Cryo-electron tomography of ASC signalling sites in pyroptotic cells

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Abstract

Inflammasomes induce cell death in response to infection, chemical entities and cell damage. Human genetics and animal models have identified the NLRP3 inflammasome as a gatekeeper of caspase-1-dependent pyroptosis. NLRP3 activation induces polymerization of ASC into a single, micron-scale perinuclear punctum, where caspase-1 is activated. These puncta have yet to be imaged at sufficient resolution to resolve their ultrastructure. Here, we apply correlative cryo-light microscopy and cryo-electron tomography to visualize ASC/caspase-1 puncta and mitochondrial membrane remodelling within NLRP3-activated cells. The puncta are composed primarily of branched ASC filaments. The N-terminal pyrin domain forms an 8-10 nm-diameter tubular core, which is decorated by the flexibly linked C-terminal caspase recruitment domain. The variable filament density allows ribosomes and *trans*-Golgi-like vesicles to permeate the network. We propose this provides structural integrity while allowing macromolecules and vesicles to diffuse in or bind with the necessary density, timing and localization.

Keywords

NLR family pyrin domain containing 3 (NLRP3); apoptosis-associated speck-like protein containing a CARD (ASC) speck; caspase-1; pyroptosis; correlative light and electron microscopy (CLEM); *In situ* cryo-electron tomography (cryo-ET); focused-ion beam (FIB) milling; inflammasome; caspase activation and recruitment domain (CARD); pyrin domain (PYD)

Running title: Cryo-ET of ASC specks and mitochondria in cell lamellae

Introduction

Inflammasomes are vital innate immune oligomeric signalling complexes that induce proteolytic activation of caspase-1, leading to secretion of proinflammatory cytokines interleukin (IL)-1 β and IL-18, and cell death by gasdermin D-driven pyroptosis. In the first step of inflammasome assembly, Nod-like receptors (NLR) sense cytosolic chemical signatures associated with microbes and cell dysfunction or damage¹. Ligand binding induces NLR oligomerization and recruitment of downstream proteins linked to the caspase-1 signalling cascade.

NLRP3 is one of the most studied inflammasome NLRs due to the large number of NLRP3 genetic variants associated with human diseases and the availability of animal disease models. At least 250 human NLRP3 protein variants have been identified, over 30 of which have been associated with disease through gain-of-function^{2,3}, including autoinflammatory diseases, degenerative disorders, dermatitis and metabolic disorders^{4,5}. NLRP3 inflammasome activation has been linked to a broad spectrum of cellular processes or cytosolic chemical signatures including potassium efflux, reactive oxygen species, calcium influx, chloride efflux, phosphatidylinositol-4-phosphate on Golgi membranes, and mitochondrial stress or damage^{6,7}. Cryo-electron microscopy (cryo-EM) single-particle image reconstructions show that ligand-bound inflammasome NLRs assemble into disk- or split washer-like oligomers⁸⁻¹¹. In addition to ligand recognition, activation of the NLRP3 and pyrin inflammasomes requires active transport along microtubules by dynein to the microtubule organizing centre (MTOC)^{12,13}. Upon activation, NLRP3 recruits the adaptor protein ASC (Apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD)). NLRP3 and ASC interact through their pyrin domains (PYDs)¹⁴. ASC then assembles into a single perinuclear punctum with a micron-scale diameter, also known as a speck, which functions as a platform for the recruitment and activation of caspase-1^{15,16}. The PYD and CARD of ASC are both required for punctum formation and signalling^{15,17,18}. Structural studies on purified ASC PYD and ASC CARD show that both

domains have a death fold and independently form helical filaments¹⁹⁻²⁴. In the cell, fluorescence microscopy imaging suggests that PYD-PYD interactions drive ASC filament formation whereas CARD-CARD interactions promote cross-linking and compaction of PYD filaments into puncta^{17,18,23-26}. In addition, ASC recruits procaspase-1 via a CARD-CARD interaction between the two proteins^{15,23}. Super-resolution light microscopy of endogenous ASC specks revealed punctate structures containing ASC, caspase 1 and NLRP3 within the same macro-assembly²⁷. These structures have yet to be imaged inside a cell at sufficient resolution to resolve the ASC or caspase-1 ultrastructure within puncta²⁶. Hence, it remains unclear how the PYD and CARD contribute to the formation of an ASC punctum inside cells.

Understanding how ASC filament formation contributes to the assembly and activation of inflammasome puncta requires structural information in the cellular context. Recent advances in the preparation of frozen vitrified cellular lamellae by focused ion-beam (FIB) milling^{28,29} have allowed the ultrastructure of multimeric protein complexes to be determined in a near-native context by cryo-electron tomography (cryo-ET). Here, we use *in situ* cryo-ET to obtain three-dimensional image reconstructions of multimeric signalling complexes in cells with active NLRP3 inflammasomes. We identify ASC/caspase-1 signalosome puncta by correlative light and electron microscopy (CLEM) and reveal their ultrastructure and pyroptotic cellular landscape in immortalised bone marrow derived macrophages (iBMDMs) by cryo-ET image reconstruction. We find that inflammasome puncta are composed of short, hollow ASC/caspase-1 filaments, extensively branched, with variable packing density allowing ribosomes and certain other cellular components to permeate the puncta. We propose that this ultrastructure of the ASC filament network functions as a signalling platform by providing structural integrity while allowing downstream signalling molecules to diffuse freely and bind at high density within the network.

Results

In situ cryo-CLEM of ASC/caspase-1 puncta

High-resolution structures of inflammasome components have been obtained from purified proteins. In a previous study in zebrafish larvae, electron tomography of ASC puncta in fixed larval sections stained with uranyl acetate showed that the puncta contained a dense filament network²⁶, but the imaging resolution was limited by chemical fixation and staining of the sample. To examine the architecture and cellular interactions of the mammalian NLRP3 signalosome in its physiological environment, we imaged ASC puncta in iBMDMs by correlative fluorescence light microscopy and *in situ* cryo-ET (cryo-CLEM). Cells expressing ASC fused with a C-terminal fluorescent protein have been used extensively to visualize ASC puncta formation³⁰. In some experimental systems, ASC overexpression can lead to puncta formation in the absence of activating inflammasome stimuli³⁰. Fluorescence microscopy of live iBMDM cells overexpressing ASC-mCerulean used in this study showed that both priming with lipopolysaccharide (LPS) and stimulation with nigericin were still required to induce ASC puncta formation (**Fig. S1a, Movie S1**). Gasdermin D cleavage observed by Western blotting and IL-1 β cytokine release measured in ELISAs confirmed that the ASC-mCerulean puncta activated pyroptotic signalling (**Fig. S1b**). Limiting the stimulation time with nigericin to 30 min allowed early-stage inflammasome signalling complexes to be captured, as indicated by colocalization of NLRP3, proteolytically activated caspase-1 and IL-1 β with ASC in the puncta, in both wild-type (WT) iBMDMs and ASC-mCerulean iBMDMs (**Fig. S1c-e**). NLRP3 and IL-1 β localization was determined by immunofluorescence. Caspase-1 localization was inferred using the carboxyfluorescein-labelled caspase-1 substrate FAM-FLICA. FAM-FLICA or its non-fluorescent analogue Z-VAD-FMK also facilitated imaging by delaying pyroptotic cell death (**Movies S1, S2**).

For high-resolution imaging, cells grown on electron microscopy grids were vitrified by plunge-freezing in liquid ethane. We used both iBMDMs overexpressing ASC-mCerulean and WT iBMDMs labelled with FAM-FLICA after NLRP3 inflammasome activation³¹. ASC/caspase-1 signalling complexes were located by cryo-fluorescence light microscopy (cryo-FM; **Fig. S2a**). Areas of the frozen cells containing an ASC or caspase punctum were FIB-milled down to lamellae 150-300 nm-thick and 15-20 μ m wide^{28,29}, with micro-expansion joints to reduce bending³² (**Fig. S2b,c**). The lamellae were imaged by cryo-CLEM, using cryo-FM to locate any remaining fluorescent signal from ASC-mCerulean or FAM-FLICA in the lamellae after milling (**Fig. 1a,b; Fig. S2d,e**). The micron-scale dimensions of the fluorescent puncta allowed cryo-ET tilt-series acquisition at regions of interest with the necessary targeting precision after correlation of the cryo-FM images with low-magnification cryo-EM maps (**Fig. S2f,g**)^{33,34}.

***In situ* Cryo-ET reveals a branched-filament ultrastructure in ASC/caspase-1 puncta**

Three-dimensional cryo-ET image reconstructions show a dense network of branched filaments in areas identified by cryo-CLEM as containing ASC-mCerulean or caspase-1 labelled with FAM-FLICA (**Figs. 1c-e, 2a, S2g**). Filaments in cells expressing ASC-mCerulean had additional density at the filament periphery, and a less distinct outline compared to filaments in cells expressing WT ASC (**Fig. 1d,e**). This suggests the additional density is primarily attributable to mCerulean, which at 27 kDa is larger than ASC (22 kDa), although other components such as caspase-1 (which was shown by light microscopy to localize to ASC specks), may also contribute to this density. A green fluorescent protein tag was similarly found to decorate cryo-ET reconstructions of amyloid-like poly-Gly-Ala aggregates with additional densities³⁵. Since mCerulean was fused to the C-terminus of ASC, the presence of mCerulean at the filament periphery supports a model in which the filaments are formed at their core by the N-terminal PYD of ASC, with the CARD located in between the PYD and mCerulean. Indeed, the cryo-EM structure of purified recombinant ASC PYD filaments and the NMR structure of full-length ASC,

taken together, suggest that the PYD forms the filament core (9-nm in diameter), with the flexibly-linked CARD increasing the filament diameter to 16-18 nm^{23,24}. In our cryo-ET reconstructions, filaments containing WT ASC had a diameter of 11-15 nm, which increased to 22-30 nm for filaments containing ASC-mCerulean (**Figs. 1d,e**). Notably, a continuous core of density 8-10 nm in diameter (**Fig. 2a-c**) was visible within the ASC-mCerulean filaments (for which the cryo-ET images were of higher quality). Although fluorescence from ASC-mCerulean and FAM-FLICA-labelled caspase-1 colocalized in iBMDMs (**Fig. S1e**), the resolution of cryo-CLEM was insufficient to determine whether any of the cryo-ET filament densities corresponded to caspase-1 or other components. We note, however, that caspase-1 (30 kDa) and procaspase-1 (45 kDa) are both larger than ASC and would therefore be expected to increase filament diameter significantly, if present with the same stoichiometry as ASC.

Filament length measurements showed that WT filaments were on average slightly shorter (191 ± 74 nm, $n = 54$) than ASC-mCerulean filaments (245 ± 116 nm, $n = 402$), with similarly broad length distributions (**Fig. 1f,g**). This difference could potentially be due to the higher levels of ASC protein expression in iBMDMs expressing ASC-mCerulean, which also express endogenous WT ASC. Analysis of the branching angles showed that the angle distributions of untagged and tagged filaments were slightly different, with larger branching angles more common in mCerulean-tagged filaments, potentially due to steric constraints from the tag (**Fig. 1h**). Our filament branching angle measurements are likely underestimated as any filaments with their axis closely aligned with the Z-axis – which would result in a large (70-90°) branching angle – would not have been detected due to the missing-wedge effect (from sample-tilt limitations). Taken together with previously published structural data from purified proteins, our cryo-ET reconstructions suggest ASC filaments form the backbone of inflammasome-induced puncta, but that the branching of these filaments results in a structural scaffold that can recruit and concentrate downstream effector proteins.

Figure 1. Cryo-ET image reconstructions of ASC-caspase 1 puncta in iBMDMs.

(a) Cryo-FM image of a lamella from an iBMDM expressing ASC-mCerulean. ASC-mCerulean fluorescence is shown (green), overlaid on a bright-field image. Scale bar, 5 μ m.

(b) Cryo-FM image shown in **(a)** correlated and overlaid on a transmission cryo-EM map of the same lamella using the EC-CLEM ICY³⁶ plugin. Scale bar, 8 μ m.

(c) Reconstructed cryo-ET volume (1.36-nm thick virtual tomographic slice), of the ASC-mCerulean punctum shown in **(a,b)**. Scale bar, 250 nm.

(d-e) Galleries of filaments within ASC-mCerulean puncta, **(d)**, or FAM-FLICA-labelled caspase-1 puncta, **(e)**, in 4-nm thick virtual tomographic slices of iBMDMs. White arrows indicate

filament axes. Pairs of yellow triangles show filament thickness. Dashed white boxes highlight ring-shaped densities. Scale bars, 50 nm.

(f-g) Filament length distribution in iBMDMs expressing ASC-mCerulean, **(f)**, and in WT iBMDMs labelled with FAM-FLICA, **(g)**.
(h) Distribution of branching angles in ASC-mCerulean puncta (from 2 tomograms), and a WT ASC punctum (1 tomogram).

For a more complete visualisation of the ASC speck ultrastructure in its cellular environment, we generated a three-dimensional model of the filament network and other cellular components including mitochondria, ER and ribosomes with IMOD³⁷. This model showed that puncta consist of several core regions of densely packed filaments separated by sparser regions, with branching filaments connecting adjacent densely packed regions (**Fig. 2a, Movies S3,S4**). Ribosomes were abundant within the filament network. Vesicles were also present, but organelles including mitochondria were excluded.

We next investigated the equilibrium dynamics of ASC-mCerulean filaments by fluorescence recovery after photobleaching (FRAP) in iBMDMs. We bleached circular regions containing ASC-mCerulean puncta and imaged the ASC-mCerulean fluorescence intensity before and after bleaching (**Fig. 2d**). Our data showed that ASC-mCerulean filament assemblies have a small mobile fraction (6.9%; **Fig. 2e**). The dissociation rate (k_{off}) of ASC-mCerulean from the imaged area was measured from the rate of fluorescence recovery as $0.023 \pm 0.001 \text{ s}^{-1}$ (**Fig. 2d,e; Dataset S1**). Most of the observed fluorescence recovery was at the periphery of puncta, suggesting that the ASC-mCerulean filaments inside the puncta are largely immobile and are not in equilibrium with ASC monomers or oligomers in the cytosol (**Fig. 2d**).

Figure 2. Visualization of the filament network and assembly dynamics of ASC puncta

(a) 3-D segmented model of the ASC-mCerulean punctum shown in Fig. 1c, generated with IMOD³⁷. Scale bar, 250 nm.

- (b) Representative instances of different filament types observed in 4-nm thick virtual tomographic slices of iBMDMs. Scale bar, 50 nm.
- (c) Average normalized density line profile of filament cross-sections, calculated from projections of 16-nm thick virtual tomographic slices of iBMDMs. Error bars show standard deviations (ASC-mCerulean, n = 25; WT, n = 13).
- (d) Fluorescence recovery after photobleaching (FRAP) of ASC-mCerulean in iBMDMs. Scale bar, 10 μ m.
- (e) FRAP curve used to calculate the ASC-mCerulean dissociation rate (k_{off}) from the bleached area. Shaded area represents $\pm$ s.e.m. (n = 8). See **Dataset S1** for source data.

***Trans*-Golgi-like vesicles are present within the ASC filament network**

How NLRP3 is activated by a broad range of different stimuli – including ion fluxes, reactive oxygen species and mitochondrial damage – remains an open question. A common feature of NLRP3 activation by these different stimuli is that the *trans*-Golgi network (TGN) is dispersed, and NLRP3 locally accumulates at foci within the dispersed TGN^{7,12}. These NLRP3 foci have been proposed to serve as nucleation sites for ASC and caspase-1 recruitment and activation⁷. Consistent with previous studies, we observed dispersion of the TGN in WT iBMDM and THP-1 cells, and in iBMDMs expressing ASC-mCerulean (**Figs. 3a,b; S3a**). Moreover, NLRP3 partially colocalized with the TGN marker TGN38 in WT iBMDMs primed with LPS, with or without nigericin stimulation (**Fig. 3a**). In stimulated iBMDMs, ASC-mCerulean puncta were located adjacent to anti-TGN38 fluorescence, but there was little direct overlap between the two markers (**Fig. 3b**). In our tomographic reconstructions, vesicles with diameters of 50 to 300 nm were observed within the ASC filament network (**Figs. 3c,d; S2g; S3b; Movies S3, S4**). We observed similar vesicles within the filament network in chemically fixed ASC-mCerulean puncta imaged by room temperature electron tomography (**Fig. S3c-e**). Notably, these vesicles had low-density lumens, a feature characteristic of TGN vesicles³⁸. Ribosomes were also found

embedded throughout the ASC filament network. Ring-shaped densities were occasionally observed within tomograms of ASC puncta, but the resolution of the reconstructions was insufficient to infer the composition of these features (**Fig. 1d**). Together, our cryo-ET and fluorescence microscopy data supports the model that the dispersed TGN may serve as a platform for ASC recruitment to multiple NLRP3 foci, which nucleate ASC polymerization, with subsequent branching of ASC filaments driving growth into a micron-scale punctum.

Figure 3. TGN dispersion and TGN-like vesicles in ASC puncta after NLRP3 stimulation

(a) Immunofluorescence microscopy of WT iBMDMs primed with LPS or stimulated with nigericin. NLRP3 partially colocalized with TGN38. The TGN was dispersed following stimulation. Scale bar, 10 μ m.

(b) Immunofluorescence microscopy of iBMDMs expressing ASC-mCerulean before and after stimulation with nigericin, or nigericin and caspase inhibitor Z-VAD-FMK. ASC-mCerulean puncta were adjacent to anti-TGN38 fluorescence, but there was little overlap between the two markers. Scale bar, 10 μ m.

(c) 1.36-nm thick virtual tomographic slices of vesicles within the ASC-mCerulean filament network in an iBMDM. Scale bar, 200 nm. Lower panel, 3-D segmentation model of the same volume.

(d) Gallery of vesicles from tomographic reconstructions of cryo-FIB-milled ASC-mCerulean iBMDMs. Scale bar, 100 nm. The histogram shows the vesicle diameter distribution.

Since NLRP3 inflammasome activation was reported to require transport of NLRP3 to the MTOC^{12,13}, we examined the localization of ASC/caspase-1 puncta relative to the MTOC. Fluorescent markers for ASC and caspase-1 did not colocalize with the MTOC components γ -tubulin, pericentrin or ninein in WT or ASC-mCerulean iBMDMs stimulated with LPS and nigericin (**Fig. S4a,b**). In stimulated THP-1 cells, ASC/caspase-1 puncta were on average closer to the MTOC, with one quarter of puncta within 2 μ m of the MTOC, but there was little direct overlap between the fluorescent markers (**Fig. S4c**).

Aberrant mitochondrial morphology during pyroptosis visualized by cryo-ET

Mitochondria function as a nexus for multiple pathogen-sensing and damage-sensing signalling pathways. Innate immune sensors of viral nucleic acids³⁹ and mitochondrial damage converge on the outer mitochondrial membrane, and induce apoptosis or pyroptosis, depending on the specific danger signal that is sensed^{40,41}. We observed fragmentation of the filamentous network of mitochondria in ASC-mCerulean iBMDMs following stimulation with LPS and nigericin, similar to AIM-2 dependent mitochondrial remodelling⁴¹, even prior to ASC punctum formation (**Fig. 4a**). Mitochondrial remodelling coincided with dissipation of the membrane potential (**Fig. 4b**, **Fig. S5a**). To visualize and analyse the morphology of mitochondria soon after inflammasome

activation we performed cryo-ET reconstructions of mitochondria following LPS priming and nigericin stimulation to induce ASC speck formation. In LPS primed iBMDMs expressing ASC-mCerulean, a 30-min stimulation with nigericin resulted in mitochondria with a more rounded overall shape and smaller size than in unstimulated cells (**Fig. 4c**). Cryo-ET reconstructions and segmentation models of WT iBMDMs 1-h post-stimulation showed that the mitochondria in these cells had an abnormal ultrastructure, with the inner membranes forming concentric tubular structures instead of the regularly spaced lamellar cristae observed in unstimulated cells (**Fig. 4c,d**). To quantify the changes in mitochondrial morphology associated with ASC-dependent signalling, four parameters were extracted and measured using Fiji⁴²: cristae spacing, inner-to-outer membrane spacing, cristae lumen width and cristae apex angles (**Fig. 5a,b**). We found the cristae spacing to be two- to fourfold smaller in pyroptotic mitochondria than in healthy mitochondria. The other three parameters were not significantly different.

Figure 4. Remodelling of mitochondrial cristae after activation of NLRP3 inflammasome.

(a) Snapshots of live ASC-mCerulean iBMDMs labelled with a MitoView mitochondrial dye.

Overlaid fluorescence and bright-field images show soon fragmentation of the mitochondrial network prior to ASC-speck formation. Scale bar 10 μ m.

(b) Flow cytometry histogram of iBMDMs stained with tetramethylrhodamine (TMRM), a dye sensitive to mitochondrial membrane potential, $\Delta\Psi_m$. Cells were stimulated with LPS and nigericin (left), or LPS and ATP (right) for 30 min.

(c) Reconstructed cryo-ET tomographic slices of an unstimulated iBMDM (left); ASC-mCerulean iBMDMs stimulated with LPS and nigericin for 30 min (centre); or WT iBMDMs, stimulated with LPS and nigericin for 1 h (right).

(d) 3-D segmentation models of the outer and inner envelope membranes (purple) and cristae membranes (pink) in the tomographic slices shown immediately above in **(c)**.

Discontinuities in the outer membranes of mitochondria during pyroptosis

During apoptosis, the Bax and Bak proteins create large disruptions in the outer mitochondrial membrane^{40,43}. Our cryo-ET reconstructions showed that mitochondria in pyroptotic iBMDMs stimulated with LPS/nigericin lacked any such large outer-membrane disruptions. We did, however, identify small discontinuities in the outer mitochondrial membrane in some of the stimulated ASC-mCerulean and WT iBMDMs (**Fig. 5c,d**). The size of these gaps varied from 10 to 22 nm (**Fig. S5b**). The presence of an intact inner membrane adjacent to the gap in the outer-membrane implied that the gap was not due to the missing wedge effect or the anisotropic resolution of cryo-ET tomograms⁴⁴.

The presence of 10-22-nm gaps in the outer membranes of mitochondria during pyroptosis raises the question of whether these gaps cause mitochondrial outer membrane permeabilization (MOMP) during inflammasome stimulation. To address this question, we measured the cryo-EM density in the intermembrane space and in an adjacent cytosolic area, where outer-membrane gaps were present. Unexpectedly, we found that the density at sites where outer membrane gaps were present was higher than in the cytosol, indicating an increase in macromolecular density at gap sites (**Fig. 5e,f**). In contrast, at sites where both membranes were intact the intermembrane space had a lower density than the cytosol, as expected. The increased cryo-ET density at gap sites spanned 10-22 nm along the Z-axis, matching the dimensions of the gaps in X and Y. We conclude that protein-rich disruptions of the outer mitochondrial membrane 10-22 nm in diameter form during pyroptosis.

Figure 5. Outer mitochondrial membrane discontinuities in pyroptotic cells.

(a) Cryo-ET reconstruction of a mitochondrion in an unstimulated WT iBMDM with closeup panels defining the following morphological parameters: inner-to-outer membrane spacing, inter-cristae spacing, crista width and crista apex angle.

(b) Quantitative analysis of the morphological parameters quantified in (a) in 3-D segmented surfaces of the mitochondrial membranes, as shown in (Fig. 4d). Error bars represent

standard deviation between measurements. Parameters were measured from four tomograms for the control and two tomograms for each of the nigericin-stimulated samples. Error bars represent standard deviation from the mean. For the crista apex angles: “control”, $n = 327$; “30 min”, $n = 187$; “1 h”, $n = 96$. For other parameters: “control”, $n = 16$; “30 min”, $n = 8$; “1 h”, $n = 8$. Statistical test: two-way ANOVA, ns, $P > 0.05$; ****, $P < 10^{-4}$. See **Dataset S3** for source data.

(c) Cryo-ET reconstructions of mitochondria in ASC-mCerulean iBMDMs stimulated with nigericin for 30 min (left) and in WT iBMDMs stimulated with nigericin for 1 h (centre, right). Dashed boxes denote outer-membrane discontinuities. Scale bars, 100 nm.

(d) Higher magnification view of a mitochondrial outer-membrane discontinuity (dashed box). Scale bar, 100 nm.

(e) Z-stack series for the boxed region in **(d)**. Arrows mark the outer-membrane discontinuity. Scale bar, 20 nm.

(f) Quantitative analysis of the cryo-ET density at the outer-membrane (OM) gap site in the Z-stack series shown in **(e)**, and at a site in the same tomogram with intact inner and outer membranes. The graph plots the average pixel density ratio $\langle \rho(\text{IMS}) \rangle / \langle \rho(\text{cyt}) \rangle$ for each Z-slice, where $\langle \rho(\text{IMS}) \rangle$ and $\langle \rho(\text{cyt}) \rangle$ are the average pixel densities of circular areas 35.4 nm in diameter, in the mitochondrial intermembrane space (IMS) and an adjacent cytosolic area, respectively. See **Dataset S2** for source data.

Discussion

NLRP3 inflammasome signalling assemblies have long been known from fluorescence microscopy imaging to form puncta, or specks, containing ASC and caspase-1. Room temperature electron tomography of ASC puncta in fixed zebrafish sections²⁶ and iBMDMs (**Fig. S3d**) showed a dense filament network within the punctum, but the chemical fixation and staining agents precluded further ultrastructure from being resolved. Our cryo-ET

reconstructions, obtained in unstained and fully hydrated conditions, show that the speck is formed of a filamentous network consisting of hollow-tube branched filaments with the dimensions predicted for ASC filaments, based on structural studies of purified ASC PYD filaments and full-length monomeric ASC^{23,24}. The identification of a filamentous network containing mCerulean-labelled ASC positively identifies the protein as the principal filament-forming component in the puncta. These data are supported by analysis of similar structures in wild-type cells labelled with a fluorescent caspase 1 substrate. The filament structure of the ASC with an mCerulean tag on the C-terminus supports the model whereby the PYD forms the filament core, and the CARD decorates the filament. The extensive filament branching visible in the reconstructions is thought to depend on CARD-CARD interactions because the CARD is required for puncta formation, whereas the ASC PYD alone forms unbranched filaments that lack the structural stability that is inherent to a branched network^{17,18,23-26}.

The density of ASC filaments varied in the cryo-ET reconstructions, with multiple core regions of densely packed filaments separated by sparser regions. This suggests that the higher-order structure of the punctum was likely seeded by multiple oligomeric assemblies, rather than growing concentrically from a single nucleation site. In contrast with huntingtin and other neurotoxic protein aggregates, which exclude other macromolecules based on cryo-ET reconstructions⁴⁵, the structural organisation of the ASC filament network allows ribosomes and small TGN-like vesicles to be retained in the puncta, or to permeate through them. Permeability of the ASC network to macromolecules could be important for efficient recruitment and release of effector, substrate and product molecules. Permeability to vesicles would allow active transport of TGN vesicles carrying activated inflammasome seed-oligomers from sites of NLRP3 activation to the site of punctum formation, as recently proposed¹². Moreover, the ribosomes within the puncta could potentially contribute to the pyroptotic signalling programme, for example by expressing proteins required for signalling, or by responding to danger signals.

Indeed, NLRP3 signalling can be activated by translational arrest through direct and indirect mechanisms⁴⁶, including binding of fungal polysaccharides to ribosomes⁴⁷.

Our cryo-ET reconstructions, in addition to revealing the novel ultrastructure of ASC within the cell, also allowed morphometric analysis of mitochondria in the inflammasome activated cellular environment. The clearest differences between mitochondria in inflammasome activated cells and unstimulated cells were a two to fourfold reduction in cristae spacing, and the appearance of protein-rich discontinuities of 10-20 nm in diameter in the outer membrane, which are distinct from BAK/BAX macropores. Permeabilization of both the plasma membrane and outer mitochondrial membrane was reported previously in cells undergoing caspase-1-dependent pyroptosis after inflammasome activation⁴¹. Pore formation in the plasma membrane by the N-terminal domain of gasdermin D (GSDMD) after cleavage by caspase-1 is required for inflammasome-dependent pyroptosis⁴⁸. The outer-membrane discontinuities in the mitochondria of inflammasome-activated cells had similar dimensions to GSDMD pores, which have an average inner diameter of 22 nm⁴⁸ (or 13-34 nm based on atomic force microscopy⁴⁹). Future studies are warranted to test the hypothesis that the outer-membrane gaps observed by cryo-ET may correspond to mitochondrial GSDMD pores.

The filament branching and packing density within ASC puncta provide structural integrity while allowing downstream signalling molecules to diffuse freely and bind at high density within the network. Although many structural details of the NLR inflammasome signalling machinery remain unclear, this study demonstrates the potential for combined cryo-CLEM and cryo-ET approaches to extract detailed, hypothesis-generating ultrastructural information for fully assembled innate immune signalling complexes in their cellular context.

Materials and Methods

Mammalian cell culture. Wild-type immortalized primary bone-marrow derived macrophages (WT iBMDMs) and ASC-mCerulean iBMDMs, kindly provided by Eicke Latz (Univ. Bonn), were cultured in Dulbecco's modified Eagle Medium high glucose (DMEM; Gibco, Waltham, MA, USA), supplemented with 10% v/v foetal bovine serum (FBS; Gibco). Human monocytic THP-1 cells (European Collection of Authenticated Cell Cultures) were grown in Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco), supplemented with heat inactivated 10% v/v FBS, 10 mM HEPES pH 7.4, 1 mM Sodium Pyruvate and 0.05 beta-mercaptoethanol. Cells were checked regularly for mycoplasma contamination by MycoAlert™ mycoplasma detection kit (Lonza) and cells were free of mycoplasma contamination.

Inflammasome stimulation. For stimulation of WT iBMDMs and ASC-mCerulean iBMDMs, cells were primed with 200 ng/ml LPS for 3 h in Opti-MEM® (Gibco) or DMEM supplemented with 10% FBS before NLRP3 stimulus was used. The following stimulus were used: 10 µM nigericin (Sigma or Invivogen) and 5 mM ATP (pH adjusted to 7.4, Sigma) for 30 min or 60 min with pan-caspase inhibitor Z-VAD-FMK (Invivogen) or carboxyfluorescein-labelled inhibitor of caspase-1 YVAD (FAM-FLICA, ImmunoChemistry Technologies) for *in situ* cryo-ET sample preparation unless otherwise specified. For stimulation of THP-1 cells, priming and stimulation were performed in RPMI Medium and conditions were listed above. To stain active caspase-1, the supernatant was removed following inflammasome stimulation; replaced with DMEM supplemented with heat-inactivated 10% v/v FBS containing 0.5x reconstituted FAM-FLICA (ImmunoChemistry Technologies).

Cytokine measurement and immunoblotting. IL-1β secretion was measured by ELISA as previously described²⁷. Briefly, supernatants were collected after inflammasome activation and BD BioSciences OptEIA was used to measure mouse IL-1β concentration in cell supernatant according to the manufacturer's instructions.

The cells were lysed in cell lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8, 1% Triton X-100, 1 mM PMSF, 10 µg/mL leupeptin, 1 µg/mL aprotinin) on ice for 10 min before centrifugation. Supernatant was collected and boiled in SDS sample loading buffer for 5 min. Following separation by 4-20% gradient SDS-PAGE, the proteins were transferred to a PVDF membrane. Membrane was blocked in 5% milk in PBS + 0.2% Tween-20. The primary antibody was rabbit monoclonal anti-mouse GSDMD (Abcam, ab209845, RRID:AB_2783550). The secondary antibody was goat anti rabbit IgG-HRP (Santa Cruz Biotechnology, sc-2004, RRID:AB_631746).

Immunofluorescence staining. For immunofluorescence, cells were plated as monolayer on µ-Slide 8-Well chamber (ibiTreat, ibidi, 80826) or 12-well chamber (removable, ibidi, 81201). Cells were primed and stimulated as above, and then fixed with 4% paraformaldehyde for 5 to 10 min at room temperature. Cells were permeabilised and blocked in 0.1% Saponin (Sigma, 47036) supplemented with 20% FBS (Gibco) or 2% Bovine Serum Albumin (Sigma, A7030) in PBS with primary antibody overnight. Washing was performed before cells were incubated with secondary antibodies. The following primary antibodies were used: rabbit anti-ASC rabbit polyclonal pAb AL177, 1:200 dilution (AdipoGen, AG-25B-0006, RRID:AB_2490440); rabbit anti-ASC monoclonal ASC/TMS1(D2W8U), 1:800 dilution (Cell Signalling Technology, 67824, RRID:AB_2799736); goat anti-NLRP3 polyclonal, 1:200 dilution (Abcam, ab4207, RRID:AB_955792); goat anti-IL-1β polyclonal, 1:500 dilution (R&D Systems, AF-401-NA, RRID:AB_416684); mouse anti-γ-Tubulin monoclonal, 1:400 dilution (Sigma-Aldrich, T6557, RRID:AB_477584); rabbit anti-TGN38 polyclonal, 1:250 dilution (Novus Biologicals, NBP1-03495, RRID:AB_1522533); rabbit anti-TOM20 FL-145 polyclonal, 1:80 dilution (Santa Cruz Biotechnology, sc-11415, RRID:AB_2207533). The Alexa Fluor secondary antibodies (488, 555, 568 and 647) used were: 488-labelled donkey anti-goat IgG (H+L) (1:500, ThermoFisher, A11055, RRID:AB_2534102); 555-labelled goat anti-rabbit IgG(H+L) (1:500, ThermoFisher,

A21428 RRID:AB_141784); 568-labelled goat anti-mouse IgG (H+L) (1:500, ThermoFisher, A11004, RRID:AB_2534072); 647-labelled goat anti-rabbit IgG (H+L) (1:500, ThermoFisher, A21244, RRID:AB_2535812). After incubation with secondary antibodies at room temperature for 1 h, cells were washed three times with blocking buffer, one time with PBS and final wash with water before mounting with ProLong Gold Antifade Mountant with DAPI (ThermoFisher).

Confocal fluorescence imaging. Single point-scanning confocal microscopy was carried out on a Zeiss (Oberkochen, Germany) LSM 780 or LSM 710 microscope using a 40x/1.3 NA Fluar or 63x/1.4 NA Plan-apochromat oil immersion objective lens. The microscope was equipped with 405, 458, 488, 514, 561 and 633 nm laser lines. Multi point-scanning confocal microscopy was carried out on Visitech (Sunderland, UK) iSIM mounted on a Nikon (Tokyo, Japan) Ti2 microscope stand using a 100x/1.49 NA SR Apo TIRF oil immersion objective lens or a 60x/1.2 NA Plan Apo VC water immersion objective lens. The iSIM was equipped with 405, 445, 488, 561 and 640 nm laser lines and Hamamatsu (Hamamatsu, Japan) ORCA-Flash4.0 V3 sCMOS cameras. Filter ranges: green (500-545 nm), red (593-624 nm). Live-cell samples were heated to 37°C and supplemented with 5% CO₂ using a microscope incubation chamber.

Live-cell imaging and Fluorescence Recovery after Photobleaching (FRAP). ASC-mCerulean iBMDMs were primed and induced as previously described with the presence of caspase-1 inhibitor (Z-VAD-FMK). Confocal FRAP was performed on a Zeiss LSM 710 microscope equipped with a 63x/1.4 NA Plan-apochromat oil immersion objective lens and a 458 nm laser line for excitation and bleaching. The sample environment was heated to 37°C and supplemented with 5% CO₂ using a microscope incubation chamber. ASC-mCerulean speck was photobleached with 100% laser power. Images were acquired at 3 s intervals. Images were collected at three pre-bleach timepoints and for 160 s post-bleaching. Movies were analysed in ImageJ/Fiji⁴² using a customised script (https://github.com/jboulanger/imagej-macro/tree/main/FRAP_Measure). Briefly, measures were normalised to account for the general

photobleaching caused by image acquisition as well as sample motion over time. Normalised fluorescence intensity measurements were obtained after background and bleaching corrections. The timeframe varied for experiments ($n = 8$). Each measurement was interpolated using the normalised fluorescence intensity measurements and imported to Python for plotting of the fluorescence intensity curve.

Quantification of Mitochondrial membrane potential. After 30 min of inflammasome stimulation as described above, iBMDMs were incubated with 20 nM TMRM (Life Technologies) for 15 min. iBMDMs were incubated for 3 h in 50 μ M of the mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP; ThermoFisher, M20036). Control samples included: untreated; TMRM+; TMRM+CCCP+; TMRM+LPS+ iBMDMs. TMRM fluorescence was quantified by flow cytometry and data were acquired on Eclipse flow cytometer (Sony Biotechnology). Quantification was set at 100,000 for cell count and a spectrum window of (FL3 (595BP)) was used to detect TMRM signal. Data were analysed and visualised using Flowjo10 (TreeStar, Ashland, Oregon, USA).

Cryo-ET sample preparation. Quantifoil finder grids (R2/1 or R2/2, Au 200 mesh grid, Quantifoil Micro Tools, Germany) were glow discharged with a 30 mA current for 30 s using Edwards Sputter Coater (S150B). Then grids were sterilized by UV irradiation for 10 min and immersed in PBS supplemented with fibronectin solution (10 μ g/ml, Sigma) overnight in 8-well or coculture wells (ibidi). Grids were washed with PBS three times. Cells were seeded in 8-well or coculture wells (ibidi) and incubated overnight at 37°C and 5% CO₂. Cells cultivated on grids were primed, induced with inflammasome stimuli and were plunge-frozen in liquid ethane using Leica EM GP2 cryo-plunger. Prior to plunging, 4 μ l of cell culture medium was added to the cell side and backside blotting was applied for 6-8 s. The chamber conditions were maintained at 37°C, 100% humidity during freezing. Grids were stored in liquid nitrogen.

Cryo-fluorescent light microscopy (Cryo-FM) and cryo-focused ion-beam (FIB) milling.

Grids were screened for cells with ASC/caspase-1 speck by light microscopy using a cryo-FM microscope (Leica EM Cryo CLEM, Leica Microsystems), equipped with a cryo-stage, an ORCA-Flash4.0 V2 sCMOS camera (Hamamatsu Photonics) and a 50x/0.9 NA HCX PL APO cryo-objective lens. Montage acquisition of grids was performed with Leica LAS X software, while recording the following channels: green (L5 filter, 50 ms), far red (Y5 filter, 20 ms), and brightfield (50 ms). Z-stacks were recorded at 0.5 μm with step size 21 for each grid square to determine the best focus for the montage. The montage was completed by stitching the best focus image from the Z-stack with Leica LAS X.

Lamellae were prepared using a Scios Dual Beam FIB scanning electron microscope (SEM; ThermoFisher) equipped with a Quorum cryo-stage (PP3010T). The milling protocol was adapted from previously published method²⁹. Grids were coated with organometallic platinum using the gas injection system (GIS; ThermoFisher) operated at RT, for 8 s, at 12 mm working distance and 25° stage tilt. A first rough milling was performed at 25° stage tilt with a 30 kV ion beam voltage and 1 nA current until the lamella thickness reached 10 μm . The micro-expansion joints were applied to improve lamella stability³² using the milling parameters listed above. Then stage was tilted to 20° for all subsequent milling steps. Rough milling steps were applied as following with 30 kV ion beam voltage: a 5 μm lamella thickness was reached with current 0.5 nA; 3 μm lamella thickness was reached with a 0.3 nA current; and 1 μm lamella thickness was obtained with a 0.1 nA current. Fine milling to a final lamella thickness of approximately 200 nm was performed with ion beam settings of 30 kV and 50 pA or 10 pA, or 10 kV and 23 pA or 11 pA.

Grids were loaded to a Linkam cryo-stage (CMS196V), and lamellae were imaged on a Zeiss microscope equipped with Axiocam 503 mono and Colibri 7-illumination module R(G/Y) CBV-UV. Z-stacks of the lamellae were acquired to identify ASC/caspase-1 specks using a 200-

300 nm step size. A projected cryo-fluorescent image was used to correlate with an SEM image using the EC-CLEM plugin from ICY³⁶. Registration between the lamella map and SEM image was achieved by transforming coordinates using edges or salient features of the lamella. The maximum projection Z-stack was then saved for correlation with SerialEM^{32,33}.

Tilt-series acquisition and tomogram reconstruction. Grids with a fluorescent lamella were transferred to a 300 kV Titan Krios electron microscope (ThermoFisher) equipped with an energy filter (Gatan). Movies were acquired with a K2 or K3 direct electron detector with SerialEM^{33,34}. Cryo-FM/EM correlation was then used to locate an area of interest for tilt-series acquisition. Briefly, a medium-magnification montage EM map (MMM) of the lamella was generated and a fluorescence map (FM) was loaded into SerialEM. The maps were correlated through recognition of geometric edges of the lamella. Transformation of coordinates yielded an overlay map which provided guidance for tilt-series acquisition. Tilt-series were collected at a nominal 32,000x or 29,000x magnification, resulting in pixel size 3.4 Å or 2.7 Å, over a tilt range of -60° to +60° with 1°, or 2° increments, a total dose of 140-240 electrons/Å² and a nominal defocus range of -4 to -8 µm. A dose-symmetric scheme was used⁵⁰. A pre-processing script was used from SubTOM (by Dustin Morado; <https://github.com/DustinMorado/subTOM>). Frames were aligned using IMOD³⁷. Reconstruction was performed by weighted back-projection, and segmentation with IMOD. The MATLAB script deconv from Warp⁵¹ was used for visualisation. Filament tracing was performed manually with IMOD and UCSF Chimera⁵². Membrane segmentation was performed with TomoSegMemTV⁵³.

Quantifications and statistical analysis. Filament lengths were measured using IMOD³⁷ from six tomograms of ASC-mCerulean iBMDMs and one tomogram of WT iBMDMs labelled with FAM-FLICA. Histograms were plotted with MATLAB. Branching angles were measured with Fiji⁴² and exported to MATLAB for plotting. The filament diameter was determined in Fiji by measuring density profiles of 20-nm lines perpendicular to the ASC filament (15 pixels in width,

ASC-mCerulean tomogram with 13.6 Å/pixel and WT ASC tomogram with 10.78 Å/pixel). The grey value profiles were normalized to their maximal individual value. Normalized grey profile were aligned and exported to Python for plotting. Similarly, diameters of trans-Golgi-like vesicles were measured in Fiji and exported to MATLAB for plotting.

Mitochondrial tomograms were loaded in Fiji⁴² and ROI manager was used to measure morphological parameters, which were compared using Two-way ANOVA and plotted with GraphPad Prism. For the inner-to-outer membrane spacing, the distance between the nearest edges of the inner and outer membranes was measured. The cristae width was measured between the inner membranes of the cristae. The spacing between cristae were measured using the perpendicular distance between the internal border of the neighbouring cristae. The inner-to-outer membrane spacing, cristae width and inter-cristae spacing were measured perpendicular to the membranes, evenly spaced for each tomogram, with thirty values per slice, for four slices per tomogram, from eight tomograms. The cristae apex angles were measured for every tip visible in ten slices per tomogram. Every value was plotted along with the average. If the end of a crista was flat, then the measurement of the two angles on either side was measured. Any measurement was only taken if the internal border of the membrane was clearly distinguishable from the matrix. All measurements were evenly spaced through each slice, to give a value representative of the whole slice, using all the mitochondria present in each image. All measurements were taken using 8 different tomograms: four control, two 30-min nigericin-treated, and two 60-min nigericin-treated mitochondria.

For measurement of the outer-mitochondrial membrane gap density, we calculated the density ratio as follows. A total of 21 virtual slices were selected in a region containing a visible outer-membrane gap. The average pixel grey value was calculated from circular areas 13.6 nm in diameter: three cytosolic areas, three inter-membrane space areas and an outer-membrane gap area. This was repeated for each of the 21 virtual slices. The ratio of average outer-

membrane gap site to cytosolic grey value, and the ratio of average inter-membrane space site to cytosolic grey value were calculated for each virtual slice. The grey area ratio was then plotted with Igor (WaveMetrics, Inc.) to show the change of protein density in Z direction.

Data availability

Representative electron tomograms were deposited in EMDB with codes EMDB with codes EMD-13585, EMD-13586, EMD-135XX and EMD-135XX. Other data are available from the corresponding authors upon request.

Code availability

SubTOM scripts and instructions are available from Github [<https://github.com/DustinMorado/subTOM>]. The FRAP ImageJ macro is available on Github [https://github.com/jboulanger/imagej-macro/tree/main/FRAP_Measure].

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Author contributions

Y.L., L.J.H., C.E.B and Y.M. conceived the experiments. Y.L., H.Z. and J.H. collected light microscopy data. Y.L. and J.B. analysed light microscopy data. Y.L., A.C.B. and C.H. prepared cryo-EM samples, performed CLEM imaging and collected cryo-ET data. Y.L. processed the cryo-ET data. Y.L. and H.A. analysed the cryo-ET data and flow-cytometry analysis. Y.L. and

730 L.J.H. performed cell-based assays. Y.L, C.E.B and Y.M. wrote the manuscript with input from
731 all authors. Project supervision, administration and funding acquisition, C.E.B. and Y.M.

732 **Competing interests**

733 C.E.B are Y.M. are consultants for Related Sciences LLC and have profits interests in Danger
734 Bio LLC. CEB is on the SAB of NodThera and Lightcast.

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737 **Cryo-electron tomography of ASC sites in pyroptotic cells**

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Supplementary Information

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Supplementary Materials and Methods

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Figures S1-S5

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Supplementary Materials and Methods

Correlative fluorescence microscopy and electron tomography of resin-embedded cells.

Correlative fluorescence microscopy and electron tomography (RT-CLEM) of resin-embedded cells was performed as described^{40,54}. Briefly, ASC-mCerulean cells were grown on carbon-coated 3 mm sapphire discs (Wohllwend GmbH) in two-well chambers (ibidi) for 24 h before priming and NLRPC-driven inflammasome stimulation as described above. Cells were stained with MitoView far-red and FLICA before high pressure freezing with an HPM100 high pressure freezing system (Leica Microsystems). Grids were imaged with a cryo-fluorescence microscope (Leica EM Cryo CLEM, Leica Microsystems) to localise cells containing ASC-mCerulean specks. Freeze substitution was performed using 0.008% uranyl acetate in acetone and embedded in Lowicryl HM20 (Polysciences) using an AFS2 (Leica Microsystems). Blocks were sectioned into 300 nm thin sections using a microtome (Leica Microsystems) equipped with a diamond knife (Diatome). The sections were collected on 200 mesh/300 mesh copper grids with carbon support (Agar Scientific Ltd). TetraSpeck 100-nm microspheres were diluted 1:100 in PBS and applied to the sections for use as fiducial markers for correlation. Sections were imaged on a Nikon Ti2 wide field microscope equipped with a Niji LED light source (Bluebox Optics, Blackwood, UK), a x100/1.49 NA Apo TIRF oil immersion objective lens and a Neo sCMOS DC-152Q-C00-FI camera (Andor Technology, Belfast, UK). Filters: mCerulean (89006 filter set, Chroma Technology, Bellows Falls, USA), fluorescein (49002 filter set, Chroma), MitoView Far Red (49006 filter set, Chroma). EM images were collected using a Tecnai F20 electron microscope (ThermoFisher) operated at 200 kV and a high-tilt tomography holder (Fischione Instruments; Model 2020). An image montage of regions of interest was acquired on a BM-Orius detector using TEM mode at 150-200 μ m defocus using SerialEM v3.8.0³⁴ at a pixel size of 1.1 nm. The correlation between the montaged map and fluorescent map was performed by image transformation of registered fiducial markers in both image modalities as previously

774 described⁵⁵. Tilt-series were acquired from approximately -60 to +60 with 1° increment at a pixel
775 size of 1.1 nm. Samples were rotated 90° to acquire dual axis tilt-series. Tomograms were
776 reconstructed and visualised with IMOD³⁷.

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Figure S1. Tracking NLRP3 inflammasome activation in iBMDMs with fluorescence microscopy and biochemical assays.

- (a) Snapshots of live iBMDMs expressing ASC-mCerulean. ASC specks formed within 10 min of NLRP3 inflammasome activation with LPS and nigericin. Merge, overlay of fluorescence and bright-field images. Scale bar, 10 μ m.
- (b) Biochemical assays of inflammasome activation in ASC-mCerulean iBMDMs: upper panel, ELISA measuring IL-1 β secretion; lower panel, Western blot showing proteolytic cleavage of GSDMD following different stimuli. FL, full-length. NTD, N-terminal domain.
- (c) Fluorescence microscopy of WT iBMDMs stimulated with LPS and either nigericin or ATP. Caspase-1 was detected with FAM-FLICA. NLRP3 and ASC were detected by immunostaining. Scale bars, 5 μ m.
- (d) Immunofluorescence microscopy of WT iBMDMs stimulated with LPS and either nigericin or ATP, with staining for IL-1 β and ASC. Scale bars, 5 μ m.
- (e) Snapshots of live ASC-mCerulean iBMDMs stained with FAM-FLICA after nigericin stimulation showing ASC-mCerulean colocalization with caspase-1 and FAM-FLICA fluorescence intensity increasing over time. Scale bar, 5 μ m.

Figure S2. Workflow for correlative cryo-light and electron microscopy (cryo-CLEM).

- (a) Cryo-fluorescence microscopy (cryo-FM) of ASC-mCerulean iBMDMs, with brightfield image overlaid. Cells were cultured on finder grids, primed with LPS, stimulated with nigericin for 30 min, labelled with MitoView and FAM-FLICA, vitrified, and then imaged. The white circle denotes one area of interest. Scale bar: 300 μ m.
- (b) Grids were transferred to a scanning electron microscope (SEM) fitted with a focused-ion-beam (FIB). The cell containing the area of interest, identified using the finder-grid markers, was imaged by SEM. Scale bar, 40 μ m.
- (c) SEM images of the area of interest before and after FIB milling: Upper panel, before FIB milling with initial target milling windows shown in brown (scale bar, 10 μ m); lower panel, side-on view of the lamella after FIB milling (scale bar, 4 μ m).
- (d) Cryo-FM image of a lamella containing an ASC speck with brightfield image overlaid. Scale bar, 10 μ m.
- (e) Low magnification transmission cryo-EM map of the lamella shown in (d). Scale bar, 7 μ m.
- (f) Medium magnification cryo-EM map overlaid with the cryo-FM map after registration and transformation. The region of interest for tilt-series acquisition is boxed. Scale bar, 1 μ m.
- (g) Reconstructed tomographic slice acquired in the area marked in (f) containing the fluorescent signal. Several short filaments and small trans-Golgi-like vesicles were visible. Scale bar, 300 nm. See **Movie S4** for a tomogram of this region.

Figure S3. Imaging of *trans*-Golgi-like vesicles within ASC puncta.

(a) Fluorescence micrographs of THP-1 cells with and without stimulation with LPS and nigericin, with TGN (anti-TGN38) immunostaining. Scale bar, 10 μ m.

(b) Two tomographic slices of ASC-mCerulean speck showing vesicles adjacent to filaments. Scale bars, 100 nm.

(c-e) CLEM of ASC-mCerulean iBMDMs stimulated with nigericin for 30 min, high-pressure frozen, chemically fixed, sectioned and imaged at room temperature (see Supplementary Materials and Methods). **(c)** Low magnification EM map overlaid with ASC-mCerulean fluorescence. Scale bar, 1 μ m. **(d)** 5.5 nm-thick tomographic slice of in the region boxed in **(c)**. Scale bar, 200 nm. **(e)** Tomographic slices showing vesicles with low-density lumens within ASC-mCerulean puncta. Scale bar, 100 nm. The histogram shows the vesicle diameter distribution.

Figure S4. Localization of ASC and caspase-1 relative to MTOC-associated components.

- (a) Fluorescence micrographs of ASC-mCerulean iBMDMs stimulated with LPS and nigericin, with γ -tubulin immunostaining. Scale bar, 10 μ m. Right, distribution of the distance between ASC-mCerulean and anti- γ -tubulin fluorescence.
- (b) Fluorescence micrographs of WT iBMDMs stimulated with LPS and nigericin. Cells were stained with FAM-FLICA, and with antibodies against γ -tubulin and ASC. Scale bar, 10 μ m. Right, distribution of the distance between ASC/FAM-FLICA and anti- γ -tubulin fluorescence.
- (c) Fluorescence micrographs of THP-1 cells stimulated with LPS and nigericin. Cells were stained with FAM-FLICA, and with antibodies against ninein and ASC. Scale bar, 10 μ m. Right, distribution of the distance between ASC/FAM-FLICA and anti-ninein fluorescence.

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iBMDM type	Nigericin stimulation (min)	Gap width (nm)	Estimated gap depth (nm)
ASC-mCerulean	30	11	11
ASC-mCerulean	30	15	12
ASC-mCerulean	30	18	14
ASC-mCerulean	30	11	14
WT	60	20	-
WT	60	17	17

Figure S5. Functional and ultrastructural analyses of mitochondria in iBMDMs stimulated with nigericin.

(a) Additional flow cytometry histograms of iBMDMs. The following controls are shown to accompany **Fig. 4b**: cells with no TMRM staining; cells stained with TMRM and a potent mitochondrial uncoupling chemical, carbonyl cyanide 3- chlorophenylhydrazine (CCCP); cells stained with TMRM only; and cells primed with LPS and stained with TMRM.

(b) Measured sizes of outer-membrane discontinuities in cryo-ET reconstructions of mitochondria in WT and ASC-mCerulean iBMDMs, as partly shown in **Fig. 5c-e**.