The adaptor ASC has extracellular and ‘prionoid’ activities that propagate inflammation

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Microbes or danger signals trigger inflammasome sensors, which induce polymerization of the adaptor ASC and the assembly of ASC specks. ASC specks recruit and activate caspase-1, which induces maturation of the cytokine interleukin 1β (IL-1β) and pyroptotic cell death. Here we found that after pyroptosis, ASC specks accumulated in the extracellular space, where they promoted further maturation of IL-1β. In addition, phagocytosis of ASC specks by macrophages induced lysosomal damage and nucleation of soluble ASC, as well as activation of IL-1β in recipient cells. ASC specks appeared in bodily fluids from inflamed tissues, and autoantibodies to ASC specks developed in patients and mice with autoimmune pathologies. Together these findings reveal extracellular functions of ASC specks and a previously unknown form of cell-to-cell communication.

The activation of most signaling receptors of the innate immune system results in the induction of gene-transcription programs that lead to the production and release of inflammatory mediators via the secretory pathway1. In contrast, the inflammasomes operate on a post-translational level. Inflammasomes trigger the proteolytic maturation of biologically inactive precursors of the interleukin 1β (IL-1β) family of cytokines and mediate the release of these active cytokines from the cytosol2. After detecting microbial substances or danger signals, the inflammasome sensor molecules, such as AIM2 or NLRP3, induce rapid polymerization of the bipartite adaptor ASC (‘apoptosis-associated speck-like protein containing a caspase-recruitment domain’; also known as PYCARD) into large helical filaments by facilitating self-interactions of the pyrin domains of ASC. The caspase-recruitment domain of ASC then assembles to nucleate the polymerization and filament formation of pro-caspase-1, which leads to its self-activation3–6. Active caspase-1 in turn induces maturation of cytokines of the IL-1 family and also mediates their release into the extracellular space4,7. By forming large filamentous signaling platforms within the cytosol of cells, ASC can provide a scaffold for the optimal recruitment and activation of caspase-1 and thereby trigger a decisive on-off signaling response8. The inflammasome sensor NLR4C, which recognizes flagellin and components of type III secretion systems, lacks a pyrin domain and can activate caspase-1 via direct self-interactions of caspase-recruitment domains. Nevertheless, ASC still forms specks upon activation of NLR4C and enhances the activation of caspase-1 and IL-1β9,10. Active caspase-1 also regulates the noncanonical release of many other proteins and triggers the inflammatory form of cell death called ‘pyroptosis’11,12. The activation of inflammasomes results in the maturation of highly proinflammatory cytokines and the death of the activated cell. Hence, such activation is thought to occur only if a potentially harmful inflammatory response is warranted, such as after tissue damage or persistent cellular invasion by bacteria or viruses. The release of IL-1 cytokines leads to the rapid recruitment of neutrophils and monocytes to the site of danger, which is important for limiting the spread of infection and for the initiation of repair after tissue damage13. Since the activation of inflammasomes results in pyroptotic cell death and thereby the release of cytoplasmic contents, it is conceivable that ASC specks could ultimately be found in the extracellular environment13.

Here we found that ASC specks were released from cells in which inflammasomes were activated and accumulated in the extracellular space. Extracellular ASC specks retained their ability to mature pro-IL-1β even after the demise of the activated cell. Extracellular ASC specks were ingested by macrophages, which resulted in lysosomal damage and IL-1β production in these cells. In mice, injection of ASC specks caused acute inflammatory reactions. Moreover, we detected extracellular ASC specks after experimental activation of inflammasomes in vivo and in bronchoalveolar lavage fluid (BALF) from...
mice and patients with inflammatory airway pathologies. Finally, autoantibodies to ASC specks developed in a small fraction of patients and mice with autoimmune pathologies, and these autoantibodies were able to increase the uptake of ASC specks by macrophages and boost IL-1β activation. Together our results identify extracellular ASC specks as an endogenous danger signal that can perpetuate inflammatory responses.

RESULTS

Accumulation of extracellular ASC specks after pyroptosis

To visualize the activation of inflammasomes, we generated mouse macrophage reporter cells stably expressing ASC fused to a fluorescent protein (ASC-FP)\(^{14}\). ASC-FP was evenly distributed in resting cells (Fig. 1a and Supplementary Movie 1), consistent with published reports of its soluble cytosolic distribution\(^3\)\(^4\). After activation of inflammasomes, ASC-FP redistributed to form a paranuclear protein speck in activated cells (Fig. 1a and Supplementary Movie 1), consistent with the ability of ASC to rapidly oligomerize after activation of inflammasomes\(^3\)\(^6\). Immunofluorescence staining with antibody to ASC (anti-ASC) revealed that endogenous ASC also formed specks after activation of inflammasomes in human peripheral blood mononuclear cells and human THP-1 monocytes (Supplementary Fig. 1a). We next activated the NLR4 inflammasome \textit{in vivo} by injecting \textit{Pseudomonas aeruginosa} (\textit{P. aeruginosa}) into the footpads of mice. \textit{Ex vivo} staining of permeabilized sections from popliteal lymph nodes of these mice revealed the appearance of ASC specks in subcapsular macrophages (Fig. 1b). These data indicated that the formation of ASC specks was a physiological cellular response to the activation of inflammasomes. Notably, confocal microscopy of ASC-FP-expressing mouse reporter macrophages and ASC-FP-expressing THP-1 human reporter monocytes also revealed the presence of ASC specks in the extracellular space following the activation of inflammasomes (Fig. 1c,d and Supplementary Movies 2 and 3). ASC specks are protein aggregates of considerable size (~1 \(\mu\text{m}\) in diameter) and therefore their appearance in cell-free supernatants can be readily quantified by flow cytometry. Using this technique, we observed a time-dependent accumulation of ASC specks in supernatants of ASC-FP-expressing THP-1 reporter cells after activation of inflammasomes (Fig. 1e). To confirm the accumulation of ASC specks in cell-free supernatants after activation of inflammasomes, we did a biochemical analysis of ASC aggregation. We were able to separate ASC specks from nuclei and other dead cell debris in supernatants of activated cells by a combination of centrifugation and filtration with 5-\(\mu\text{m}\) filters that retain nuclei but allow the smaller ASC specks to pass\(^5\) (Supplementary Fig. 1b). By this method, we generated cell-free supernatants of resting cells or cells in which inflammasomes were activated, then chemically crosslinked ASC with disuccinimidyl suberate (DSS) in these supernatants prior to assessing its aggregation by immunoblot analysis for ASC. This method confirmed the presence of not only monomeric species of ASC (~22 kilodaltons in size), but also dimers, trimers and higher order oligomers (Fig. 1f). To investigate whether the activation of other inflammasomes can also trigger the accumulation of ASC specks in cell-free supernatants, we treated cells with poly(dA:dT) or anthrax lethal toxin to activate the AIM2 inflammasome or NLRP1 inflammasome, respectively. ASC specks accumulated in cell-free supernatants of cells in which the NLRP1, NLRP3 and AIM2 inflammasomes were activated (Fig. 1g,h). These data showed that activation of all the inflammasomes we tested led to the accumulation of ASC specks in the extracellular space.

We next assessed whether the accumulation of extracellular ASC specks is a result of inflammasome-mediated cell death. Indeed, the appearance of extracellular ASC specks in cell-free supernatants correlated with the release of lactate dehydrogenase from activated cells (Fig. 1l). Consistent with the induction of cell death after activation of inflammasomes and the proposal that destabilization of the plasma membrane is a key feature of pyroptotic cell death\(^11\), ASC-FP-expressing reporter macrophages failed to exclude propidium iodide shortly after the formation of ASC specks triggered by the activation of inflammasomes (Fig. 1j and Supplementary Movie 4). Thus, ASC specks were probably not actively released but instead remained in the extracellular space after cellular demise. However, since pyroptosis also induces membrane disintegration, it is conceivable that extracellular fluid might also have access to ASC specks shortly after pyroptotic cell death. To test this hypothesis, we added antibody to green fluorescent protein (anti-GFP) directly conjugated to Alexa Fluor 555 to the supernatants and activated NLRP3 in ASC-FP-expressing reporter cells. Live-cell time-lapse microscopy showed that fluorescent antibody-gated positively stained ASC-FP-specks shortly after their appearance in activated cells (Fig. 1k and Supplementary Movie 5). Hence, these data confirmed that the formation of ASC specks preceded cell death and that extracellular fluid promptly gained access to ASC specks within remnants of pyroptotic cells.

ASC specks remain active in the extracellular space

Since the accumulation of ASC specks in cell-free supernatants of cells in which inflammasomes were activated correlated with cell death, we next investigated whether caspase-1 is required for this process. By flow cytometry, we found that the accumulation of ASC specks in cell-free supernatants obtained from cells after activation of inflammasomes was much lower in caspase-1-deficient (\textit{Casp1}\(^{-/-}\)\textit{ASC-FP-expressing macrophages than in wild-type ASC-FP-expressing cells (Fig. 2a). Similarly, we did not detect the accumulation of ASC specks in supernatants of wild-type ASC-FP-expressing macrophages in which inflammasomes were activated and caspase-1 activity was inhibited with \textit{z-YVAD-fmk} (Fig. 2b). We used DSS to crosslink ASC in filtered cell-free supernatants and in lysates of resting macrophages or in macrophages in which inflammasomes were activated. We then assessed ASC by immunoblot analysis and confirmed that caspase-1 activity was dispensable for the formation of ASC specks in cells, yet it was required for the accumulation of ASC specks in cell-free supernatants (Fig. 2c).

To assess the formation of ASC specks from endogenous sources and to monitor the extracellular accumulation of ASC specks after activation of inflammasomes, we stimulated wild-type, ASC-deficient \textit{(Pycard}\(^{-/-}\}; called ‘\textit{Asc}-/-’ here), NLRP3-deficient (\textit{Nlrp3}\(^{-/-}\)) or \textit{Casp1}\(^{-/-}\)\textit{macrophages with lipopolysaccharide (LPS) and ATP. We then isolated endogenous ASC specks from either cell-free supernatants or cell pellets of the activated cells and probed for ASC and NLRP3 by immunoblot analysis. We found that endogenous ASC specks assembled in wild-type and in \textit{Casp1}\(^{-/-}\) cells but not in \textit{Nlrp3}\(^{-/-}\) cells (Supplementary Fig. 2a, consistent with the fact that NLRP3 signaling is upstream of ASC. However, we detected the accumulation of ASC specks in cell-free supernatants of wild-type macrophages but not in \textit{Casp1}\(^{-/-}\) macrophages in which inflammasomes were activated (Supplementary Fig. 2a). Together these findings confirmed that caspase-1-mediated cell death was required for the accumulation of extracellular ASC specks formed from endogenous ASC.

We hypothesized that one function of extracellular ASC specks might be to promote further processing of caspase-1 and IL-1β in the extracellular space. Indeed, it is known that pro-caspase-1 and pro-IL-1β can accumulate in supernatants of macrophages in which inflammasomes are activated\(^15\)\(^,\)\(^16\) and thus it appeared plausible that...
ASC specks might further promote the maturation of caspase-1 and IL-1β. Fluorescent ASC specks spontaneously assembled upon heating of cytosolic extracts to 37°C, which allowed us to prepare recombinant ASC specks from macrophages (Supplementary Fig. 2b). Immunoblot analysis and flow cytometry revealed that these recombinant ASC specks contained NLRP3, ASC and caspase-1 (Supplementary Fig. 2c,d). Furthermore, confocal microscopy of antibody-stained endogenous proteins in activated THP-1 monocytes confirmed that both extracellular ASC specks and intracellular ASC specks contained both NLRP3 and ASC (Supplementary Fig. 2e). NLRP3 was present in the core of the endogenously formed ASC specks (Supplementary Fig. 2e,f), similar to published findings obtained with inflammasomes reconstituted in vitro with purified proteins. Of note, microscopy of cells in which inflammasomes were activated revealed that only a portion of the NLRP3 was associated with ASC specks, although all cellular ASC was recruited to the speck (Supplementary Fig. 2f). To determine whether ASC specks can activate pro-IL-1β or pro-caspase-1 in cell-free systems, we next isolated cytosols from LPS-primed Asc−/−...
macrophages and incubated them at 37 °C in presence or absence of recombinant ASC specks purified from ASC-FP-expressing cells. This analysis showed that processing of pro-caspase-1 and pro-IL-1β into their mature forms occurred only in Asc−/− cytosols incubated with ASC specks (Fig. 2d). Of note, we did not detect processing of IL-1β or caspase-1 in the cytosol of LPS-primed, ATP-activated Asc−/− macrophages (Fig. 2d).

To assess whether ASC specks are also able to process mature IL-1β from its precursor form when it is released from cells, we next generated cell-free supernatants from resting macrophages or macrophages in which inflammasomes were activated. To additionally assess the role of caspase-1 present on the speck in this process, we isolated ASC specks from wild-type or Casp1−/− macrophages and incubated them at 37 °C with cell-free supernatants of macrophages in which inflammasomes were activated and incubated the mixture (protocol, Supplementary Fig. 3b). We subsequently pelleted the Casp1−/− ASC specks by centrifugation, washed them and assessed caspase-1 in the pellets. Immunoblot analysis with anti-GFP served as a loading control, since ASC specks were able to trigger lysosomal damage. To directly assess whether the uptake of exogenous ASC specks induces the activation of inflammasomes in phagocytes, we incubated macrophages expressing

Extracellular specks are a danger signal

Phagocytosis of extracellular peptide aggregates, such as amyloid-β and islet amyloid polypeptide, can activate the NLRP3 inflammasome by inducing lysosomal damage. Since ASC specks are large protein aggregates, this raised the possibility that they could also be sensed as a danger signal by phagocytic cells of the immune system. Indeed, we observed that macrophages ingested ASC specks, which remained stable in phagolysosomal compartments of cells over several hours before being degraded (Fig. 3a,b). To investigate whether the ingestion of ASC specks results in lysosomal damage, similar to what is observed with crystals or peptide aggregates, we next assessed phagolysosomal stability by incubating mouse macrophages with fluorescent dextran, alone or together with fluorescent ASC specks or the lysosome-damaging peptide LeuLeu-OMe (L-Leucyl-L-leucine methyl ester). Incubation of macrophages with ASC specks or LeuLeu-OMe caused lysosomal swelling and leakage of fluorescent dextran into the cytosol (Fig. 3c,d), which suggested that ASC specks were able to trigger lysosomal damage.
ASC tagged with the red fluorescent protein mCherry (ASC–mCherry) together with ASC specks containing ASC tagged with cyan fluorescent protein (ASC–mCerulean) so that we could simultaneously track both the added aggregated ASC specks and the soluble ASC inside macrophages. We observed that uptake of ASC–mCerulean specks by ASC–mCerulean–expressing macrophages resulted in the formation of mCerulɪn speck aggregates within these cells (Fig. 3e). Consistent with that observation, ASC specks added to bone marrow–derived macrophages (BMDMs) induced IL-1β in a dose- and time-dependent manner, but we did not find such structures in such material obtained from ASC–mCherry–expressing macrophages or a mock preparation of ASC–mCherry–expressing macrophages (Mock; 200 µg/ml) or cholesterol crystals (Chol; 250 µg/ml). (g) ELISA of IL-1β in supernatants of BMDMs primed as in f and then left untreated (--) or treated for 1–12 h (horizontal axis) with ASC specks or a mock preparation as in f (key). Data are representative of three independent experiments (a–c) or are pooled from two independent experiments with at least five microscopic fields per condition (d; mean and s.d.) or eight fields (e; mean and s.d.), four independent experiments (f; mean and s.e.m.) or two independent experiments (g; mean and s.d.).

ASC specks have ‘prionoid’ activities

While analyzing the uptake of purified ASC–mCerulean specks added to recipient ASC–mCerulean–expressing reporter macrophages by confocal microscopy (Fig. 3e), we noticed that ASC–mCerulɪn was recruited to a fraction of the ingested ASC–mCerulean specks (Fig. 4a). We noted that after longer incubation periods, ASC–mCerulean specks had recruited all the soluble cytosolic ASC of the recipient ASC–mCerulɪn–expressing reporter macrophages (Fig. 4b). These data suggested that in addition to inducing the release of IL-1β from macrophages, extracellular ASC specks might also aggregate cytosolic soluble ASC from recipient cells after their escape from phagosomes. A similar ‘seeding’ activity is commonly observed with prion or ‘prionoid’ (prion-like) proteins, which are able to aggregate their cytosolic soluble counterparts once they escape endocytic vesicles and gain access to the host cell cytosol26–28. We next investigated whether ASC specks in cells or purified from activated cells display prion-like features, similar to what has been shown with other purified ASC proteins5,6. Imaging of fluorescent ASC specks by confocal scanning microscopy and super-resolution stimulated emission depletion microscopy revealed the presence of long fibrillar structures (Fig. 4c,d). We next generated recombinant ASC specks from wild-type cells or, as a control, subjected Asc−/− cells to the same protocol. Electron microscopy of ASC speck material isolated from wild-type cells revealed the presence of fibrillar structures, but we did not find such structures in such material obtained from Asc−/− cells (Fig. 4e,f). Furthermore, ASC speck preparations obtained from ASC–FP–expressing reporter cells and stained with gold–labeled anti–GFP identified the structures as ASC specks (Fig. 4g). Cryo–electron microscopy further confirmed the presence of fibrillar structures in ASC speck preparations isolated from macrophages (Supplementary Fig. 4a).

Most proteins that form prions have the ability to promote their own polymerization in a process called ‘seeding’, in which preformed fibers provide templates for fiber elongation. This process potentially drives prion infectivity and the development of associated diseases26,29,30. To assess the propensity of purified ASC specks isolated from cells to act as a ‘seed’ for the further aggregation of soluble ASC in more detail, we added ASC–mCerulean specks to soluble cytosolic extracts of ASC–mCerulɪn–expressing cells and examined the recruitment of the soluble ASC–mCerulɪn to ASC–mCerulean specks over time. This analysis revealed that ASC specks recruited soluble ASC within seconds (Supplementary Fig. 4b and Supplementary Movie 6), which led to the formation of an outer layer of ASC–mCerulɪn around a core of ASC–mCerulean (Supplementary Fig. 4c). Moreover, we were also able to monitor the rapid recruitment of soluble ASC–mCerulɪn to ASC–mCerulean specks by flow cytometry (Supplementary Fig. 4d). Of note, ASC was specifically required for nucleation of its soluble counterpart, since soluble protein of the crystallizable fragment Fc tagged with mCherry was not aggregated by incubation with ASC–mCerulean specks (Supplementary Fig. 4e). Hence, these data suggested that ASC specks isolated from cells acted as a ‘seed’ to further aggregate soluble ASC in a cell-free system or within cytosols after...
ASC specks escaped from the phagosome. These features are shared with other prionoid proteins, and these data suggested that ASC specks were able to ‘infect’ bystander cells and propagate the nucleation of soluble ASC.

ASC specks induce inflammatory responses in vivo

Activation of the NLRP3 inflammasome in vivo results in the recruitment of neutrophils and inflammatory monocytes by a mechanism largely dependent on the receptor for IL-1 (IL-1R)\(^{20,21}\). To assess the ASC speck–induced inflammatory response in vivo, we injected fluorescent ASC specks or, as a control, fluorescent beads into the ears of left untreated (None) or primed with LPS and activated with poly(dA:dT); below, z-stacks of images above (different field). Scale bars, 4.9 \(\mu\)m. (d) Stimulated emission-depletion microscopy of ASC specks in ASC-mCerulean–expressing immortalized macrophages incubated overnight (a) or for 36 h (b) with ASC-mCerulean specks assembled in vitro; arrowheads indicate ASC-mCerulean specks that recruited ASC-mCherry (open) or not (filled); top and bottom rows indicate independent experiments with the same conditions, and columns present split channels. Right (b), 10× magnification of area outlined at left. Scale bars, 2.6 \(\mu\)m. (c) Confocal microscopy of ASC-mCerulean–expressing macrophages left untreated (None) or primed with LPS and activated with poly(dA:dT); below, z-stacks of images above (different field). Scale bars, 4.9 \(\mu\)m. (e) Electron microscopy of ASC specks prepared from a wild-type immortalized macrophage or a mock preparation of an Asc\(^{−/−}\) immortalized macrophage.

Scale bars, 100 nm. (f) Electron microscopy of ASC-mCerulean specks assembled in vitro; right, magnification of areas outlined at left. Scale bars, 1 \(\mu\)m (left) or 0.5 \(\mu\)m (right).

(g) Electron microscopy of ASC-mCerulean specks isolated from LPS-primed, nigericin-activated immortalized macrophages stained with anti-GFP directly conjugated to gold nanoparticles (arrows) 10 nm in diameter. Data are representative of at least two independent experiments.

ASC specks into wild-type mice promoted the development of a sterile peritonitis characterized by the recruitment of neutrophils and inflammatory monocytes (Fig. 5c). The recruitment of these inflammatory cells after the injection of ASC specks was partially impaired in Nlpr3\(^{−/−}\) mice and was entirely blocked in IL-1R-deficient (Il1r1\(^{−/−}\)) mice (Fig. 5d), which indicated that ASC specks elicited the maturation of IL-1\(β\) in vivo. Furthermore, these data suggested that ASC specks were able to induce activation of caspase-1 in an NLRP3-independent manner, probably due to the propensity of ASC specks to directly recruit ASC and activate caspase-1 in cells that ingested ASC specks. Consistent with NLRP3-independent activation of caspase-1 in vivo, the in vitro IL-1\(β\) response to ASC specks was not significantly lower in Nlpr3\(^{−/−}\) BMDMs than in wild-type cells, but it was dependent on ASC and caspase-1 (Fig. 5f). Thus, ASC specks caused inflammatory responses in vivo and in vitro, and the activation of IL-1\(β\) was not fully dependent on the activation of inflammasomes by NLRP3.

Extracellular specks accumulate in inflammatory disease

We next investigated whether the activation of inflammasomes in vivo led to the appearance of extracellular ASC specks. Immunostaining of fixed and permeabilized lymph node sections revealed that ASC specks were present in most subcapsular macrophages after infection with P. aeruginosa (Fig. 1b). To investigate whether such infection would also induce the appearance of extracellular ASC specks in vivo, we modified the detection protocol and first infected mice by injection of P. aeruginosa into the footpad, followed by injection of anti-ASC, 4 h later, via the same route. This procedure could stain ASC specks appearing in the extracellular space (in the absence of detergents). We then collected the draining popliteal lymph nodes and visualized...
extracellular ASC specks in driving inflammation in vivo. (a) Confocal microscopy of whole-mount ear skin from lysozyme-GFP reporter mice 4 h after subdermal injection of ASC-mCerulean specks (5 µg in 10 µl of PBS; bottom row) or 10 µl of phycocerythrin-conjugated Calibrate beads (top row); right, 2.5x (top) or 5x (bottom) magnification of area outlined at left. Scale bars, 100 µm. (b) Confocal microscopy of whole-mount ear skin from wild-type mice 4 h after subdermal injection of ASC-mCerulean specks, probed with anti-Ly6G to stain neutrophils and with anti-CD31 to stain endothelial and stromal cells (ECs); right and left (bottom row) represent independent experiments with similar conditions; outlined areas indicate deposition of ASC specks. (c) Gating strategy (top) and quantification (right) of neutrophils (CD11b+Gr-1+F4/80−) and inflammatory monocytes (CD11b+Ly6C+Ly6G+F4/80−) obtained by peritoneal lavage of wild-type mice 16 h after intraperitoneal injection of PBS, silica crystals (250 µg) or ASC specks (200 µg). Each symbol (right) represents an individual mouse (n = 9 per group); small horizontal lines indicate the mean (± s.e.m.). Bottom left, microscopy of cells from outlined areas in plots above. Original magnification, x100. *P = 0.0018 (unpaired two-tailed Student’s t-test), and **P = 0.0023, ***P = 0.0021 and ****P = 0.0006 (Mann-Whitney test). (d) Quantification (as in c) of cells obtained by peritoneal lavage of wild-type mice given injection of PBS (n = 4) or wild-type mice (n = 15), Nlrp3−/− mice (n = 8) or Il1r−/− mice (n = 4) given injection of ASC specks (200 µg). NS, not significant; *P = 0.0081 and **P = 0.0040 (Mann-Whitney test). (e) ELISA of IL-1β in supernatants of LPS-primed wild type, Nlrp3−/−, Asc−/− or Casp1−/− inflammatory macrophages left untreated (−) or stimulated with ASC specks (100 µg/ml) assembled in vitro. *P < 0.05 (Mann-Whitney test). Data are representative of two independent experiments (a,b) or are pooled from three independent experiments (c,e); mean and s.e.m. or two independent experiments (d); mean and s.d.)

Our data showing a role for extracellular ASC specks in driving inflammatory responses opened the possibility that ASC specks might be able to accumulate in inflamed tissues. Chronic obstructive pulmonary disease (COPD) is an inflammatory airway disease in which activation of inflammasomes is thought to have a role.33,34 COPD develops because of chronic inflammatory responses to noxious particles or gases in lung tissues. To test the hypothesis that ASC specks accumulate during chronic lung inflammation, we made use of a mouse model of smoke-induced COPD, in which mice develop hallmark features of the human disease.35 We exposed mice to air or cigarette smoke 5 d a week for a total of 8 weeks, after which we collected BALF. We were able to quantify ASC specks by flow cytometry after staining them with two different monoclonal antibodies to ASC, each labeled with a different fluorescent dye (Fig. 6b). By this method, we observed that mice that inhaled cigarette smoke for 8 weeks had significantly more extracellular ASC specks in their BALF than did mice exposed to air for the same period (Fig. 6c). These data indicated that ASC specks may be part of a chronic inflammatory response to smoke-induced damage to cells and tissues. We next attempted to identify ASC specks in BALF from healthy human subjects or patients with COPD, pneumonia or pulmonary hypertension. Untreated BALF samples from patients contained a large number of cells (Fig. 6d). Therefore, we removed cells by centrifugation at 400g and further filtered the supernatants through 5-µm filters, similar to the protocol established to separate ASC specks from cellular debris in cell culture supernatants (Supplementary Fig. 1b). This procedure yielded cell-free BALF, yet the smaller material was still available for further analysis in the filtrate (Fig. 6d). To investigate whether extracellular ASC specks found in BALF obtained from humans was an active state (i.e., containing ASC monomers, dimers and oligomers), we pelleted the filtrates by centrifugation and crosslinked the ASC in the filtrate with DSS (Fig. 6c). This analysis revealed that BALF
from patients with COPD and pneumonia contained extracellular preassembled ASC specks, while BALF from patients with pulmonary hypertension or from healthy donors did not (Fig. 6e). As a positive control, we used cell-free supernatants of THP-1 cells in which the NLRP3 inflammasome was activated (Fig. 6e). Together these data showed that ASC specks were present in the extracellular space in lungs from patients with inflammatory pulmonary disease and suggested that these speck could be part of the chronic inflammatory response observed in such patients.

**Anti-ASC opsonizes ASC specks and increases inflammation**

We next sought to investigate whether extracellular ASC specks modify the inflammatory response after activation of inflammasomes in vivo. We injected anti-ASC or purified immunoglobulin G (IgG) into mice intravenously and subsequently induced peritonitis by intraperitoneal injection of silica crystals. The systemically available anti-ASC should bind to the extracellular ASC specks that appear after in vivo activation of inflammasomes and thereby alter the biological response. Indeed, mice treated with anti-ASC before activation of inflammasomes by intraperitoneal injection of silica crystals had much more recruitment of neutrophils and inflammatory monocytes to the peritoneum than did mice treated with the IgG control antibody (Fig. 7a). These data confirmed that ASC specks were released after activation of inflamasomes in vivo and became accessible to anti-ASC. The enhanced inflammatory response in vivo to silica crystals in the presence of anti-ASC indicated that anti-ASC might opsonize ASC specks and thereby induce enhanced inflammation. To assess this, we preincubated fluorescent ASC specks with anti-ASC or anti-GFP and compared their uptake by macrophages. We found that antibody-opsonized ASC specks were taken up more efficiently by macrophages than were ASC specks that had not been opsonized by antibodies (Fig. 7b–d). Consistent with those results, opsonization of ASC specks by antibodies enhanced the release of IL-1β from macrophages (Fig. 7e). Together these data suggested that anti-ASC contributed to the inflammatory responses by opsonizing ASC specks.

Several autoantibodies have been shown to have inflammatory properties, as they can increase the cellular uptake of inflammatory material (such as DNA or RNA) as immunocomplexes. In fact, this inflammatory nature is believed to be part of the pathogenesis of autoantibody-mediated inflammation36. Therefore, it is conceivable that patients with autoimmune diseases have autoantibodies to extracellular ASC. To test this hypothesis, we obtained serum from patients with autoimmune disease and high titers of antinuclear antibody (ANA)37 and screened the serum by flow cytometry to assess the presence of antibodies that react to ASC specks. Indeed, among the ANA+ patients tested, 18% had antibodies that reacted to ASC specks (Supplementary Fig. 6a). We then used an enzyme-linked immunosorbent assay (ELISA) to confirm that the serum was positive for anti-ASC specks (Supplementary Fig. 6a). Next, we assessed whether antibodies to ASC specks would also develop in mice with experimental lupus37. Similar to results obtained for serum from patients with autoimmune disease, we also detected autoantibodies reactive to ASC specks in serum from mice with experimental lupus (Supplementary Fig. 6b). These data suggested that ASC specks might be opsonized by autoantibodies and that this might contribute to the inflammatory...
response. To test this hypothesis, we incubated ASC specks with serum from autoimmune mice before adding them to LPS-primed BMDMs and found that they boosted IL-1β responses in these recipient cells to an extent similar to that achieved by purified commercially available anti-ASC (Fig. 7g). We did not observe this effect when we incubated ASC specks with serum from healthy control mice (Fig. 7g). Together these data suggested that antibodies to ASC were present in inflammatory diseases and might potentially exacerbate the effects of extracellular ASC specks.

**DISCUSSION**

The activation of inflammasomes induces the rapid polymerization of ASC and caspase-1 into a fibrillar signaling platform. Similar processes can be found in other signaling pathways, and it is thought that these functional protein fibrils favor ‘digital’ on-off responses that lead to the cleavage and release of caspase-1 substrates. Since the activation of caspase-1 also results in cell death, each activated cell can respond only once. Thus, the inflammatory output of the individual cell in which inflammasomes are activated would thus depend solely on the available cellular pool of caspase-1 substrates. Such a one-time response would greatly limit the possible inflammatory output of cells in which inflammasomes are activated. Our study has revealed previously unappreciated extracellular activities of ASC specks that persisted even after the demise of the activated cell. The ability of ASC specks to mature caspase-1 and its substrates in the extracellular space might contribute to a ‘depot effect’ of inflammasomes and lead to prolonged inflammatory activity. Furthermore, we found that ASC specks were recognized as a danger signal akin to other aggregated substances; this cell-to-cell transfer of aggregated ASC leading to new cellular signaling represents an additional cell-to-cell communication mechanism.

The seeding activity and fibrillar structure of ASC specks is reminiscent of that of prion proteins, which propagate from cell to cell in an aggregated protein state. Prion proteins can induce the conversion of correctly folded proteins to prions that accumulate and eventually cause diseases. Some prion proteins misfold into a characteristic amyloid fold. However, our extensive attempts to identify amyloid folds in ASC specks were negative, which suggests that ASC specks are not true amyloid proteins but instead have prion-like activities. Another feature of prions is their resistance to proteases.

**Figure 7** Antibodies to ASC specks develop in autoimmune diseases. (a) Quantification of peritoneal neutrophils and inflammatory monocytes in peritoneal lavage fluid from C57BL/6 mice given iv injection of purified rabbit IgG (100 µg) or polyclonal antibody to ASC (100 µg) and, 2 h later, given ip injection of PBS or silica crystals (250 µg). Each symbol represents an individual mouse (IgG + PBS, n = 3; anti-ASC + PBS, n = 3; IgG + silica, n = 4; anti-ASC + silica, n = 7); small horizontal lines indicate the mean (± s.d.). *P = 0.0061 (Mann-Whitney test) and **P = 0.0028 (unpaired two-tailed Student’s t-test). (b) Fluorescence microscopy of BMDMs incubated with untreated (UT) ASC-mCerulean specks (top) or ASC-mCerulean specks coated with monoclonal antibody to GFP (1 µg) (middle) or polyclonal antibody to ASC (1 µg) (bottom); right, 5x magnification of area outlined at left. Scale bars, 90 µm. (c) Frequency of macrophages (treated as in b) containing ASC-mCerulean ASC specks ingested by phagocytosis. (d) Quantification of mCerulean-positive (ASC-speck+) lysosomes in cells treated as in b. Each symbol represents an individual cell; small horizontal lines indicate the mean (± s.d.). (e) ELISA of IL-1β in supernatants of LPS-primed BMDMs left untreated or stimulated with ASC specks precoated with anti-ASC (1 µg) and assembled in vitro. (f) Flow cytometry analyzing the distribution of recombinant ASC-mCerulean specks (top), the fluorescence of ASC-mCerulean specks (middle) or polyclonal antibody to ASC (1 µg) (bottom); right, 5x magnification of area outlined at left. Scale bars, 500 µm. (g) ELISA of IL-1β in cell-free supernatants of LPS-primed wild-type BMDMs left untreated (–) or incubated for 6 h (UT) or with ASC specks alone (UT) or with ASC specks coated with polyclonal anti-ASC (Anti-ASC) or serum from mice with pristane-induced SLE (Autoimmune; n = 4) or control mice (n = 4 per group). Each symbol represents a technical replicate. Data are from one experiment representative of two independent experiments (a,c,d,f,g); mean and s.d. of three fields per condition (c) or 20 cells per condition (d); mean and s.d. in (g) or one experiment representative of three independent experiments (b) or are pooled from three independent experiments (e; mean and s.e.m. of triplicates).
Indeed, we observed that ASC specks remained visible for prolonged periods of time after injection into tissues. The deposition of ASC specks in tissues induced the recruitment of neutrophils. After their recruitment to the endangered tissues, neutrophils can amplify tissue inflammation as they degranulate and release various inflammatory mediators, enzymes and danger signals. Of note, neutrophils can carry ASC and caspase-1 in secretory vesicles and tertiary granule compartments, which are released upon activation. Since ASC specks can recruit soluble ASC and activate caspase-1, neutrophil-derived ASC or caspase-1 may thereby further contribute to the inflammatory milieu.

It is conceivable that an extracellular function of inflammasomes has evolved to enhance and to focus the inflammatory tissue reaction to the site of injury. On the other hand, the failure to appropriately clear ASC specks in inflamed tissues could lead to the accumulation of ASC specks and the subsequent perpetuation of immune responses. In agreement with this hypothesis, the presence of ASC and other inflammasome components in cerebrospinal fluid correlates with poor outcomes in patients with brain injury. Typically, large doses of aggregated or crystalline substances are required for activation of the NLRP3 inflammasome. We thus hypothesize that the NLRP3-activation threshold, combined with the ability of macrophages to degragate subthreshold amounts of ASC specks, would limit the spread of inflammation and lead to a concentration-dependent effect. Additionally, the activity of caspase-1 is sensitive to oxidation, which could block the enzymatic function of extracellular specks. Consistent with the proposal of an activation threshold by which extracellular ASC specks are recognized by cells, antibody-coated ASC specks were ingested more efficiently than were untreated ASC specks. The coating of ASC specks with anti-ASC resulted in more production of IL-1β by recipient cells in vitro and an enhanced inflammatory response to the activation of inflammasomes in vivo.

Our findings of autoantibodies to ASC specks in a large percentage of patients with autoimmune disease warrants further studies of larger populations. Additionally, whether autoantibodies to ASC specks are associated with inflammatory pathologies remains to be investigated. The finding that treatment with anti-ASC altered the in vivo inflammatory response to crystals suggests that extracellular ASC specks appear after the activation of inflammasomes and opens the possibility that therapies directed against ASC specks could modulate inflammation. So far, only inflammasome effector molecules, such as IL-1β and IL-18, or their receptors have been targeted for pharmaceutical intervention. Indeed, this idea is supported by studies of models of traumatic brain injury and spinal cord injury showing that anti-ASC therapy is able to reduce the activation of caspase-1 and IL-1β and result in reduced tissue damage. However, as shown by our in vivo studies with anti-ASC, different antibodies can have contrasting effects, which warrants careful design and selection of the antibodies to ASC for future therapeutic use. For example, modern antibody-engineering techniques allow the production of antibodies that do not interact with Fc receptors. In addition, antibodies that interfere with the ASC speck-mediated maturation of extracellular caspase-1 or IL-1β could be generated, and hence different activities of ASC specks could be targeted therapeutically.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

B.S.F., L.B. and W.K. designed and did experiments and analyzed data; J.M.R., A.S., G.E., M.S.M., B.G.M., P.B. and S.H. did experiments; C.B., M.N., A.A.-A., S.H. and A.A. did and analyzed data from electron microscopy and cryo–electron microscopy; D.D.N., T.E., P.B., A.M.-R., A.A. and W.K. analyzed data and provided critical suggestions and discussions throughout the study; S.Z. provided BALF samples from patients with COPD; L.B., M.E. and R.E.S. provided serum samples from patients with autoimmune disease; B.J., A.G.J. and P.M.H. provided the mouse model of COPD; B.S.F. and E.L. designed the study; and B.S.F., D.D.N. and E.L. wrote the paper.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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Animal model of COPD. The animal model of COPD has been described. Wild-type BALB/c mice were exposed to cigarette smoke (twice per day and five times per week for 8 weeks) via a custom-designed and purpose-built nose-only directed-flow inhalation and smoke-exposure system (CH Technologies) housed in a fume and laminar flow hood. Each exposure lasted 75 min. At the end of the treatment, BALF samples were harvested from mice. ASC specks in cell-free supernatants or BALF were quantified on a MACSquant analyzer (Miltenyi Biotec) after subtraction of the number of A488 A647 events in BALF stained with anti-ASC (2E1-7-488 and TMS-1-A647) from the number of A488 A647 events in BALF stained with IgG1 isotype-matched control antibody (02-6100-A488 and A647). All antibodies were labeled with the Zenon kit (noted above). Debris-sized events were gated on the basis of the forward- and side-scatter distribution of beads 0.7–0.9 μm in diameter (Spherotech) and 6 μm in diameter (BD Biosciences). Data were analyzed by FlowJo X 10.0.7 software.

Cells. Inflammflammatory macrophages stably transduced with constructs for the expression of mCerulean- or mCherry-tagged ASC have been described. BMDMs were obtained by culture of bone marrow cells from 6- to 8-week-old C57BL/6 mice in DMEM supplemented with 10% FBS, 10 μg/ml Ciprofay-500 and 40 ng/ml M-CSF (R&D Systems). Six days later, BMDMs were collected and plated. Immortalized BMDMs were cultured in DMEM supplemented with 10% FBS and 10 μg/ml Ciprofay-500. Human peripheral blood mononuclear cells were purified from whole blood over Ficoll density gradients (GE Healthcare); erythrocytes were lysed in red cell lysis buffer (Miltenyi Biotec) and were seeded in RPMI medium supplemented with 10% FBS and 10 μg/ml Ciprofay-500. The monocytic cell line THP-1 was cultured in RPMI-1640 medium supplemented with 10% FBS and 10 μg/ml Ciprofay-500. For stimulation assays, THP-1 cells were treated overnight with 100 nM phorbole 12-myristate 13-acetate, then were primed for 2 h with 1 μg/ml of LPS and were subsequently activated with 10 μM of nigericin as indicated in the figures. For the stimulation of mouse macrophages, cells were primed for 3 h with 200–250 ng/ml of LPS and were activated with 5 mM ATP or 10 μM nigericin.

Production and purification of ASC specks. ASC specks were assembled in vitro by incubation of cytosolic extracts for 45 min at 37 °C (Supplementary Fig. 2c) or were purified from LPS-primed cells activated with ATP or nigericin, as described. The ASC specks were further passed through a ‘cushion’ of 50% Percol in CHAPS buffer (20 mM HEPES-KOH, pH 7.5, 5 mM MgCl2, 0.5 mM EGTA, 0.1 mM PMSF and 0.1% CHAPS) for purification as described (Supplementary Fig. 2d). For imaging by electron microscopy, fluorescent ASC specks were further sorted by flow cytometry before analysis. For investigation of the oligomerization of ASC, ASC specks were washed with 0.5 ml CHAPS buffer and were chemically crosslinked for 30 min at room temperature with 2 mM DSS (Pierce) prior to immunoblot analysis.

Immunoblot analysis. Proteins were separated by 4–12% SDS-PAGE in precast gels (Novex, Invitrogen) with MES or MOPS buffer (Novex, Invitrogen). Proteins were then transferred onto PVDF membranes (Millipore) and nonspecific binding was blocked with 3% BSA in Tris-buffered saline containing 0.1% Tween-20, followed by overnight incubation with specific primary antibodies (identifed above). Membranes were then washed and were incubated with the appropriate secondary antibodies (coupled to IRDye 800CW or IRDye 680RD; 1:15,000 dilution; LI-COR Biosciences), and immunoreactivity was observed by near-infrared detection, followed by analysis with an Odyssey CLx imaging system (LI-COR Biosciences).

Confocal microscopy. A Leica TCS SP5 SMD confocal system (Leica Microsystems) was used for confocal laser-scanning microscopy. For live imaging, the temperature was maintained at 37 °C with 5% CO2 through the use of an environmental control chamber (Life Imaging Services and Solent Scientific). Images were acquired at various time points with a 63x objective, followed by analysis with the Leica Application Suite Advanced Fluorescence imaging platform, version 2.2.1 (Leica Microsystems), or Velocity 6.01 software. A Leica TCS STED microscope was used for stimulated emission-depletion imaging. ASC specks purified from activated macrophages

ONLINE METHODS

Reagents. Ultrapure LPS (from Escherichia coli strain 0111:B4), was from Invivogen, nigericin was from Invitrogen, and ATP was from Sigma-Aldrich. Silica crystals were from US Silica. Antibodies to ASC were as follows: monoclonal anti-ASC (1:500 dilution; 63902; TMS-1; BioLegend), monoclonal anti-ASC (1:1,000 dilution; 04-147; 2E1-7; Millipore), polyclonal rabbit anti-ASC (gift from A. Marshak) polyclonal anti-ASC (1:1,000 dilution; AL-258; 2E1; Sigma-Aldrich) and polyclonal anti-ASC (1:200 dilution; sc-22514-R; N-153; Santa Cruz). Mouse monoclonal antibody to NLRP3 (1:1,000 dilution; Cryo-2; AG-20B-0014-C100) and to caspase-1 p20 (1:1,000 dilution; casper-1; AG-20B-0042-C100) were from Alpha Diagnostic. Polyclonal antibody to caspase-1 were from R&D Systems. The ELISA kit for mouse IL-1β (ZS-B11; Invitrogen) was used for direct labeling of 1 μg IgG1 monoclonal antibody to ASC (identified above) or same amount of a purified IgG1 isotype-matched control antibody (02-6100; Invitrogen). Purified rabbit IgG (100 μg; 02-6102; Invitrogen) was used as a control for injection into mice for comparison with rabbit polyclonal antibody to ASC (AL177; Adipogen). Anti-GFP (1:2,000 dilution; D11122) was from Invitrogen. ATTO 647N-conjugated goat anti-rabbit (1:500 dilution; 40839) was from Sigma. The following combinations of antibodies to mouse tissues were used for the identification of neutrophils and inflammatory monocytes by flow cytometry: phycoerythrin–anti-CD11b-PE (M1/70; eBioscience), allophycocyanin–anti-Ly-6G (anti-Gr-1; RB6-8C5; eBioscience) and Alexa Fluor 450–anti-Ly6C (HL1; eBioscience) and the isotype-matched control antibodies phycoerythrin–conjugated rat IgG2b (eB149/10H5; eBioscience), allophycocyanin–conjugated rat IgG2b (eB149/10H5; eBioscience) and Alexa Fluor 488–conjugated rat IgG2a (eBR2a; eBioscience). Dead cells that stained positively for 7-aminocoumarin D (559925; BD Pharmingen) were gated out and excluded from the analysis. Alexa Fluor 647–conjugated antibody to mouse Ly6G (1A8; BioLegend) was used for the identification of neutrophils, anti-F4/80 (BM8; eBioscience) was used for the identification of macrophages, and anti-CD31 (MEC 13.3; BD Biosciences) and anti–collagen IV (ab19808; abcam) were used for the identification of endothelial-stromal cells, all by confocal microscopy. Phycoerythrin-labeled Calibrite beads (349502; BD Biosciences), and polystyrene particles 0.7–0.9 μm in diameter (PP-08-10; Spherotech) were used as a reference for flow cytometry. Phycoerythrin-labeled beads were also used as control for experiments involving injection into ear skin whole mounts.

Patients. This study was in compliance with the Declaration of Helsinki (1989) of the World Medical Association. Bronchoalveolar lavage (BAL) specimens were obtained from patients undergoing bronchoscopy and BAL for routine medical diagnostics. Written informed consent was obtained from each subject before enrollment. Samples were obtained from a total of twelve Caucasian subjects in this study, with COPD (n = 4), pneumonia (n = 4) or pulmonary hypertension (n = 2) and healthy donors (n = 2). Subjects were recruited from the Department of Internal Medicine of the University of Bonn, Germany. COPD was diagnosed in accordance with the current guidelines of the American Thoracic Society, European Respiratory Society and World Association of Sarcoidosis.

Serum samples from a total of 80 patients with autoimmune disease and an antinuclear antibody tested on HEp2 cells (a derivate of HeLa human cervical carcinoma cells), with a serum dilution of 1:2,560 or greater, were obtained from the Department of Immunology and Rheumatology of Hannover Medical School, Germany. Written informed consent was obtained from all patients. Serum was screened for reactivity to purified ASC specks on the basis of flow cytometry (Fig. 2f). Serum samples from a total of seven healthy donors served as controls.

Mice. Mice were housed in pathogen-free conditions and were handled in accordance with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health and the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School. Asc−/− and Nlrp3−/− mice have been described. C57BL/6j mice (6–8 weeks of age; female) were from Jackson Laboratories. Nlrp3−/− mice were from Millenium Pharmaceuticals.
expressing ASC-mCerulean were stained with polyclonal anti-GFP (1:1,000 dilution) followed by secondary antibody to ATTO 647N (1:500 dilution).

Analysis of crystal- or ASC speck–induced lysosomal damage. Lysosomal stability was assessed as described21,22. Cells were seeded in eight-well glass-bottomed dishes and were allowed to adhere overnight. Cells were then treated for 4 h with 40 µg/ml of Alexa Fluor 647–coupled dextran (10 kDa; Invitrogen) in the presence of inflammasome activators or fluorescent ASC specks. Cells were washed twice with PBS, were fixed with 4% formaldehyde and were imaged by confocal microscopy as described above.

Bacterial infection. Mice were given injection in the footprint of 30 µl of PBS containing 1 × 10^7 colony-forming units of P. aeruginosa46. Four hours after injection, the draining popliteal lymph nodes were collected, then were fixed for 12 h with PLP buffer (0.05 M phosphate buffer containing 0.1 M l-lysine, pH 7.4, 2 mg/ml NaIO 4 and 10 mg/ml parafomaldehyde) and then were dehydrated in 30% sucrose before being embedded in Tissue-Tek OCT Compound freezing medium (Sakura Finetek). Sections 30 µm in thickness were cut on a CM3050S cryostat (Leica), then the sections were made to adhere to Superfrost Plus slides (VWR) prior to being stained (antibodies identified above). Slides were then mounted with Fluornount G (Southern Biotech) and were imaged on an LSM 710 confocal microscope (Carl Zeiss Microimaging). For the detection of extracellular ASC specks in vivo, mice were given injection of PBS or P. aeruginosa as described above, then were given another injection 4 h later of 1 µg rabbit polyclonal anti-ASC (gift from A. Marashk). 30 min after the antibody injection, lymph nodes were harvested and processed as described above, except that only secondary staining was done (with Alexa Fluor–conjugated chicken anti-rabbit; 1:500 dilution; A21442; Invitrogen) to reveal the presence of extracellular ASC specks.

Recruitment of neutrophils in ear skin whole mounts. Mice were given subcutaneous injection in the ear of 10 µl of phycoerythrin-labeled Calibrate beads (BD Biosciences) or 5 µg of fluorescent ASC specks in 10 µl PBS. Four hours later, mice were killed and their ears were excised and divided into dorsal and ventral halves, which were fixed in 1% paraformaldehyde (Electron Microscopy Sciences) and stained with antibodies (identified above) diluted in washing buffer consisting of 1× PBS, 1% BSA and 0.025% (vol/vol) Triton X-100 (Sigma-Aldrich). For identification of neutrophils, ventral ear whole mounts were stained with Alexa Fluor 647–anti-Ly6G; macrophages were stained with Alexa Fluor 488–anti-F4/80, and endothelial-stromal cells were stained with anti-CD31 (all antibodies identified above). An LSM 710 confocal microscope equipped with a 20× Plan Apochromat objective with a numerical aperture of 0.8 (Carl Zeiss Microimaging) was used for confocal microscopy of 1-µm optical slices of skin whole mounts.

Assay of the activity of ASC specks. Immortalized Asc−/− macrophages were primed with LPS as indicated (Fig. 2d) for the induction NLRP3 and pro-IL-1β. Cells were then lysed in CHAPS buffer (described above) by being passed 25 times through a syringe fitted with a 20-gauge needle. Lysates were centrifuged at 19,000g for 8 min to obtain crude lysates before ultracentrifugation at 100,000g for 30 min at 4 °C to obtain cytosolic samples. The cytosolic samples were incubated for 1 h at 37 °C with ASC specks assembled in vitro or a corresponding volume of PBS. For analysis of the activity of ASC specks in supernatants of activated wild-type BMDMs, BMDMs were primed for 3 h with LPS (500 ng/ml) and were then activated for 40 min with ATP (2.5 mM) or nigericin (5 µM). Cell-free supernatants of those BMDMs (10 µl) were then fractionated by SDS-PAGE and analyzed by immunoblot for caspase-1 (AL177; Adipogen) or anti-IL-1β (R&D Systems).

Assessment of the recruitment of pro-caspase-1 to ASC specks. ASC-mCerulean specks (50 µg) generated from wild-type or Casp1−/− immortalized macrophages expressing ASC-mCerulean were incubated for 4 h with cell-free supernatants (180 µl) generated as described above or with serum-free DMEM. The ASC specks were pelleted for 8 min at 2,500g and were washed twice with PBS, then were fractionated by SDS-PAGE and analyzed by immunoblot for caspase-1 and GFP (antibodies identified above).

Flow cytometry. For assessment of the recruitment of inflammatory cells to the peritoneal cavity, 6- to 8-week-old C57BL/6 female mice were given intraperitoneal injection of PBS alone or 200 µg of silica crystals or 20–200 µg of ASC specks in 200 µl PBS. After 16 h, peritoneal lavage cells were harvested and were preincubated with 10 µl of the monoclonal antibody 2.4G2 (for blockade of the receptor FcγRIIB/III; Miltenyi Biotech) before being stained with fluorescence-conjugated antibody to CD11b, Ly-6G, Ly6C or F4/80 or fluorescence-conjugated isotype-matched control antibodies (all identified above). Dead cells were excluded on the basis of their staining with 7-amino-actinomycin D. The absolute number of neutrophils (CD11b+Ly-6G+Ly6C−) and inflammatory monocytes (CD11b+Ly6C−Ly6G+F4/80+) was determined by flow cytometry as described21. Data were analyzed with FlowJo X 10.0.7 software.

Transmission electron microscopy of cytosolic lysates of wild-type or Asc−/− immortalized macrophages. ASC specks assembled in vitro from cytosolic contents of wild-type immortalized macrophages (10 µl) were placed on Formvar carbon-coated copper grids. After removal of excess liquid, grids were washed three times with water before being stained for 1 min with 2% aqueous uranyl acetate. Samples were analyzed on a Philips CM12 electron microscope (transmission electron microscope with an acceleration voltage of 80 keV).

Transmission electron microscopy of purified ASC specks. Purified ASC specks from supernatants of ATP-activated ASC-mCerulean–expressing immortalized macrophages (5 µl) were placed on Formvar carbon-coated copper grids. After removal of excess liquid, grids were washed twice with water before being stained for 20 s with 2% aqueous uranyl acetate. Samples were analyzed on a JEM-2200FS transmission electron microscope (JEOL) operated at an acceleration voltage of 200 keV and equipped with a Titan TemCam-F416 (TVIPS). Magnification ranged from ×8,000 to ×20,000, corresponding to the pixel size at a ‘specimen level’ (pixel resolution) of 1.47–0.58 nm.

Cryo–transmission electron microscopy of purified ASC specks. ASC specks assembled in vitro from ASC-mCerulean–expressing immortalized macrophages and sorted by flow cytometry (5 µl) were applied to glow-discharge unit (Pelco easiGlow; Ted Pella) with Quantifoil grids (with a hole diameter of 3.5 µm and spacing between holes of 1 µm). Samples were frozen in liquid ethane through the use of an automated plunge-freezing machine (Vitrobot Mark IV; FEI). Samples were stored in liquid nitrogen until being imaged. Plunge-frozen samples of ASC specks were imaged under ‘low-dose’ conditions (diminished electron-beam illumination) in a cryo–transmission electron microscope (Titan Krios; FEI) operated at an accelerating voltage of 300 kV. Images were recorded on a Falcon II direct electron detector (FEI). Magnification ranged from ×6,500 to ×14,000 corresponding to pixel size at a specimen level (pixel resolution) of 1–0.5 nm.

Statistics. Prism 6.0 software (GraphPad Software) was used for statistical analysis. Unpaired two-tailed Student’s t-tests were used after data fulfilled the criteria of normal distribution and equal variance; otherwise, Mann–Whitney U-tests were used. A P value of < 0.05 was considered significant. No specific method of randomization was used for the generation of samples or in animal experiments. Animal models were assessed by researchers not ‘blinded’ to mouse identity, and mice were arbitrarily assigned identification numbers during experimentation.

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