{PYD} and ASC\textsuperscript{PYD} do not co-assemble, yet the recognition entails at least one to be oligomeric. Overall, our biochemical experiments agree with the computational predictions.

**Simulations to identify key interfaces that govern the recognition and distinction between AIM2\textsuperscript{PYD} and ASC\textsuperscript{PYD}**. Our observations thus far suggest that there exist distinct interactions that underpin individual assemblies and those that mediate the recognition between AIM2\textsuperscript{PYD} and ASC\textsuperscript{PYD}, such as the type 2 interface for the heterotypic recognition. The side chains at the filament interfaces are poorly conserved between AIM2\textsuperscript{PYD} and ASC\textsuperscript{PYD} (Supplementary Fig. 4A), indicating that diverse
In vitro and in cellulo experiments corroborate in silico predictions. Mutations that abolish the self-assembly of AIM2PYD decreases the dsDNA-binding activity of AIM2FL, as oligomerization is couple to signal recognition. Nevertheless, most of such AIM2FL mutants still assemble into filaments on the dsDNA scaffold. Also of note, AIM2 filaments can self-perpetuate its assembly by accelerating the polymerization of nascent monomers. Thus, to test our simulation results, we generated Rosetta predicted mutations on AIM2FL and first confirmed filament formation on dsDNA (Supplementary Fig. 5; all mutants formed filaments except N73L). We then determined whether dsDNA-bound AIM2FL mutants could accelerate the polymerization of FRET donor/acceptor-labeled AIM2PYD or ASCPYD (Fig. 5A–C and Supplementary Fig. 6; see also ref. 19).

As predicted from our simulation, L11A, A36D/R, and I46D were significantly more defective in accelerating the polymerization of AIM2PYD than that of ASCPYD (Fig. 5A and Supplementary Fig. 6). We also tested D23K as it appeared to enhance the interaction with ASC, while disrupting AIM2–AIM2 interactions (Fig. 4A). We found that D23K–AIM2FL did not enhance the interaction with ASCPYD but was more defective in inducing the polymerization of AIM2PYD (Fig. 5A and Supplementary Fig. 6). These results consistently suggest that the lateral surface residues of AIM2PYD preferentially, but not exclusively, promote homotypic assembly. Next, again consistent with Rosetta predictions, E21K and M75D were significantly more defective in inducing the polymerization of ASCPYD than that of AIM2PYD (Fig. 5B and Supplementary Fig. 6), corroborating that the type 2b surface of the AIM2PYD filament recruits ASCPYD (N73L–AIM2FL failed to induce any filament formation consistent with the lack of self-assembly (Fig. 5, and Supplementary Figs. 5 and 6)). Notably, M75A–AIM2FL (null in Rosetta mutagenesis) retained the WT-like activity (Supplementary Fig. 6), supporting the idea that a simple alanine-scanning approach is inadequate due to the redundancy in assembly code.

We then used the above Rosetta-based approach to identify mutations at the type 2a surface of ASCPYD that would selectively disrupt the interaction with AIM2PYD (Supplementary Fig. 7A–C). Previously, we found that the ASCPYD filament accelerates the assembly of AIM2PYD via a positive feedback loop. Thus, we

ΔΔGs for AIM2PYD•AIM2PYD (both top and bottom docking; Fig. 4A) vs. ΔΔGs for AIM2•ASC complexes (ASCPYD docking on the top pockets of AIM2PYD; Fig. 4A). We found that the vast majority of mutations are deleterious for both AIM2PYD•AIM2PYD and AIM2PYD•ASCPYD interactions (the upper right quadrant in Fig. 4A), suggesting that the a.a. selection has already been optimized for the self-assembly and recognition. Nonetheless, we identified 88 mutations at nine unique side chains, resulting in ΔΔG(ΔΔG>AIM2PYD>10 and ΔΔG<10 (i.e., mutations that would selectively disrupt AIM2PYD•AIM2PYD interactions without abolishing AIM2PYD•ASCPYD interactions; boxed area in Fig. 4B and listed in Supplementary Fig. 4B). Interestingly, all these side chains were found on the lateral type 1 and type 3 interfaces, but none at the axial type 2 interfaces (Fig. 4B and Supplementary Fig. 4B). Next, to identify mutations that would selectively disrupt AIM2PYD•ASCPYD interactions, we looked for those resulted in ΔΔG(AIM2PYD) < 10 and ΔΔG(AIM2PYD) > 10, respectively (Fig. 4A). We found that the vast majority of mutations at ten unique AIM2PYD side-chain positions, all but one located on the type 2b surface (Fig. 4B and Supplementary Figs. 4B and 5B).

These results consistently suggest that the lateral surface residues of AIM2PYD preferentially, but not exclusively, promote homotypic assembly. Next, again consistent with Rosetta predictions, E21K and M75D were significantly more defective in inducing the polymerization of ASCPYD than that of AIM2PYD (Fig. 5B and Supplementary Fig. 6), corroborating that the type 2b surface of the AIM2PYD filament recruits ASCPYD (N73L–AIM2FL failed to induce any filament formation consistent with the lack of self-assembly (Fig. 5, and Supplementary Figs. 5 and 6)). Notably, M75A–AIM2FL (null in Rosetta mutagenesis) retained the WT-like activity (Supplementary Fig. 6), supporting the idea that a simple alanine-scanning approach is inadequate due to the redundancy in assembly code.

We then used the above Rosetta-based approach to identify mutations at the type 2a surface of ASCPYD that would selectively disrupt the interaction with AIM2PYD (Supplementary Fig. 7A–C). Previously, we found that the ASCPYD filament accelerates the assembly of AIM2PYD via a positive feedback loop. Thus, we

ΔΔGs for AIM2PYD•AIM2PYD (both top and bottom docking; Fig. 4A) vs. ΔΔGs for AIM2•ASC complexes (ASCPYD docking on the top pockets of AIM2PYD; Fig. 4A). We found that the vast majority of mutations are deleterious for both AIM2PYD•AIM2PYD and AIM2PYD•ASCPYD interactions (the upper right quadrant in Fig. 4A), suggesting that the a.a. selection has already been optimized for the self-assembly and recognition. Nonetheless, we identified 88 mutations at nine unique side chains, resulting in ΔΔG(ΔΔG>AIM2PYD>10 and ΔΔG<10 (i.e., mutations that would selectively disrupt AIM2PYD•AIM2PYD interactions without abolishing AIM2PYD•ASCPYD interactions; boxed area in Fig. 4B and listed in Supplementary Fig. 4B). Interestingly, all these side chains were found on the lateral type 1 and type 3 interfaces, but none at the axial type 2 interfaces (Fig. 4B and Supplementary Fig. 4B). Next, to identify mutations that would selectively disrupt AIM2PYD•ASCPYD interactions, we looked for those resulted in ΔΔG(AIM2PYD) < 10 and ΔΔG(AIM2PYD) > 10, respectively (Fig. 4A). We found that the vast majority of mutations at ten unique AIM2PYD side-chain positions, all but one located on the type 2b surface (Fig. 4B and Supplementary Figs. 4B and 5B).

These results consistently suggest that the lateral surface residues of AIM2PYD preferentially, but not exclusively, promote homotypic assembly. Next, again consistent with Rosetta predictions, E21K and M75D were significantly more defective in inducing the polymerization of ASCPYD than that of AIM2PYD (Fig. 5B and Supplementary Fig. 6), corroborating that the type 2b surface of the AIM2PYD filament recruits ASCPYD (N73L–AIM2FL failed to induce any filament formation consistent with the lack of self-assembly (Fig. 5, and Supplementary Figs. 5 and 6)). Notably, M75A–AIM2FL (null in Rosetta mutagenesis) retained the WT-like activity (Supplementary Fig. 6), supporting the idea that a simple alanine-scanning approach is inadequate due to the redundancy in assembly code.

We then used the above Rosetta-based approach to identify mutations at the type 2a surface of ASCPYD that would selectively disrupt the interaction with AIM2PYD (Supplementary Fig. 7A–C). Previously, we found that the ASCPYD filament accelerates the assembly of AIM2PYD via a positive feedback loop. Thus, we

ΔΔGs for AIM2PYD•AIM2PYD (both top and bottom docking; Fig. 4A) vs. ΔΔGs for AIM2•ASC complexes (ASCPYD docking on the top pockets of AIM2PYD; Fig. 4A). We found that the vast majority of mutations are deleterious for both AIM2PYD•AIM2PYD and AIM2PYD•ASCPYD interactions (the upper right quadrant in Fig. 4A), suggesting that the a.a. selection has already been optimized for the self-assembly and recognition. Nonetheless, we identified 88 mutations at nine unique side chains, resulting in ΔΔG(ΔΔG>AIM2PYD>10 and ΔΔG<10 (i.e., mutations that would selectively disrupt AIM2PYD•AIM2PYD interactions without abolishing AIM2PYD•ASCPYD interactions; boxed area in Fig. 4B and listed in Supplementary Fig. 4B). Interestingly, all these side chains were found on the lateral type 1 and type 3 interfaces, but none at the axial type 2 interfaces (Fig. 4B and Supplementary Fig. 4B). Next, to identify mutations that would selectively disrupt AIM2PYD•ASCPYD interactions, we looked for those resulted in ΔΔG(AIM2PYD) < 10 and ΔΔG(AIM2PYD) > 10, respectively (Fig. 4A). We found that the vast majority of mutations at ten unique AIM2PYD side-chain positions, all but one located on the type 2b surface (Fig. 4B and Supplementary Figs. 4B and 5B).

These results consistently suggest that the lateral surface residues of AIM2PYD preferentially, but not exclusively, promote homotypic assembly. Next, again consistent with Rosetta predictions, E21K and M75D were significantly more defective in inducing the polymerization of ASCPYD than that of AIM2PYD (Fig. 5B and Supplementary Fig. 6), corroborating that the type 2b surface of the AIM2PYD filament recruits ASCPYD (N73L–AIM2FL failed to induce any filament formation consistent with the lack of self-assembly (Fig. 5, and Supplementary Figs. 5 and 6)). Notably, M75A–AIM2FL (null in Rosetta mutagenesis) retained the WT-like activity (Supplementary Fig. 6), supporting the idea that a simple alanine-scanning approach is inadequate due to the redundancy in assembly code.

We then used the above Rosetta-based approach to identify mutations at the type 2a surface of ASCPYD that would selectively disrupt the interaction with AIM2PYD (Supplementary Fig. 7A–C). Previously, we found that the ASCPYD filament accelerates the assembly of AIM2PYD via a positive feedback loop. Thus, we
generated Rosetta predicted mutations on full-length ASC (ASCFL) and tested their capacity for inducing the polymerization of FRET-labeled ASCP2D or AIM2P2D. We used ASCFL as the C-terminal CARD would promote the polymerization of ASCP2D even if mutations were too deleterious. We found that L61S and G37E were significantly more defective in accelerating the assembly of AIM2P2D than that of ASCP2D, corroborating that the type 2a surface of ASCP2D recognizes AIM2P2D (Fig. 5C and Supplementary Fig. 8). AIM2FL showed large puncta, as previously reported43 (Fig. 5D, N73L). We also imaged G37E- and L61S-ASCFL-mCherry plus WT AIM2FL-eGFP, which cannot oligomerize as observed from WT (Fig. 5D, L11A, D23K, and A36D); N73L-AIM2FL-eGFP, which cannot oligomerize in vitro and in cellulo experiments consistently support our in silico predictions, in which unique lateral and axial interfaces dictate their recognition and distinction.

We next probe the interactions among AIM2FL and ASCFL WT and mutants in HEK293T cells. WT AIM2FL (ASCFL) tagged with C-terminal eGFP or mCherry colocalized in the same complexes as expected (Supplementary Fig. 8). AIM2FL showed filamentous complexes that often tangled up into speck-like clusters, while ASCFL displayed large puncta, as previously reported43 (Fig. 5D left, Supplementary Fig. 8A–C). AIM2FL-eGFP mutants colocalized with WT AIM2FL-mCherry (Supplementary Fig. 8B), likely due to assembling/binding on the same (transfected) dsDNA strands as WT. Interestingly, when AIM2FL-eGFP and ASCFL-mCherry were co-transfected, ASCFL filaments further expanded as if ASCFL assembles from multiple AIM2FL foci (Fig. 5D, (+WT AIM2FL)). When we co-transfected AIM2FL-eGFP mutants and WT ASCFL-mCherry, those that preferentially decreased the ability to interact with AIM2P2D still resulted in expanded ASCFL complexes as observed from WT (Fig. 5D, L11A, D23K, and A36D). By contrast, ASCFL stayed as a single punctum when co-transfected with AIM2FL mutants that failed to accelerate the polymerization of ASCP2D (E21K and M75D; Fig. 5D, E21K and M75D); N73L-AIM2FL-eGFP, which cannot oligomerize (Supplementary Figs. 5 and 8A), also failed to interact with ASCFL-mCherry. The ASCFL mutants still showed large puncta and also colocalized with WT (Supplementary Fig. 8C). However, WT AIM2FL-eGFP failed to colocalize or induce the expansion of these mutants when co-transfected (Fig. 5E). Together, our in vitro and in cellulo experiments consistently support our in silico predictions, in which unique lateral and axial interfaces within homologous filaments dictate their recognition and distinction.
**Strategies for signaling by assembly.** The AIM2PYD filament displays higher stability than either the ASCPYD filament or AIM2PYD-ASCPYD complex (Fig. 2), which would ensure homotypic assembly of the receptor filament especially within the dsDNA scaffold. On the other hand, the interaction between AIM2PYD and ASCPYD is more favorable than homotypic ASCPYD interactions at a specific axial pole (Fig. 2). In addition, AIM2PYD and ASCPYD recognize each other only when at least one is oligomeric (Fig. 3B). Of note, electrostatic surface analyses suggest that the charge complementarity is reversed at the type 1 interface for AIM2 and ASC, likely indicating that the heterologous interactions between the monomers are not favorable (i.e., the type 1a surface of AIM2PYD is largely basic, whereas that of ASCPYD is acidic; Supplementary Fig. 9A). Importantly, our in silico, in vitro, and in cellulo experiments consistently demonstrate that the directional interaction at the type 2 interface is most critical (Figs. 2–5). The surface area of the type 2 interface is much smaller than that of the type 1 interface in homomeric PYDs (Fig. 6A). However, because of the axial location, the type 2b surfaces become as accessible as the type 1 surfaces once AIM2PYD assembles into a filament (Fig. 6B). Moreover, electrostatic surface analyses suggest that the bottom of the AIM2PYD filament is unfavorable for interacting with the ASCPYD filament due to highly positively charged surfaces (Supplementary Fig. 9B). We propose that such conditional scaffolding by the upstream filament not only ensures proper signal transduction orders, but also maximizes signal amplification (Fig. 6C). For instance, inflammasomes assemble in a digital fashion and entail cell death

**Future directions.** Our successful implementation of Rosetta to decode the specificity of the AIM2-ASC inflammasome suggests that our approach can be broadly applied to other homologous signaling filaments. However, we noted that Rosetta was correct at ~50% in predicting energetically important mutations (Fig. 5), indicating that there is room for improvement. Nonetheless, given that precisely pinpointing the role of individual residues is intrinsically challenging, we find the Rosetta suite to be an excellent tool for decoding the specificity of the filamentous assemblies.

**Discussion.** Inflammasomes transduce signals by assembling supramolecular structures

**Strategies for homotypic filament assembly.** Our experiments consistently show that the assembly of individual filaments occur bidirectionally, with lateral type 1 and type 3 interfaces (especially type 1) of AIM2PYD favoring homotypic interactions, while still supporting the recognition of ASCPYD. The lateral type 1 surfaces are the largest in any PYDs (Fig. 6A left), which would be ideal for recognizing other homotypic protomers to initiate assembly without any prescribed directionality. The lack of directionality in homotypic assembly would then allow AIM2 to maximally benefit from one-dimensional random diffusion on pathogenic dsDNA,

resulting in a timely response by the upstream receptor (Fig. 6A right). Interestingly, the bidirectional assembly is in contrast to other cytoskeletal and signaling filaments such as actin

and B-cell lymphoma 10 (BCL10)

Considering that both actin and BCL10 filaments originate from cell membranes/defined borders

it is tempting to speculate that the bidirectional assembly of inflammasome filaments have evolved to take full advantage of no immediate boundaries in the cytosol.

**Fig. 6 Strategies for signal transduction by the AIM2-ASC inflammasome.** A Left: a surface representation of AIM2PYD monomer. The buried surface area in the filament for each interface type is indicated. Right: a scheme describing the advantages of bidirectional homotypic assembly. B The top surface view of the AIM2PYD filament. Each solvent accessible interface is colored in red with the calculated surface area. C A scheme describing the advantages of unidirectional signal transduction by the AIM2-ASC inflammasome.
found for AIM2 apply in other receptors and how well Rosetta fares in answering these questions.

**Methods**

**Protein expression and purification.** Human AIM2FL (residues 1–343), AIM2PYD (residues 1–94) SF94C for fluorophore labeling, ASCPYD (residues 1–92) were cloned into the pET28b vector (Novagen) with a N-terminal MBP tag and TEV recognition site. For cryo-EM, we followed a construct including ~20 a.a. in the unstructured linker region (residues 1–117) resulted in well-separated filaments (denoted as AIM2NSD, Fig. 1A). ASCFL was cloned with the MBP tag at both N- and C-termini with the TEV recognition site flanking MBP and ASCFL. All protein expression was conducted in Escherichia coli with an overexpressing affinity (MBP/amylose), cation exchange, and followed by size-exclusion chromatography. Proteins were then concentrated and stored at –80 °C, see also ref. 17,18 (all primers generated for this study are listed in Supplementary Table 2).

**Cryo-EM sample preparation.** A total of 5 µl sample of 2.75 µM AIM2RN (cleaved for 30 min with 6 µM TEVp) was applied to Lacey grids, followed by automatically blotting for 1.5 s and plunge freezing, using the FEI Vitrobot Mark III with an exposure time of 42 electrons/A² at a total dose of 12 s and a total exposure time of 24 s and an axial rise of 14 Å per subunit per rotation of 53.3° and an axial rise of 14 Å per subunit) converged. The resolution of the entire reconstruction was estimated by the FSC between two independent half subsets. Neutralizing ions were added with ~200 Cl and ~200 K to a box size of 13 nm × 13 nm × 13 nm.

Following initial energy minimization and equilibration, a second step of equilibration was performed in the NPT ensemble with a 2 fs timestep for 50 ns. A Nose-Hoover thermostat was used to maintain a temperature of 300 K with a 1 ps coupling time constant. The protein and solvent were coupled to separate temperature baths. A Parrinello-Rahman isotropic barostat with a 5 ps coupling time constant was used to maintain a pressure of 1 bar. Particle Mesh Ewald (PME) with a 1.2 nm cutoff radius, a 0.12 nm Fourier spacing, and cubic interpolation of 4 was used for electrostatics. Van der Waals interactions had a 1.2 nm cutoff radius. A LINCS algorithm was used for bond constraints and XYZ periodic boundary conditions were enforced.

Following the second step of equilibration, well-tempered Metadynamics (MetaD) simulations were performed using GROMACS 4.5.1 patched with PLUMED2 (ref. 62), using a CHARMM36 force field. The collective variable (CV) was the distance between the center-of-mass of the pocket and the center-of-mass of the ligand (residue 60). The backbone RMSD stayed mostly constant during the course of simulation (Supplementary Fig. 3A). Gaussians of energy were deposited along the trajectory in this CV space. Gaussians had an initial hill height of 1 kJ/mol and a width of 0.05 nm. Gaussians were deposited every 40 fs. A bias factor of 2 was used to adjust the hill heights according to the well-tempered MetaD scheme. Gaussians were saved to a grid with a bin spacing 0.01 nm. The entire simulation was complete when the chain was completely dissociated from the pocket, i.e., the CV distance exceeded 5 nm. Positional restraints were placed on every alpha-carbon in the pocket to prevent dissociation of the pocket protomers during the entirety of the MetaD simulations. The sum of all deposited Gaussians was computed to represent the dissociation free energy.

**Polymerization assays.** A total of 100 nM of AIM2FL was cleaved by 6 µM TEVp for 20 min in a 384-well plate. After cleavage, 150 nM of linearized plasmid dsDNA (~5 kbp, binding site normalized) was added and allowed to bind for 30 min. To start the assay, 2 µM FRET mix of MBP-tagged AIM2PYD or ASCPYD was added to the same well containing TEVp. Each experiment consisted of a control well with no AIM2FL, one with AIM2FL WT, and multiple AIM2FL mutants for both AIM2PYD and ASCPYD wells. AIM2 and ASC samples were run at the same time to ensure proper statistical analyses. Half-times for polymerization were calculated and converted to apparent kinetic rates. The no AIM2FL control and AIM2FL WT control were used to normalize the kinetics for each mutant into an activity ratio scaling from 0 (no AIM2FL present) to 1 (AIM2FL WT activity).

P values were calculated using Student's t test for paired samples. The same strategy was used for ASCPYD WT and mutants (0.5 µM, precleaved by TEVp for 30 min) inducing the polymerization of FRET-labeled AIM2PYD or ASCPYD.

**nsEM.** AIM2FL bound to dsDNA was prepared in the same manner as for the polymerization assays (100 nM protein, 150 nM dsDNA, linear plasmid ~5 kilobases). The samples were applied to carbon coated grids and imaged in a FEI Titan 80-300 electron microscope.

**Imaging recombiant AIM2PYD and ASCPYD filaments.** Filament assembly of Aim2FL- or Dlight659-labeled MBP-AIM2PYD and MBP-ASCYPYD (1 µm each or 3 µM of nascent proteins for Fig. 3A) was induced by removing MBP by TEVp as indicated in figure legends. For preassembly, the AIM2PYD or ASCPYD filament was cleaved and incubated for 30 min prior to adding nascent proteins. Images were then taken using a Zeiss AxioSkop 50 with a Zeiss Axiocam HRC camera and an Axio Observer inverted microscope with LSM700 confocal module.

**Imaging AIM2FL-6GFP/mCherry and ASCFL-6GFP/mCherry in HEK293T cells.** AIM2FL- and ASCFL-6GFP variants were cloned into the pCMV6 vector harboring eGFP or mCherry. To preserve native PYD-PYD interactions, the fluorescent proteins were positioned at the C-terminus of AIM2FL or ASCFL. Plasmids were then transiently transfected into HEK293T cells using lipofectamine (0.5 µg each plasmid, Invitrogen). After 12 h, cells were fixed with 4% paraformaldehyde and mounted on glass slides using ProLong Gold Antifade Mountant with DAPI (Thermo Fisher). Cells were then imaged using the same confocal microscope as the recombinant proteins.
References

1. Broz, P. & Dixit, V. M. Inflammasomes: mechanism of assembly, regulation and signalling. Nat. Rev. Immunol. 16, 407–420 (2016).

2. Bandera, A. et al. The NLRP3 inflammasome is upregulated in HIV-infected antiretroviral therapy-treated individuals with defective immune recovery. Front. Immunol. 9, 214 (2018).

3. Maruzuru, Y. et al. Herpes simplex virus 1 VP22 inhibits AIM2-dependent inflammasome activation to enable efficient viral replication. Cell Host Microbe 23, 254–265 e7 (2018).

4. Kim, S. et al. The AIM2 inflammasome regulates Th1 cell responses in infection, inflammation, and autoimmunity: role in DNA sensing. J. Immunol. 199, 435–444 (2015).

5. Lu, A. W. & Wu, H. Structural mechanisms of inflammasome assembly. FEBS J. 282, 435–444 (2015).

6. Bami, S. et al. The use of anakinra in the treatment of secondary hemophagocytic lymphohistiocytosis. Pediatr. Blood Cancer 67, e28581 (2020).

7. Liljeström, P. et al. Crystallographic and molecular modelling studies of the C-terminal domain of the human NAIP/NLRC4 inflammasome. Acta Crystallogr. D Biol. Crystallogr. 66, 686–691 (2010).

8. Qi, M. et al. AIM2 promotes the development of non-small cell lung cancer by modulating mitochondrial dynamics. Oncogene 39, 2707–2723 (2020).

9. Yamaoka, M. et al. AIM2 promotes the development of non-small cell lung cancer by modulating mitochondrial dynamics. Cancer Cell 29, 1–15 (2016).

10. Leitinger, N. et al. AIM2 regulates adaptive immunity and pyroptosis as a sensor of cytosolic DNA. Nature 460, 149–154 (2009).

11. Singhal, S. et al. AIM2 facilitates caspase-1 activation in response to cytoplasmic DNA. Nat. Immunol. 13, 407–415 (2012).

12. Lee, J. W. et al. AIM2 regulates innate immunity and inflammation in response to cytoplasmic DNA. Nat. Immunol. 13, 407–415 (2012).

13. Li, Y. et al. Cryo-EM structures of ASC and NLRC4 CARD inflammasomes reveal nucleated polymerization. Science 350, 404–409 (2015).

14. Tenebaum, H. et al. The AIM2 inflammasome mediates inflammatory responses to cytosolic DNA. Nature 471, 91–95 (2011).

15. Bami, S. et al. The use of anakinra in the treatment of secondary hemophagocytic lymphohistiocytosis. Pediatr. Blood Cancer 67, e28581 (2020).

16. Matyszewski, M. et al. Cryo-EM structure of the NLRC4(CARD) filament provides insights into how symmetric and asymmetric multimerization structures drive inflammasome assembly. J. Biol. Chem. 293, 20240–20248 (2018).

17. Matyszewski, M. et al. Cryo-EM structure of the NLRC2-ASC inflammasome reveals nucleated polymerization. Science 350, 404–409 (2015).

18. Tenebaum, H. et al. The AIM2 inflammasome mediates inflammatory responses to cytosolic DNA. Nature 471, 91–95 (2011).

19. Bami, S. et al. The use of anakinra in the treatment of secondary hemophagocytic lymphohistiocytosis. Pediatr. Blood Cancer 67, e28581 (2020).

20. Matyszewski, M. et al. Cryo-EM structure of the NLRC4(CARD) filament provides insights into how symmetric and asymmetric multimerization structures drive inflammasome assembly. J. Biol. Chem. 293, 20240–20248 (2018).

21. Zhang, L. et al. Cryo-EM structure of the activated NAIP2-NLRC4 inflammasome reveals nucleated polymerization. Science 350, 404–409 (2015).

22. Tenebaum, H. et al. The AIM2 inflammasome mediates inflammatory responses to cytosolic DNA. Nature 471, 91–95 (2011).

23. Bami, S. et al. The use of anakinra in the treatment of secondary hemophagocytic lymphohistiocytosis. Pediatr. Blood Cancer 67, e28581 (2020).

24. Matyszewski, M. et al. Cryo-EM structure of the NLRC4(CARD) filament provides insights into how symmetric and asymmetric multimerization structures drive inflammasome assembly. J. Biol. Chem. 293, 20240–20248 (2018).

25. Tenebaum, H. et al. The AIM2 inflammasome mediates inflammatory responses to cytosolic DNA. Nature 471, 91–95 (2011).

26. Matyszewski, M. et al. Cryo-EM structure of the NLRC4(CARD) filament provides insights into how symmetric and asymmetric multimerization structures drive inflammasome assembly. J. Biol. Chem. 293, 20240–20248 (2018).

27. Zhang, L. et al. Cryo-EM structure of the activated NAIP2-NLRC4 inflammasome reveals nucleated polymerization. Science 350, 404–409 (2015).

28. Tenebaum, H. et al. The AIM2 inflammasome mediates inflammatory responses to cytosolic DNA. Nature 471, 91–95 (2011).

29. Bami, S. et al. The use of anakinra in the treatment of secondary hemophagocytic lymphohistiocytosis. Pediatr. Blood Cancer 67, e28581 (2020).
Acknowledgements

We thank Drs. Jeffrey Gray and L. Mario Amzel for discussion, and Drs. Brendan Antiochos and Shuai Wu for cell culture experiments. This work was supported by American Cancer Society Research Scholars Grant (RSG-15-224-01DMC), NSF CAREER award (MCB1845003), and NIH R01GM129342 to J.S., NIH R35GM122510 to E.H.E. Computational resources were provided by Maryland Advanced Research Computing Center at Johns Hopkins University.

Author contributions

M.M. and J.S. conceived the project and designed experiments. M.M., W.Z., Z.M., J.L., and N.M. performed experiments. M.M., W.Z., Z.M., J.L., N.M., A.Y.L., E.H.E., and J.S. interpreted data. M.M., W.Z., N.M., and J.S. wrote the paper which other authors commented on.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-23045-8.

Correspondence and requests for materials should be addressed to J.S.

Peer review information Nature Communications thanks Qian Yin and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access

This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021