The CARD plays a critical role in ASC foci formation and inflammasome signalling

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INTRODUCTION

Innate immunity comprises the first line of defence against invading pathogens. The innate immune response is initiated by TLRs (Toll-like receptors), by NLRs (nucleotide-binding domain, leucine-rich repeat-containing receptors) and other sensors such as AIM2 (absent in melanoma 2), which recognize PAMPs (pathogen-associated molecular patterns) and DAMPs (damage-associated molecular patterns). Members of the NLR family are potent inducers of key defence pathways, including initiation of the NF-κB (nuclear factor κB) response, activation of inflammatory caspases and release of pro-inflammatory cytokines [1,2]. Failure of correct NLR signalling results in various autoimmune disorders including Crohn’s disease [NOD (nucleotide-binding oligomerization domain-containing) 2] [3], Blau syndrome (NOD2) [4], Vitiligo [NLRP (NLR family, PYD domain-containing) 3 or AIM2 agonists in RAW264.7 cell reconstitution assays. Analogously, we show that productive formation of the Salmonella typhimurium-induced NLRC4 (NLR family CARD domain-containing protein 4) inflammasome is dependent on ASC–CARD-mediated platform formation. Thus the results of the present study depict a central role of CARDs in the formation of ASC signalling platforms and provide an important tool for investigation of CARD-dependent networks.

Key words: caspase 1, caspase activation and recruitment domain (CARD), death domain, innate immunity, inflammasome, inflammatory cytokine, nucleotide-binding domain, leucine-rich repeat-containing receptor (NLR), NLR family CARD domain-containing protein 4 (NLRC4), Salmonella, signalling platform formation.

The CARD (caspase activation and recruitment domain) architecture allows the formation of ASC/caspase 1 foci. The lack of foci formation for ASC CARD mutants correlates with a loss of IL-1β (interleukin 1β) processing in response to NLRP (NLR family, PYD domain-containing) 3 or AIM2 agonists in RAW264.7 cell reconstitution assays. Analogously, we show that productive formation of the Salmonella typhimurium-induced NLRC4 (NLR family CARD domain-containing protein 4) inflammasome is dependent on ASC–CARD-mediated platform formation. Thus the results of the present study depict a central role of CARDs in the formation of ASC signalling platforms and provide an important tool for investigation of CARD-dependent networks.
omitting the need for an adaptor protein [17]. Yet, an amplifying function for ASC has been proposed in the context of responses to *Salmonella* infection [15,18], further underlining the necessity to obtain insight into the CARD-dependent signalling mechanism of ASC.

To gain insight into the role of the CARD system in ASC-mediated innate immunity, we adapted the BiFC (bimolecular fluorescence complementation) assay [19], allowing us to investigate and visualize productive ASC foci formation (Figure 1A) in living cells. Briefly, the BiFC technology uses complementary fragments of Venus, which represents an improved yellow fluorescent protein. These fragments do not reconstitute spontaneously, but, when fused to interacting proteins, the split fragments associate and restore a functional fluorescent signal. Using this assay, we were able to distinguish between non-functional assemblies, which show complementation of the fluorescent signal, but lack of platform formation, and functional assemblies representing intact inflammasome-like foci. In the present study, we show that the PYD domain of ASC is not sufficient for ASC foci formation, but also requires the presence of the CARD. Unexpectedly, when expressed in combination with caspase 1 CARD, the CARD of ASC is sufficient to support productive foci assembly. We furthermore identified surface residues on the CARDs of ASC and caspase 1, mutations of which interfered with ASC inflammasome formation. We then tested the effect of ASC mutants on inflammasome formation in RAW264.7 murine macrophage cells to delineate the importance of CARD-dependent oligomerization in the NLRP3 and AIM2 signalling pathways as well as in the NLRC4 response to *Salmonella* infection.

Figure 1 Oligomeric foci formation visualized by the BiFC system

(A) Schematics of BiFC assay. Lack of interaction between proteins bearing complementary fragments (VC and VN) of Venus–GFP leads to a lack of fluorescence. Diffuse fluorescence indicates proximity of binding partners due to binding or loose assemblies. Foci formation indicates defined productive oligomerization and hence signalling platform formation. (B–D) BiFC assay results exemplifying different outcomes in correspondence to above depicted schematics. HEK-293T cells were transiently transfected with VN- and VC-fusions. Cells were visualized for fluorescence 24 h post-transfection. (B) Example of the lack of interaction using VC–ASC CARD and VN–caspase 4 CARD. (C) Diffuse fluorescence observed after co-transfection of VN–ASC PYD and VC–ASC PYD. (D) Foci formation observed after co-transfection of VN–ASC full-length and VC–ASC full-length. (E) Fluorescent microscopy images showing BiFC of VN–ASC CARD and VN–caspase 1 CARD leading to a diffuse fluorescence pattern. (F) Fluorescent microscopy images showing BiFC of VC–ASC CARD and VN–caspase 1 CARD resulting in foci formation. (G) HeLa cells were transfected with VN–caspase 1 CARD and VC–ASC CARD. Fluorescent microscopy images showing perinuclear localization of ASC CARD/caspase 1 CARD foci with nuclei visualized by DAPI (4′,6-diamidino-2-phenylindole) staining. For each BiFC experiment outlined here and below all VC–ASC constructs were tested for auto-activation by co-expression with empty VN–vector and VN–caspase 1 constructs by co-expression with empty VC–vectors respectively.
EXPERIMENTAL

Models

A model for the CARD of caspase 1 (UniProt entry P29466) was created using MODELLER [20] on the basis of the structure of the Iceberg CARD (PDB code 1DGN). The Figures for the caspase 1 CARD model and for ASC CARD [based on the ASC structure (PDB code 2KN6)] were prepared using PyMOL (http://www.pymol.org).

Construction of plasmids and mutagenesis

CARDs of human caspase 1 (UniProt entry P29466) and 4 (UniProt entry P49662) were fused to N-terminal Venus fragments of the pVN vector. CARDs of human ASC (UniProt entry Q9ULZ3) and NLRC4 (UniProt entry Q9NPP4) were fused to C-terminal Venus fragments of the pVCC vector. The pVN and pVCC vectors were provided by Dr Gordon Mills (MD Anderson Cancer Center, Houston, TX, U.S.A.). Point mutations in caspase 1 and ASC were generated using QuikChange® site-directed mutagenesis (Stratagene). All mutants were verified by DNA sequencing analysis.

BiFC assay

HEK (human embryonic kidney)-293T or HeLa cells were transfected with VN (Venus N-terminus)- and VC (Venus C-terminus)-fusion constructs using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were incubated at 37°C for 20 h. Fluorophore formation in living cells was imaged using fluorescence microscopy. Images were acquired using an AMG EVOS digital inverted-fluorescence microscope. For DNA staining, cells were fixed with 4% formaldehyde, washed three times with 1× PBS, permeabilized with 0.1% Triton X-100 and labelled with DAPI (4,6-diamidino-2-phenylindole).

Cell culture and stable cell lines

HEK-293T, HeLa and RAW264.7 cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with glutamine, antibiotics and 10% FBS (fetal bovine serum). Stable WT (wild-type) ASC and mutant ASC RAW264.7 cells were generated using lentiviral infection. Full-length WT ASC and mutant ASC were subcloned into the pRDI292 expression plasmid. Lentivirus was produced by co-expression of pRDI with and mutant ASC were subcloned into the pRDI292 expression plasmid. Lentivirus was produced by co-expression of pRDI with and mutant ASC were subcloned into the pRDI292 expression plasmid.

RESULTS

The CARD of ASC is crucial for the formation of ASC inflammasomes

The ability of ASC to assemble into so-called ‘foci’ is key for inflammasome activation. Owing to its widely known insolubility upon overexpression in bacterial systems, the use of recombinant ASC or its domains for monitoring oligomer formation in vitro is limited. We therefore adapted the BiFC assay for qualitatively investigating the mechanism of ASC foci formation in living cells. The BiFC assay is based on two split fragments of the Venus fluorescence protein. The fragments termed VN and VC are fused to proteins, which constitute potential binding partners. Upon interaction between the binding partners, the fluorescent fragments are brought in close proximity restoring a functional fluorescent signal (Figure 1A) to result in what we term a ‘diffuse’ fluorescence signal (Figures 1C and 1E). However, if the proteins not only undergo interaction, but form productive signalling platforms, this diffuse signal condenses to intense speck-like foci (compare Figures 1C and 1D), representing a clear readout to observe and test this event. Therefore this system is appropriate to investigate ASC foci formation, which was previously observed to result in focused fluorescent platforms when ASC was fused to full-length GFP [21]. Furthermore, this assay has the advantage of not only following foci formation of full-length ASC, but also visualizing productive platform formation in the light of specific domains and combinations thereof. When the BiFC technology was applied to full-length VN–ASC and VC–ASC, we indeed observed the formation of foci (Figure 1D), making this approach an attractive tool to investigate the CARD system and its implications in ASC-mediated inflammatory responses.

We next examined the role of each domain of the bipartite adaptor ASC in the process of inflammasome formation. Commonly, only the PYD domain of ASC is regarded as the driving force for ASC inflammasome formation [13], whereas the role of the ASC CARD is thought to be restricted to recruitment and binding of caspase 1 via homotypic CARD–CARD interactions. However, when we investigated this in the
Figure 2  Mutational analysis of caspase 1 CARD surfaces with respect to foci formation with WT ASC CARD

(A) BiFC results of HEK-293T cells transfected with either WT or mutant VN–caspase 1 CARD together with WT VC–ASC CARD. Either lack of fluorescence or foci formation was observed for different caspase 1 mutants. The mutants R10E, D27R, E41R, K42E, R55E and D59R led to loss of binding, whereas the mutants R15E and R45D showed no effect on foci formation. F, foci; N, no interaction. (B) Cell lysates were immunobotted for VN–GFP (depicting caspase 1 CARD) and β-actin as a loading control showing similar expression levels of mutant caspase 1 constructs compared with WT caspase 1.

BiFC system, we observed that a combination of VN– and VC–ASC PYD resulted in a diffuse fluorescent signal (Figure 1C). This result was surprising as it indicates that the PYD domains exhibit affinity towards each other, but fail to form minimal ASC platforms. Similarly, VN– and VC–ASC CARD showed diffuse fluorescence, indicating a CARD–CARD attraction (Figure 1E). The observed foci formation in the context of full-length ASC, and absence of foci when either domain was expressed alone, points to a necessity for both domains and thus an active role of the CARD in driving ASC-dependent inflammasome formation.

Given the importance of the ASC CARD in inflammasome assembly, we further investigated the relation to its downstream target caspase 1 CARD, particularly with regard to foci formation. Surprisingly, co-expression of VC–ASC CARD with VN–caspase 1 CARD resulted in platform formation (Figure 1F). The resulting foci were similar to foci formed in the BiFC assay using full-length ASC. Furthermore, these ASC–CARD/caspase 1–CARD foci also showed the same attributes, namely the presence of one distinct perinuclear focus per cell (Figure 1G), as previously observed for endogenous ASC inflammasomes [12]. The formation of foci solely by the CARDs of these two proteins points to an active role of the caspase 1 CARD in productive platform formation.

ASC and caspase 1 CARD platforms are highly oligomeric and involve multiple surfaces on ASC and two main areas on caspase 1 CARD

The observed foci formed by ASC/caspase 1 CARDs in our BiFC assay allowed us to interrogate surfaces both on ASC and caspase 1 CARDs with respect to platform assembly. Using the structure of ASC CARD [22] and a model of caspase 1 CARD based on the Iceberg NMR structure [23] (Supplementary Figures S1A and S1B at http://www.biochemj.org/bj/449/bj4490613add.htm), we mutated several charged solvent-exposed residues covering all surfaces on both domains. In a first set of experiments we tested caspase 1 CARD mutants using the BiFC assay and transiently expressed WT and mutant VN–caspase 1 CARD together with WT VC–ASC CARD to investigate their ability to form functional platforms. A subset of mutants (R10E, D27R, E41R, K42E, R55E and D59R) of the caspase 1 CARD not only abolished foci formation, but also resulted in loss of fluorescence, indicating abrogation of interaction (Figure 2A). This loss does not stem from reduced expression levels of the mutants owing to misfolding or other factors, as shown in Figure 2(B). These experiments constitute a broad extension of a previous study, which reported that the D27G mutation of caspase 1 CARD interrupts ASC/caspase 1 CARD signalling [24]. We furthermore show that all mutants leading to a loss of foci formation and interaction reside in two main surfaces (Figure 2A). The first key surface is created by helices 1, 3, and 4 (Arg<sup>10</sup>, Lys<sup>42</sup>, Arg<sup>55</sup> and Asp<sup>59</sup>), whereas the second area required for foci formation is located in helices 2 and 3 (Asp<sup>27</sup> and Lys<sup>42</sup>) of the caspase 1 CARD. The mutations E8R, R15E on helix 1, R33E in the loop connecting helices 2 and 3, R45D in helix 3, and Q67R, Y75E and E78R on helix 5 showed no effect on foci formation (Figure 2A and Supplementary Figure S1C).

Next, VC–ASC CARD WT or mutants were co-transfected with WT VN–caspase 1 CARD. Mutants covering several surfaces (R125D, E130R, D134R, Y137E, E144R, R160E and D191R)
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Figure 3 Mutational analysis of ASC CARD surfaces with respect to foci formation with WT caspase 1 CARD

(A) HEK-293T cells were transiently transfected with VN–caspase-1 CARD and VC–ASC CARD WT or ASC CARD mutants respectively. At 24 h after transfection, foci formation was analysed using fluorescence microscopy. Representative images from fluorescent microscopy are shown. The ASC mutants R125D, E130R, D134R, Y137E, E144R, R160E and D191R led to diffuse phenotype, whereas the mutants D134A, Y146E, Q145A and R150E resulted in foci formation. D, diffuse staining; F, foci. (B) Immunostaining of total cell lysates showing proper expression of fusion proteins. Cell lysates were probed for ASC and for β-actin as a loading control. (C) Cells transfected with either WT or mutants (E130R, D134R, E144R, Q145A and Y137E) VC–ASC CARD and WT VN–caspase 1 (Casp1) CARD were analysed in the BIFC assay at 24 h post-transfection and then lysed by adding native PAGE lysis buffer (Invitrogen) and sonication. Total lysates were applied to native PAGE and immunoblotted for ASC and VN–GFP (depicting caspase 1 CARD). Only the mutants causing a ‘diffuse’ phenotype showed the presence of low-order ASC–caspase 1 CARD assemblies, which were able to enter the gel matrix. Highly-oligomeric assemblies with very low mobility are shown above. Cell lysates were also probed for β-actin as a loading control (bottom panel). WB, Western blot.

lost the ability to form foci, whereas mutants D143A, Q145A, Y146E and R150E showed no effect (Figure 3A). Again, as in the case for caspase 1 CARD, all ASC CARD mutants show similar or greater expression compared with the WT CARD (Figure 3B). This points to the involvement of multiple surfaces both on ASC and caspase 1 (Supplementary Figures S1A and S1B). However, in the case of ASC mutants, diffuse fluorescence was still observed, suggesting low-order associations, but failure to propagate productive platform formation.

To further investigate the nature of the assemblies showing diffuse fluorescence, we lysed cells transfected with VC–ASC (WT and mutants) and VN–caspase 1 constructs and subjected them to native PAGE. For this purpose, cells were resuspended in lysis buffer and subjected to sonication. Notably, whole-cell lysates were used for native PAGE, since centrifugation leads to precipitation of intact ASC foci (results not shown). Immunoblotting for ASC and caspase 1 constructs showed that only cells containing ‘diffuse’ ASC mutants showed a presence of low-oligomeric assemblies migrating in the native PAGE, whereas cells containing functional foci of WT ASC–CARD and caspase 1 CARD completely lacked these low-oligomeric arrangements (Figure 3C). Thus low-oligomeric assemblies retaining solubility probably do not represent active signalling platforms.
CARD-dependent ASC foci correlate with caspase 1-mediated IL-1β processing downstream of both NLRP3 and AIM2

To verify our findings from the BiFC assay and to further investigate the importance of CARD-mediated oligomerization in inflammasome activation, we tested WT and ASC mutants for their ability to mediate NLRP3 and AIM2 signalling under more physiological conditions. We chose the RAW264.7 macrophage cell line, which lacks endogenous ASC, and generated stable cell lines reconstituted with either full-length WT ASC or ASC mutants. Stable cell lines were challenged using divergent stimuli that have been shown to trigger NLRP3 inflammasome formation consisting of: (i) ATP, a danger signal that triggers potassium release and Ca2+ mobilization [25]; (ii) PGN (peptidoglycan), a known PAMP derived from bacterial cell walls [26]; and (iii) MSU (monosodium urate) mimicking the reported effect of urea crystals in gout [27]. As expected, RAW264.7 cells expressing WT ASC resulted in inflammasome activation and subsequent release of processed IL-1β upon treatment with LPS (lipopolysaccharide) and ATP (Figure 4A). The addition of zVAD-fmk (benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone) prevented IL-1β processing, showing the caspase-dependency of this process. In contrast, RAW cells expressing ASC mutants E130R, D134R or E144R, which led to the diffuse phenotype lacking productive foci formation, produced levels of IL-1β similar to the negative control (RAW cells lacking ASC) following treatment with all NLRP3 stimuli (Figures 4A and 4B). This defect did not result from a difference in pro-IL-1β synthesis, since immunoblotting showed equivalent pro-IL-1β levels in all cell lines (Figure 4A). These findings confirm the crucial role of the ASC CARD for NLRP3–ASC inflammasome signalling and caspase 1 activation in a cellular context.

To further expand our investigation we included the PAMP-sensing factor AIM2, which also forms ASC-dependent caspase 1-activating inflammasomes. AIM2 contains an N-terminal PYD domain, but in contrast with NLR proteins, features a C-terminal HIN200 (haemopoietic interferon-inducible nuclear antigens with 200 amino acid repeats) domain that senses double-stranded DNA in response to bacterial or viral infection [28–30]. Most importantly, AIM2 lacks the NACHT domain typical for NLRs, which is thought to form AAA+-type ring structures and was reported to serve as an oligomerization unit [31] that drives inflammasome formation. Thus AIM2 inflammasomes constitute an attractive tool to further investigate the role of CARD-dependent ASC oligomerization in this inflammatory signalling pathway.
Therefore we tested the effect of ASC CARD mutants on AIM2 signalling using poly-da:dT [poly(deoxyadenylic-deoxythymidylic) acid], a double-stranded DNA analogue and specific AIM2 activator. The results of the present study show that the ASC mutants that led to a diffuse phenotype in the BiFC system also exhibited significant reduction of IL-1β production upon AIM2 activation, whereas WT ASC showed robust IL-1β secretion (Figure 4C). Thus, similar to the NACHT-containing NLRs, AIM2 signalling requires a functional ASC CARD, pointing to an important role of the CARD in driving the signalling process.

A crucial role of ASC CARD in NLRC4-mediated S. typhimurium infection

Next we used the insights obtained about the role of the ASC CARD in the oligomerization process to further investigate the elusive function of ASC in Salmonella-induced cytokine activation mediated by NLRC4. In contrast with NLRP proteins, NLRC4 contains an N-terminal CARD, which in principle allows direct interaction with CARD-containing targets, omitting the need for a PYD/CARD-containing ASC adaptor. NLRC4 is a known sensor for flagellin during S. typhimurium and Legionella infections and mediates caspase 1 activation resulting in IL-1β processing [32,33]. The N-terminal CARD of NLRC4 has been shown to directly interact with the CARD of caspase 1 [17]. Conversely, a previous study described the formation of NLRC4-dependent foci, which incorporate ASC and caspase 1 upon Salmonella infection [15]. However, no evidence of a direct interaction between NLRC4 and ASC has been described and the exact role of ASC in the NLRC4 inflammasome complex is still not fully understood.

Therefore we tested the effect of ASC on the interaction of VN–NLRC4 CARD and VC–caspase 1 CARD. To this end, we expressed the CARDs of VN–NLRC4 and VC–caspase 1 in the presence or absence of full-length ASC. Indeed, co-expression of full-length Myc-tagged ASC together with the VN–NLRC4 CARD and VC–caspase 1 CARD caused a change from diffuse staining to punctate fluorescent foci (Figure 5A). We then tested the ASC mutants E130R and D134R, which are CARD mutants we found to cause diffuse fluorescence patterns in the preceding sections, therefore lacking the ability to propagate productive platform formation. Indeed, when used to replace WT ASC in co-expression experiments with the VN- and VC-tagged CARDs of NLRC4 and caspase 1, the foci described above were lost and a diffuse pattern was observed (Figure 5A). Taken together, these findings indicate and visualize that the ASC platform is crucial for productive NLRC4 inflammasome foci formation, and support the idea that efficient IL-1β processing is ASC dependent. To verify and extend the function of ASC as pertains to NLRC4 inflammasome signalling, we infected RAW264.7 cells stably expressing WT or ASC mutants (E130R and D134R) with S. typhimurium. In line with foci formation observed in the BiFC assay, WT ASC led to robust IL-1β release upon Salmonella infection (Figure 5B). Consistent with the fact that ASC platform formation is required for efficient caspase 1 activation and thus cleavage of pro-IL-1β, RAW cells expressing the ASC mutants E130R and D134R showed an approximately 10-fold reduction in the response to Salmonella infection, similar to the response observed for the empty control (Figure 5B). This reduction did not result from a difference in pro-IL-1β synthesis, since immunoblotting revealed equivalent pro-IL-1β levels in all cell lines (Figure 5B). Furthermore, the requirement of ASC for NLRC4-mediated IL-1β secretion was seen for different multiplicities of infection (Supplementary Figure S2 at http://www.biochemj.org/bj/449/bj4490613add.htm). Thus, in agreement with the results of the BiFC assay, the infection experiments also revealed a key role of ASC and its CARD in NLRC4-mediated response to Salmonella infection.

DISCUSSION

In the present study we apply a combination of a fluorescence-based protein complementation assay technology and physiological readouts to depict the role of the ASC CARD in ASC-mediated inflammasome signalling pathways. We show a significant role of the ASC CARD in ASC foci formation and find that the PYD is insufficient to drive platform formation. Moreover, we show that the ASC CARD in isolation is capable of forming foci in combination with the CARD of its target caspase.
1. This unexpected involvement in foci formation may point to a general role of the caspase 1 CARD or other death domain family proteins in augmenting signalling platform assembly in a ‘target feedback mechanism’. Additionally, owing to their ability of solely forming foci, CARDs may also directly interact with other factors driving inflammasome formation such as scaffold proteins [14]. To our knowledge our NLRC4/ASC/caspase 1 BiFC assay is the first system visualizing a direct interaction of ASC with NLRC4/caspase 1 as well as its absolute requirement for foci formation.

Using the BiFC assay as a qualitative tool for visualizing foci formation, we performed an extensive mutational screen covering surface residues on both ASC and caspase 1 CARDs and find that multiple surfaces are involved in ASC CARD-dependent foci formation, whereas two main surfaces are required for the CARD of caspase 1. Subsequently, we reconstituted a macropathage cell line that is naturally devoid of asc with full-length ASC WT or CARD mutants, and observe that the lack of foci formation of ASC CARD mutants in the BiFC system correlated with loss of IL-1β secretion. This correlation was observed for both NLRP3- and AIM2-dependent activation of caspase 1, emphasizing a critical role of the ASC CARD in functional inflammasome formation. Finally, our system enabled us to depict and visualize the elusive function of ASC in NLRC4 inflammasome formation upon Salmonella infection. Using the CARDs of NLRC4, ASC and caspase 1 in a three-component BiFC ‘bridge’ assay, we show that ASC is key for productive foci formation. When tested in a physiological context, ASC mutants that fail to propagate foci formation in the BiFC system resulted in a 10-fold reduction of secreted IL-1β upon Salmonella infection similar to cells lacking ASC. These findings reveal an important bridging function of ASC in the Salmonella-mediated NLRC4-caspase 1 inflammasome.

Taken together the present study not only depicts a new key role for the CARD in the formation of highly oligomeric functional ASC inflammasomes, but also sheds new light on the function of ASC in NLRC4-dependent Salmonella infection. It furthermore represents a model for the investigation of signalling platforms that rely on productive oligomerization for signal transduction. We predict that the approach used here may be applicable to investigations of other large multimeric protein complexes such as plaque-forming disorders stemming from aberrant aggregation events, as described for Alzheimer’s, Huntington’s and Parkinson’s diseases.

REFERENCES


AUTHOR CONTRIBUTION

Martina Proell, Motti Gerlic, John Reed and Stefan Riedl designed experiments; Martina Proell, Motti Gerlic, John Reed, Peter Mace and Stefan J. Riedl analysed data; Martina Proell, John Reed and Stefan Riedl wrote the paper.

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SUPPLEMENTARY ONLINE DATA

The CARD plays a critical role in ASC foci formation and inflammasome signalling

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Figure S1 Model of caspase 1 CARD and structure of ASC CARD: mutational analysis

(A and B) Shown is a model of caspase 1 CARD based on the structure of Iceberg [1] (PDB code 1DGN) and the structure of ASC CARD [2] (PDB code 2KN6). Residues targeted for mutational analysis are shown in stick representation. Residues that resulted in a loss of foci formation in the BiFC assay are highlighted in yellow, whereas residues that showed no effect are depicted in grey (helices are annotated). (C) BiFC results of HEK-293T cells transfected with either WT or mutant VN–caspase 1 CARD together with WT VC–ASC CARD. The caspase 1 mutations E8R, R33E, and Q67R showed no effect on binding or foci formation.

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Figure S2  *Salmonella* dose response

RAW cells stably expressing either WT ASC, ASC mutants E130R, D134R, E144R, or empty vector control were infected with different doses of *Salmonella* (MOI (multiplicity of infection) of 20 and 2 respectively). Supernatants were analysed for secreted IL-1β using ELISA (means ± S.D.; n = 3).

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