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Apoptosis-Associated Speck-like Protein Containing a CARD Forms Specks but Does Not Activate Caspase-1 in the Absence of NLRP3 during Macrophage Swelling

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Apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) is a key adaptor molecule required for the inflammatory processes. ASC acts by bridging NLRP proteins, such as NLRP3, with procaspase-1 within the inflammasome complex, which subsequently results in the activation of caspase-1 and the secretion of IL-1β and IL-18. In response to bacterial infection, ASC also forms specks by self-oligomerization to activate caspase-1 and induce pyroptosis. Hitherto, the role of these specks in NLRP3 inflammasome activation in response to danger signals, such as a hypotonic environment, largely has been unexplored. In this article, we report that, under hypotonic conditions and independently of NLRP3, ASC was able to form specks that did not activate caspase-1. These specks were not associated with pyroptosis and were controlled by transient receptor potential vanilloid 2 channel–mediated signaling. However, interaction with NLRP3 enhanced ASC speck formation, leading to fully functional inflammasomes and caspase-1 activation. This study reveals that the ASC speck can present different oligomerization assemblies and represents an essential step in the activation of functional NLRP3 inflammasomes. The Journal of Immunology, 2015, 194: 1261–1273.

Inflammation is a response against infection that is initiated by the activation of pattern recognition receptors (PRRs) on macrophages. PRRs are triggered by both pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), resulting in the production of proinflammatory cytokines (1). Signaling through PRRs of the TLR family induces synthesis of the inactive forms of IL-1β and IL-18, two key proinflammatory cytokines that depend upon the activity of caspase-1 for their maturation and secretion.

The adaptor molecule apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (CARD) (ASC or Pycard) is essential for caspase-1 activation in response to diverse disease- and pathogen-associated signals (2). ASC is a cytosolic protein that contains a CARD and an N-terminal pyrin domain (PYD). ASC can control the activation of caspase-1 by two mechanisms: as a molecular adaptor between PYD and CARD-containing signaling molecules within the inflammasome, a cytosolic PRR multiprotein complex, or via the oligomerization of ASC monomers into a cytosolic speck called the pyroptosome (2, 3). The pyroptosome is responsible for a specific type of cell death called pyroptosis, a mechanism mediated by caspase-1 activation through which macrophages infected with intracellular pathogens die rapidly, facilitating pathogen clearance (4).

According to the current model for inflammasome activation, conformational changes in cytosolic PRRs of the nucleotide-binding and leucine-rich repeat receptor (NLR) family lead to an interaction with caspase-1 (2). ASC is suggested to be necessary in bridging NLRP3 or AIM2 with procaspase-1 to form an inflammasome. In this situation, ASC has been found to self-assemble into fiber-like structures from oligomers of NLRP3 or AIM2, culminating with the production of large protein aggregates that amplify the activation of caspase-1 (5, 6). Other NLRs, such as NLRC4 and NLRP1, can interact directly with caspase-1 through their CARDs. However, recent studies revealed that ASC is also necessary for caspase-1 activation within NLRC4 and NLRP1 complexes (7–10). In these situations, ASC assembles into...
a ring-like structure surrounding NLRC4 oligomers (11). These data suggest that ASC is essential for inflammasome activation in general and that it could present different oligomerization assemblies.

The NLRP3 inflammasome is activated by diverse DAMPs and PAMPs. Its activation by DAMPs is central for sterile inflammatory responses and for amplification of infection-induced inflammation (2). Endogenous DAMPs activating NLRP3 can be classified into two main categories: (1) soluble activators, such as extracellular ATP acting through P2X7 receptors, or a decrease in extracellular osmolarity sensed by transient receptor potential (TRP) channels; or (2) particulate crystalline factors, such as monosodium urate (MSU) or cholesterol crystals (12–15). We reported recently that the NLRP3 inflammasome adopts an inactive preassembled conformation that changes into an active form following variation in the concentration of intracellular potassium ions (15). ASC is required for NLRP3-induced caspase-1 activation in response to extracellular hypotonicity and compacts preassembled NLRP3 complexes, accelerating their conformational change upon activation (15).

ASC represents an important target for the negative regulation of the inflammasome by endogenous proteins. Inflammasome dominant-negative regulators function by binding to ASC through the PYD or CARD, inhibiting ASC-dependent processes (16). ASC may also be a target for drugs blocking IL-1β release, such as cytokine release inhibitory drug 3, which targets ASC oligomerization during NLRP3 inflammasome activation (17, 18). Furthermore, ASC plays a key role in the development of different sterile inflammatory responses involving the NLRP3 inflammasome, including gout, type 2 diabetes, atherosclerosis, metabolic syndrome, chronic obstructive pulmonary disease, spinal cord injury, and Alzheimer’s disease (12, 13, 19–23).

Although ASC is accepted to be critical for inflammasome activation, the different oligomerization states of ASC during inflammasome activation and its real-time assembly kinetics with NLRP3 have not been addressed. In this study, we assessed ASC oligomerization and speck formation in response to hypotonicity and other NLRP3 activators, as well as the relationship between ASC speck formation and the activation of caspase-1. We discovered that ASC oligomerization detected by chemical cross-linking and ASC speck formation detected by immunocytochemistry represent different states of ASC assembly driven by NLRP3. In response to low osmolarity or extracellular ATP stimulation, ASC formed specks, as detected by immunofluorescence, independently of NLRP3 and without activation of caspase-1. These specks were not observed when NLRP3-deficient cells were activated by nigericin, MSU crystals, or Escherichia coli. However, ASC oligomerization in response to hypotonicity or ATP (as detected by protein cross-linking and caspase-1 activation) required NLRP3 that localized inside the speck. Our data also revealed that ASC speck formation was controlled through TRP vanilloid 2 (TRPV2) and TGF-β–activated kinase 1 (TAK1) following a decrease in extracellular osmolarity.

Materials and Methods

Cells and reagents

E. coli LPS 055:B5, ATP, and nigericin were from Sigma-Aldrich; human rILN-γ was from PeproTech; 5Z-7-oxozeaenol was from Tocris; MSU crystals were from InvivoGen; caspase-1 inhibitor Ac-YV AD-AOM and fluorescent caspase-1 substrate z-YVAD-AOM–were from Merck-Millipore; and mouse monoclonal anti-V5 and DAPI, as well as Phalloidin–Texas Red, were from Invitrogen. Abs for ELISAs were from R&D Systems; caspase-1 p10 rabbit polyclonal, IL-1β, and ASC were from Santa Cruz Biotechnology; mouse monoclonal anti-NLRP3 and mouse monoclonal anti-caspase-1 p20 were from Adipogen; anti-GFP was from Calbiochem; and mouse monoclonal anti-flag was from Sigma-Aldrich. All HRP-conjugated secondary Abs were from DAKO Cytomation, and fluorescent-conjugated secondary Abs were from Jackson Immuno.

All animal experiments were carried out under the Animals (Scientific Procedures) Act 1986. Peritoneal and bone marrow macrophages were obtained from male C57BL/6J mice or from Nlrp3−/− or double Casp-1–/–Casp-11−/− mice, as previously described (14, 24, 25). THP-1 and HEK293 cells were maintained in RPMI 1640 and DMEM:F12 (1:1) media, respectively, supplemented with 10% FCS, 2 mM Glutamax, and 1% pen/ streptomycin (Gibco). Lipopolysaccharide (2 μg/ml) (Invitrogen) was used for transfecting HEK293 cells per the manufacturer’s instructions. Biochemical/immunocytochemistry experiments were performed 48 h after transfection. Differentiation and priming of THP-1 cells were performed by overnight incubation in media supplemented with LPS and IFN-γ (100 ng/ml each), and mouse macrophages were primed with LPS (100 ng/ml) for 4 h. Cells were stimulated for different lengths of time with an isotonic solution (300 milliosmole [mOsm]) consisting of NaCl (147 mM), HEPES (10 mM), glucose (13 mM), CaCl2 (2 mM), MgCl2 (1 mM), and KCl (2 mM); hypotonic solution (90 mOsm) was achieved by diluting the solution 1:4 with distilled sterile water. For TRPV2 gene silencing, THP-1 cells were infected with recombinant lentivirus vectors expressing GFP, random nontargeting short hairpin RNA (shRNA), or TRPV2 shRNA; experiments were performed 7 d postinfection with GFP+ sorted cells (15).

Immunocytochemistry and microscopy

Macrophages stimulated on coverslips were washed twice with PBS, fixed with 4% formaldehyde, 4% sucrose in PBS for 5 min at room temperature, and then washed three times with PBS. Cells were blocked with 0.5% BSA and permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature before incubating with rabbit anti-ASC (1:500), mouse anti-caspase-1 (1:200), or mouse anti-NLRP3 (1:1000) for 1 h at 37°C. Cells were washed and incubated with the appropriate fluorescence-conjugated secondary Ab (1:200) for 2 h at room temperature, rinsed in PBS, and incubated for 5 min with 300 nM DAPI. To stain for active caspase-1, macrophages were activated with hypotonic solution for 45 min at 37°C, washed, and incubated with the fluorochrome inhibitor of caspase-1, green fluorescent peptide 5-carboxyfluorescein-Tyr-Val-Ala-Asp-fluoromethyl ketone (FAM-YVAD-fmk [FLICA]; Immunocytochemistry Technologies), according to the manufacturer’s recommendations. Cells were fixed with 4% formaldehyde, blocked, permeabilized, and stained for ASC or NLRP3, as described above. All coverslips were mounted on slides with ProLong Gold Antifade Reagent (Invitrogen). Images were acquired with a Delta Vision RT (Applied Precision) restoration microscope using a CoolSNAP HQ (Photometrics) camera, with a 60×/1.42 Plan Apo or 100×/1.40 U PLAN Apo objective, a Z optical spacing of 0.2 μm, and the 488/560, 546/650, and 555/671-nm filter set (Chroma 86000v2) or with an Axiovert 25 microscope equipped with 488 and 100× objectives and an AxioCam HRC camera (both from Zeiss). Where mentioned in the text, raw images were deconvoluted using Softworx software, and maximum-intensity projections of these deconvoluted images are shown in the results.

ASC oligomerization

Visualization of ASC oligomerization was performed as previously described (3). Briefly, cells were lysed in buffer A (20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 320 mM sucrose) by passing the lysate through a 21-gauge needle 30 times. The cell lysate was centrifuged, and the supernatant was filtered using a 3-μm filter. The supernatant was diluted with one volume of CHAPS buffer (20 mM HEPES-KOH [pH 7.5], 5 mM MgCl2, 0.5 mM EGTA, 0.1 mM PMSF, 0.1% CHAPS) and then centrifuged to pellet ASC oligomeric complex structures. ASC oligomers were cross-linked using 2 mM NHS-Diazirine (Pierce) for 30 min at 4°C in CHAPS buffer. After quenching with 20 mM Tris (pH 8.8), UV irradiation (using a 365-nm UV bulb) was performed for 10 min at 4°C before adding sample buffer.

Commmunoprecipitation, Western blotting, ELISA, caspase-1 activity, and K+ determination

Detailed methods used for Western blot analysis, caspase-1 activity, and ELISAs were described previously (26, 27). For communoprecipitation (colP), equal amounts of protein (500–750 μg) were incubated with 2 μl (1.5 μg) anti-GFP (Calbiochem) for 1 h at 4°C on a rotating wheel. After adding Protein Gagarose beads (20 μg/sample) for 1 h at 4°C, the beads were washed three times (5 min at 4°C) in lysis buffer. Proteins were eluted by heating samples (5 min at 80°C) in the presence of reducing agent and loading sample buffer (Invitrogen). Proteins were separated on 4–12% gradient Bis-Tris gels (Invitrogen) and transferred to a nitrocellulose
membrane (Millipore). Intracellular K⁺ was quantified from THP-1 cell lysates by indirect potentiometry on a Cobas 6000 with ISE module (Roche).

**Flow cytometry for extracellular ASC speck determination**

For the detection of extracellular particles of ASC, 0.5 ml cell-free culture supernatants of stimulated macrophages was incubated for 1 h with 1 μg rabbit polyclonal anti-ASC (Adipogen). Particles were centrifuged and washed before incubation with the secondary Ab, Alexa Fluor 647–conjugated goat anti-rabbit IgG (Life Technologies). Samples were washed before being analyzed by flow cytometry, with a FACSCanto and FACSDiva software (both from BD Biosciences), by gating for small particles or cell debris based on forward scatter versus side scatter, adjusted to display correct separation of leukocyte populations. Gated events are presented on plots showing the percentage of positive particles relative to all small gated particles.

**Bioluminescence resonance energy transfer measurements**

Transfected HEK293 cells were plated on a polyl-lysine-coated 96-well plate; after adhesion, cells were washed with PBS with calcium and magnesium, and readings were performed immediately after the addition of 5 μM coelenterazine-H substrate (Invitrogen) in isotonic or hypotonic solution. Readings also were performed during incubation in a buffer containing 120 mM glycerol with 140 mM NaCl or 140 mM KCl or following stimulation of the cells with nigericin. Signals were detected with M coelenterazine-H substrate (Invitrogen) in isotonic or hypotonic solution. Readings also were performed during incubation in a buffer containing 120 mM glycerol with 140 mM NaCl or 140 mM KCl or following stimulation of the cells with nigericin. Signals were detected with two filter settings (Renilla-lucerase (Luc) filter [485 ± 20 nm] and YFP filter [530 ± 25 nm]) at 37°C using the Mithras LB940 plate reader (Berthold Biotechnologies). The bioluminescence resonance energy transfer (BRET) ratio was defined as the difference between the emission at 530 nm/485 nm of cotransfected R-Luc and YFP fusion proteins and the emission at 530 nm/485 nm of the R-Luc fusion protein alone. Results are expressed in miliBRET units. For saturation curves, a constant amount of R-Luc–tagged protein was transfected with increasing quantities of YFP–tagged proteins. The amounts of YFP– and R-Luc–tagged proteins were determined by reading on separate plates at 530 nm after excitation at 485 nm and reading at 485 nm in the presence of coelenterazine-H, determined by reading on separate plates at 530 nm after excitation at 485 nm and reading at 485 nm in the presence of coelenterazine-H, respectively. BRET signals were determined as described above. BRET experiments were performed using the ARPEGE Pharmacology Screening-Interactome platform facility at the Institute of Functional Genomics (Montpellier, France).

**Quantitative RT-PCR analysis**

Detailed methods used for quantitative RT-PCR were described previously (28). Specific primers were purchased from QIAGEN (QuantiTect Primer Assays); for each primer set the efficiency was >95%, and a single product was seen on melt-curve analysis. Relative expression levels were calculated using the 2−ΔΔCt method normalizing to GAPDH expression levels for each treatment, and the fold increase in expression was relative to the smallest expression level.

**Lactate dehydrogenase–release measurements**

The presence of lactate dehydrogenase (LDH) in the medium was measured using the Cytotoxicity Detection kit (Roche), following the manufacturer’s instructions. It was expressed as the percentage of the total amount of LDH in the cells.

**Statistical analysis**

Data are presented as the mean ± SEM from the number of assays indicated (from at least three separate experiments). Data were analyzed, using Prism (GraphPad) software, by an unpaired two-tailed Student t test to determine the difference between two groups or by one-way ANOVA with the Bonferroni multiple-comparison test to determine the differences among more than two groups.

**Results**

**ASC oligomerizes in response to cell swelling**

We recently described that hypotonicity activates the NLRP3 inflammasome (15). The aim of this study was to investigate the involvement of ASC in this process. Biochemical cross-linking revealed that hypotonic solution induced the oligomerization of ASC, mainly into dimers, but also into higher-order complexes (Fig. 1A). As expected, the NLRP3 activator nigericin also induced oligomerization of ASC (Fig. 1A). ASC oligomerization is known to result in pyroptosis, a specific type of cell death driven by ASC-dependent activation of caspase-1 (3, 10). Kinetics of LDH release into macrophage supernatants after a 1-h exposure to hypotonic solution revealed that the release of LDH was negligible compared with unstimulated macrophages (Fig. 1B). However, LDH release increased up to 20% after 3 h in hypotonic solution (Fig. 1B). In parallel, we measured the release of IL-1β as an indicator of caspase-1 activation, because inhibition of caspase-1 abrogated the release of IL-1β (Fig. 1B). After 20 min in hypotonic solution, ~40% of the releasable IL-1β was detected in the supernatants of macrophages. The maximum release of IL-1β under these conditions was achieved after 40 min of stimulation with hypotonic solution (Fig. 1B). Pyroptosis is also characterized by cell swelling (3, 10), and we found that macrophage cell swelling induced by hypotonicity was followed by a regulatory volume decrease (RVD), when cells returned to their initial size (Supplemental Fig. 1A). We reported recently that RVD is an essential process for NLRP3 inflammasome activation (15). The absence of ASC or irreversible inhibition of caspase-1 did not influence cell swelling or LDH kinetics of macrophages (Supplemental Fig. 1A). These results suggest that ASC oligomerization and IL-1β release are not coupled with cell death at early time points of stimulation by hypotonic solution.

To study macrophage viability at longer times after ASC speck formation, macrophages were incubated for 1 h in isotonic or hypotonic solution and then exposed to a normal isotonic cell culture media (Supplemental Fig. 1B). After 16 h, macrophages pretreated with hypotonic solution demonstrated no apparent cell death, as measured by the level of LDH in the supernatant (Fig. 1C), although they still contained ASC oligomers (Fig. 1D). Prolonged gene expressions for IL-1β, IL-12p40, IL-6, and cyclooxygenase-2 induced by LPS also were similar between hypotonic- or isotonic-pretreated macrophages (Fig. 1E), suggesting that LPS priming was not affected by the formation of ASC specks.

**ASC oligomerization requires TRPV2 signaling and NLRP3**

We then studied the effect of cell swelling and RVD modulators on ASC oligomerization. K⁺ efflux during cell swelling is important for RVD and for IL-1β processing and release (Supplemental Fig. 2A–C). This is in agreement with previous work showing that intracellular K⁺ depletion is an important step for NLRP3 inflammasome activation (15, 29). Prevention of K⁺ efflux and RVD, using a glycerol-KCl solution, resulted in a reduction in ASC oligomerization (Fig. 2A). The importance of RVD for ASC oligomerization also was confirmed by using 5-Nitro-2-(3-phenylpropylamino)benzoic acid, a blocker of swelling activated Cl⁻ channel, which impaired RVD (Supplemental Fig. 2D), caspase-1 activation, and ASC oligomerization (Fig. 2B). Similarly, glyburide, a NLRP3 inhibitor (30), was able to prevent RVD (Supplemental Fig. 2D) and ASC oligomerization (Fig. 2B).

Activation of the NLRP3 inflammasome during hypotonic stimulation involved TRP-channel signaling, whereas TRPM7 controls RVD, and TRPV2 is activated during RVD (15). ASC oligomerization induced by hypotonic solutions was abolished by the broad-spectrum TRP channel inhibitors La³⁺ and 2-Aminoethyldiphenyl borate (Fig. 2B). Furthermore, effective TRPV2 gene silencing in THP1 macrophages (Supplemental Fig. 3A, 3B) resulted in a reduction in both ASC oligomerization and IL-1β release caused by hypotonic solution (Fig. 2C). THP1 infection with control lentivirus carrying a scramble shRNA sequence did not impair ASC oligomerization or IL-1β release induced by hypotonicity (Supplemental Fig. 3C). High concentrations of...
Mg^{2+} inhibit TRPM7 (31), consistent with our discovery that decreasing the Mg^{2+} concentration enhanced ASC oligomerization (Fig. 2D).

During RVD in macrophages, TRP channels modulate an increase in intracellular Ca^{2+} that activates TAK1 kinase, which ultimately signals to the NLRP3 inflammasome (15). When intracellular Ca^{2+} was chelated by 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis-acetoxymethyl ester (BAPTA-AM) or when TAK1 activation was inhibited, hypotonic stimulation failed to induce ASC oligomerization and maturation of IL-1β (Fig. 2E). NLRP3 was found to be necessary for ASC oligomerization, because ASC appears monomeric in macrophages deficient in NLRP3 that were exposed to hypotonic solution (Fig. 2F). Altogether, these results suggest that ASC oligomerization could be an important step in signaling for NLRP3 inflammasome formation and that the presence of NLRP3 is ultimately required for ASC oligomerization.

**ASC forms specks during hypotonic stimulation**

Under resting conditions, endogenous ASC was homogeneously distributed throughout the cytosol of macrophages (Fig. 3A, Supplemental Fig. 3D). After 40 min in a hypotonic solution, ASC completely relocated and formed specks (Fig. 3A, Supplemental Fig. 3D). ASC specks were present in 23.2 ± 3.3% of THP-1 cells and in 27.8 ± 4.2% of mouse primary bone marrow–derived macrophages (BMDMs) exposed to hypotonic solution. Almost all (87.5 ± 7.9%) THP-1 cells containing ASC specks also contained active caspase-1 (Fig. 3B). These results suggest that ASC specks are important for the activation of caspase-1 following stimulation with hypotonic solution.
TRPV2 translocates to the plasma membrane during RVD and induces cell permeabilization (15). Macrophage plasma membrane permeabilization was associated with ASC speck formation, because we found that 79.6 ± 2.5% of cells with ASC specks were positive for Lucifer yellow (Fig. 4A). Consistently, TRPV2 gene silencing in THP1 cells resulted in a reduction in ASC speck formation induced by hypotonicity (Fig. 4B). Finally, ASC specks caused by hypotonic stimulation also were reduced when either intracellular Ca\(^{2+}\) was chelated by BAPTA-AM or when TAK1 was inhibited by 5Z-7-oxozeaenol (Fig. 4C). Unexpectedly, NLRP3 deficiency did not affect ASC speck formation after hypotonic stimulation (Fig. 4D), suggesting that NLRP3 is key for ASC oligomerization and caspase-1 activation but not for ASC speck formation.

We next investigated whether other NLRP3 activators could induce ASC speck formation in the absence of NLRP3. As expected, in macrophages from wild-type mice, stimulation with extracellular ATP, nigericin, uric acid crystals, or *E. coli* caused oligomerization of ASC, as detected by the appearance of higher m.w. complexes of ASC after chemical cross-linking (Fig. 5A). Similar to hypotonicity, all of these NLRP3 activators failed to induce ASC oligomerization and IL-1\(\beta\) maturation in the absence of NLRP3 (Fig. 5A). However, NLRP3 deficiency did not affect ASC speck formation after ATP stimulation, but it completely prevented it after nigericin, uric acid crystal, or *E. coli* challenge (Fig. 5B, 5C). These data suggest that NLRP3 activation in response to different stimuli could depend upon different dynamics of ASC oligomerization.

A kinetic analysis of ASC oligomerization induced by hypotonicity in wild-type macrophages showed that ASC cross-linking, IL-1\(\beta\) release, and caspase-1 activation occurred after a 10-min stimulation (Fig. 6A–C). However, although strong ASC oligomerization depended upon NLRP3, Western blots from purified cross-linked ASC oligomer proteins (ASC Oligomer), total cell extracts (Cells), or cell supernatants (Sups) of THP-1 cells primed with LPS and IFN-\(\gamma\) (100 ng/ml each, 16 h). (A) ASC Western blots from cells incubated for different times (0, 20, or 40 min) with two hypotonic solutions: 120 mM glycerol with 140 mM of NaCl (Na\(^+\) Gly) or 140 mM of KCl (K\(^+\) Gly). (B) ASC and caspase-1 Western blots from THP-1 cells activated as in (A) and treated as indicated with La\(^3+\) (La, 2 mM), 2-Aminoethoxydiphenylborate (2-APB; 100 \(\mu\)M), 5-Nitro-2-(3-phenylpropy lamino)benzoic acid (NPPB; 100 \(\mu\)M), or glyburide (100 \(\mu\)M). (C) ASC and IL-1\(\beta\) Western blots from uninfected THP-1 macrophages (control cells) or cells infected with GFP and non-sense shRNA-lentivirus (LV-GFP) or lentivirus coding for GFP and TRPV2 shRNA (LV-shRNA) and subsequently stimulated as in (A). (D) ASC Western blots from THP-1 cells activated as in (A) and treated with different concentrations of MgCl\(_2\), as indicated. (E) ASC, TAK1, phospho-TAK1 (P-TAK1), and IL-1\(\beta\) Western blots of THP-1 cells primed as in (A) and treated with BAPTA-AM (100 \(\mu\)M) or 5Z-7-oxozeaenol (OXO; 100 \(\mu\)M), as indicated. (F) ASC, NLRP3, caspase-1, and IL-1\(\beta\) Western blot of BMDMs from wild-type (WT) or NLRP3-deficient (Nlrp3\(^{-/-}\)) mice primed with LPS (1 \(\mu\)g/ml, 4 h) and incubated for 1 h with either an isotonic or hypotonic solution. Western blots are representative of two to four independent experiments.
merization was detected at early time points of stimulation, IL-1β release and caspase-1 activation were almost negligible after 10 min of stimulation. As expected, a deficiency in NLRP3 resulted in the absence of ASC cross-linking, IL-1β release, and caspase-1 activation at all time points examined after hypotonic stimulation (Fig. 6A–C). ASC specks were found inside both wild-type and NLRP3-deficient macrophages after 10 min of hypotonic stimulation (Fig. 6D). The percentage of cells with ASC specks decreased over the time of stimulation; this occurred significantly faster in wild-type macrophages compared with Nlpr3<sup>−/−</sup> macrophages (Fig. 6D). Recent reports show that ASC specks are released from macrophages after formation (27, 32), and we found extracellular ASC specks after hypotonic stimulation in both wild-type and NLRP3-deficient macrophages (Fig. 6E). We observed that the majority of cells containing ASC specks at early time points after stimulation had more than one speck/cell (Fig. 6F). When multiple ASC specks were present in a cell, they were smaller in size compared with specks formed at later time points after stimulation (Fig. 6F). Compared with wild-type macrophages, NLRP3-deficient macrophages had more cells containing multiple ASC specks at late time points of stimulation (Fig. 6F). These data suggest that the large end point speck found in the majority of wild-type macrophages is an aggregate of smaller ASC specks formed quickly after stimulation. The formation of this big speck appears to be dependent on NLRP3 and could be the caspase-1–activating platform.

Characterization of ASC and NLRP3 protein dynamics inside the ASC speck

To further study the interaction and dynamics of ASC and NLRP3 complex assembly inside the speck during hypotonic stimulation, we labeled the C terminus (Ct) of ASC with YFP and labeled the Ct of NLRP3 with Luc to record BRET (Fig. 7A). Prior to stimulation, ASC-YFP and NLRP3-Luc proteins expressed in HEK293 cells are in spatial proximity to each other, as indicated by a high specific saturated BRET signal (Fig. 7B), and they also colocalized in the cytosol of the cell (Fig. 7C). The noninteracting protein β-arrestin–YFP gave a nonspecific linear increase in the BRET signal with NLRP3-Luc (Fig. 7B). Similarly, nonspecific BRET due to random collision was found when ASC-YFP was coexpressed with the NLRP3-Luc construct truncated at the PYD (NLRP3-ΔPYD, Fig. 7B). NLRP3-APYD also was unable to colocalize within the ASC speck (Fig. 7D). These results indicate that the BRET signal between ASC-YFP and NLRP3-Luc was due to a specific interaction of ASC and NLRP3 through the PYD.

However, a physical interaction between ASC and NLRP3 was not evident by colP experiments in resting conditions (Fig. 7E, 7F). After hypotonic cell stimulation, ASC and NLRP3 did coimmunoprecipitate through specific PYD interaction (Fig. 7E, 7F). This strong interaction after hypotonic stimulation also was evidenced by a fast reduction (∼30%) in the magnitude of the net BRET signal (Fig. 7G), suggesting that the NLRP3 leucine-rich repeat domain and the ASC CARD separate from each other during inflammasome activation. Such a conformational change was not observed when K<sup>+</sup> efflux and RVD were prevented by a glycerol-KCl solution (Fig. 7H). Similarly, NLRP3 activation by nigericin resulted in a fast reduction in the magnitude of the net BRET signal induced by nigericin rebounded quickly (Fig. 7I). These data support the idea that the dynamics of ASC and NLRP3 complex assembly are different during hypotonic and nigericin stimulation. Nigericin-induced conformational changes among ASC and NLRP3 were abolished when nigericin was applied in an extracellular buffer containing high K<sup>+</sup> (Fig. 7J). As a control, stimulation of cells expressing a YFP-Luc fusion protein resulted in no variation in the net BRET signal (Fig. 7K).

**ASC speck formation is associated with the initial activation of the NLRP3 inflammasome**

To further elucidate the role of ASC specks during NLRP3 inflammasome activation, we studied the subcellular localization of endogenous ASC, NLRP3, and active caspase-1 by immunofluorescence in THP-1 macrophages. Under resting conditions, ASC and NLRP3 were homogenously distributed throughout the cytoplasm of macrophages, and no FLICA staining was detected (Fig. 8A). Active caspase-1 was detected 20 min after a decrease in extracellular osmolarity and localized with NLRP3 close to the ASC speck (Fig. 8A). Because FLICA impairs caspase-1 auto-cleavage, the localization of caspase-1 that we observed could represent an accumulation of inhibited caspase-1 that could not be released from the polymerizing ASC speck. After 40 min in hypotonic solution, the NLRP3 and FLICA signals were found throughout the cytoplasm (Fig. 8A). We also found that, after hypotonicity, caspase-1 was located in the ASC speck in wild-type, but not in Nlpr3<sup>−/−</sup> or Casp1-Casp11<sup>−/−</sup> macrophages (Fig. 8B), suggesting that NLRP3 was important for recruiting and activating caspase-1 into the ASC oligomer. Consistent with these observations, we found NLRP3 in the purified ASC oligomeric
fraction after hypotonic stimulation (Fig. 8C). Similar to murine BMDMs, kinetic analysis during THP-1 macrophage swelling revealed that ASC oligomerization occurred 15 min after the hypotonic challenge (Fig. 8D), which was before active caspase-1 or mature IL-1β could be detected in the cell supernatants (Fig. 8D). Caspase-1 and mature IL-1β release were detected after 25 min of hypotonic stimulation [i.e., at a time when ASC oligomerization was already maximal (Fig. 8D)].

Altogether, our data suggest that hypotonic extracellular media induced the formation of ASC specks that oligomerized independently of NLRP3 but did not activate caspase-1. Therefore, interaction of these ASC specks with NLRP3 PYD is necessary to activate caspase-1 in response to hypotonic stimulation.

**Discussion**

In this study, we found that hypotonic solutions, acting as a danger signal in macrophages, induced a relocalization of ASC into specks...
that were associated with NLRP3 inflammasome assembly and caspase-1 activation. NLRP3 was not necessary for ASC speck formation in response to hypotonicity or extracellular ATP; however, it was required for caspase-1 recruitment to the ASC speck and its activation. These results show that certain types of ASC specks were not detected by chemical cross-linking and that ASC specks formed in the absence of NLRP3 were generally smaller in size. Conventionally, ASC specks detected by immunocytochemistry are considered to represent the same event as ASC oligomers detected by chemical cross-linking (3), with ASC recognized as an adaptor protein for the NLRP3 inflammasome (2) required for cell swelling–induced caspase-1 activation (15). We found that hypotonic stimulation induced ASC speck formation independently of NLRP3 without oligomerization of ASC, as detected by chemical cross-linking. In the absence of assembly with NLRP3, these ASC specks failed to activate caspase-1 or IL-1β release.

The ASC speck is the initial structure for pyroptosis, a specific type of cell death driven by caspase-1. ASC specks and pyroptosis are often triggered by infection with intracellular pathogens or by exposure to noninfectious agents like PAMPs or DAMPs (imiquimod, MSU crystals, ATP, α-toxin, or lipopeptides) (3, 10, 14). One of the consequences of caspase-1 activation is cell swelling, which could end with pyroptosis (3). Our data demonstrate that ASC speck formation and caspase-1 activation are a result, rather than a cause, of cell swelling followed by a RVD process induced.
by hypotonic solutions. As such, a deficiency in ASC, as well as caspase-1 inhibition, did not alter cell swelling and RVD in response to low extracellular osmolarities. Although hypotonic stimulation induced ASC specks, ASC oligomerization, and activation of caspase-1, we found that macrophages remain viable in hypotonic solutions; the ASC specks are a platform for caspase-1 activation independent of functional NLRP3. In fact, ASC-induced cell death recently was found to be independent of caspase-1 catalytic activity (33); rather, it is due to a change in the expression of proteins involved in pyroptotic cell death by microbe-infected macrophages. Our study also revealed that ASC oligomerization in response to hypotonic stimulation was irreversible, and macrophages maintained their viability and functionality in these conditions.

Macrophages infected with *Salmonella* activate both NLRP3 and NLRC4 via their recruitment into ASC specks (9, 11), and this mechanism was proposed as an alternative pathway for inflammasome activation. However, it is unknown whether ASC specks also form a molecular platform for DAMP activation of NLRP3. When expressing ASC and NLRP3 in HEK293 cells, we found ASC in close proximity to NLRP3 during resting conditions. Upon hypotonic stimulation, there was a decrease in net BRET signal relative to the total number of cells containing ASC specks; $n > 1000$ cells from two independent experiments. (A) Monochromatic images of BMDMs treated as in (A) for 10 or 45 min and stained for ASC (left panels). Arrowheads and arrows indicate multiple ASC aggregates or a single ASC aggregate in the cell, respectively. Scale bars, 2 μm. Bar graph shows the percentage and SEM of cells containing single or multiple ASC specks relative to the total number of cells containing ASC specks; $n > 1000$ cells from two independent experiments (right panel). *$p < 0.05$, ***$p < 0.001$.

These results suggest that ASC oligomerization is also caused, in part, by the overexpression of the two proteins. We proposed previously that this molecular interplay most likely occurs by a compacting or hiding of the NLRP3 PYD inside the inflammasome complex (15). In this study, we found that, as expected, the NLRP3 PYD is crucial for its interaction with the ASC speck. This protein compaction could explain the lack of NLRP3 staining in the ASC speck (by immunocytochemistry) after hypotonic stimulation, whereas NLRP3 localization in the ASC speck was strongly suggested by the coIP experiments. This dual activity of ASC—its self-association into specks and its interaction with NLRP3—was suggested recently when the structure of the PYD of ASC was found to contain two binding sites: one important for self-association and the other for NLRP3 interaction (34). The decrease in net BRET signal also could reflect a potential dissociation of ASC and NLRP3 during hypotonic stimulation, although a physical interaction between them was observed by coIP after stimulation. In resting conditions, maintaining inflammasome proteins in close proximity does not determine inflammasome activation. Thus, we were able to find NLRP3 and ASC colocalization and positive BRET signal but were not able to identify an association by coIP.

Our data show that cell activation by decreasing extracellular osmolarity or by extracellular ATP induced a rapid assembly of ASC into specks, independent of functional NLRP3, because it occurred in *Nlrp3<sup>−/−</sup>* macrophages and by coexpression of ASC with NLRP3-ΔPYD, which was unable to localize into the ASC speck.
FIGURE 7. NLRP3 interacts with the ASC speck. (A) Schematic representation showing NLRP3 BRET donor and ASC acceptor. (B) BRET saturation curve for HEK293 cells transfected with a constant quantity of NLRP3-Luc-Ct (solid lines) or NLRP3-ΔPYD-Luc-Ct (dashed line) and increasing amounts of the BRET acceptor ASC-YFP (● and △) or β-arrestin–YFP (○). (C) HEK293 cells transfected with NLRP3 (red) and ASC-YFP (green). Nuclei stained with DAPI (blue). Arrowheads denote ASC specks with NLRP3. Scale bar, 10 μm. (D) Quantification of NLRP3 and NLRP3-ΔPYD in the ASC speck. Data represent average and SEM of n ≥ 36 cells from three independent experiments. (E) NLRP3-flag and ASC-V5-YFP coIP after HEK293 cells were incubated for 40 min in isotonic or hypotonic solution. Western blots are representative of three independent experiments. (F) NLRP3-flag or NLRP3-ΔPYD-flag and ASC-V5 coIP after HEK293 cells were incubated for 40 min in hypotonic solution. Western blots are representative of two independent experiments. (G) Kinetics of net BRET signal in HEK293 cells transfected with the BRET donor NLRP3-Luc-Ct and the acceptor ASC-YFP in response to isotonicity (gray circles) or hypotonic solution. Data are average and SEM of three or four independent experiments. (H) Percentage of net BRET signal reduction in cells transfected as in (G) after 20 min in a solution with 120 mM glycerol and either 140 mM NaCl (Na+-Gly) or 140 mM KCl (K+-Gly). Data are average and SEM of four independent experiments. (I) Kinetics of net BRET signal recorded as in (G) in response to vehicle (gray circles) or nigericin (20 μM, black circles). The arrow indicates when nigericin was injected. Data are average and SEM of three independent experiments. (J) Percentage of net BRET signal reduction in cells treated as in (I) after a 2-min stimulation with nigericin in normal or high extracellular K⁺ concentration. Data are average and SEM of two independent experiments. (K) Kinetics of net BRET signal in HEK293 cells transfected with a Luc-YFP fusion construct (BRET control protein) in response to isotonicity (white circles), hypotonicity (light gray circles), 120 mM glycerol solution with 140 mM NaCl (Na⁺-Gly, dark gray circles), or nigericin (black circles). The arrow indicates when stimulation was induced. Data are average and SEM of three independent experiments. ***p < 0.001.
speck. Other NLRP3 activators, such as nigericin, MSU crystals, and E. coli infection, were unable to induce ASC specks in the absence of NLRP3. These data suggest different dynamics of ASC and NLRP3 complex assembly during activation. Such differences also were suggested by the BRET experiments in which differences were observed when cells were activated by hypotonicity or nigericin.

Initial small ASC specks formed in response to hypotonicity are independent of NLRP3. However, aggregation of these specks over time in a single spot and oligomerization of ASC, as revealed by cross-linking, require the presence of NLRP3, which is crucial for caspase-1 activation. Later conformational changes in the ASC speck could serve as a mechanism to spread active caspase-1 through the cytosol, as was described for pyroptosis (10). Current models for NLRP3 inflammasome activation suggest that, upon macrophage stimulation by danger signals, NLRP3 oligomerizes and then recruits ASC in fiber-like structures that amplify caspase-1 activation (5, 6). Our results also suggest that NLRP3 is necessary to form a compact ASC structure, probably through the tight oligomerization of ASC. A recent study also showed that, in response to Salmonella, NLRP3 and NLRC4 localize to the same ASC speck (11), suggesting a central role for ASC specks in inflammasome formation and/or activation. Our results show that, after hypotonicity or ATP stimulation, small ASC specks appear in wild-type and Nlrp3<sup>−/−</sup> macrophages, and the presence of NLRP3 is important for developing ASC specks to form large irregular structures that recruit and activate caspase-1. In the absence of NLRP3, ASC specks fail to recruit and activate caspase-1. Although ASC can self-aggregate and activate caspase-1 when macrophages are infected with different bacteria or when over-expressed in HEK293 cells (27), we cannot rule out that, in macrophages deficient in NLRP3, inactive ASC specks formed in response to hypotonicity could be induced by another receptor. However, our data could suggest an alternative model for inflammasome assembly in response to hypotonicity or ATP stimulation: the small inactive ASC specks may represent aggregates that will assemble with NLRP3 to form an inflammasome. Following NLRP3 activation, NLRP3 would interact with these small ASC specks and induce ASC oligomerization detected by cross-linking, leading to the formation of a single ASC speck where caspase-1 would be activated. However, this model does not seem to be extendable to all NLRP3 activators, because our experiments point out that nigericin, MSU crystals, and E. coli activation do not induce ASC speck formation in the absence of NLRP3. Therefore, we cannot exclude that formation of small ASC specks and NLRP3 inflammasome assembly with ASC might represent two different processes in which ASC specks observed in the absence of NLRP3 might be involved in an inflammasome-independent function. In fact, it is known that ASC modulates immune cell functions via expression of Dock2 and Rac activation independently of NLRP3 inflammasome and caspase-1 (35). Moreover, currently available methods do not allow direct mea-

FIGURE 8. ASC speck formation is associated with the initial assembly of the NLRP3 inflammasome. (A) High-resolution images of THP-1 macrophages primed with LPS and IFN-γ (100 ng/ml each, 16 h) and subsequently subjected to hypotonic extracellular solution for different times, stained for ASC (blue, second row), NLRP3 (red, third row), and active caspase-1 using FLICA reagent (green, top row). Arrowheads denote ASC speck localization. Scale bar, 10 μm. (B) Images of wild-type (WT), Nlrp3<sup>−/−</sup>, or Casp1-Casp11<sup>−/−</sup> BMDMs primed with LPS (1 μg/ml, 4 h), subjected to hypotonicity (90 mOsm, 45 min), and stained for ASC (green) and caspase-1 (red). Scale bar, 10 μm. Arrowheads denote ASC speck. (C) NLRP3 Western blots on purified ASC oligomeric fraction (ASC Oligomer) and total cell extracts (Cells) in THP-1 cells treated as in (A). Western blots are representative of two independent experiments. (D) ASC, IL-1β, and caspase-1 Western blots of purified cross-linked ASC oligomer proteins (ASC Oligomer), total cell extracts (Cells), and cell supernatants (Sups) in THP-1 cells treated as in (A). Western blots are representative of two independent experiments.
measurement of NLRP3 activation; they only allow the downstream consequences of inflammasome activation to be measured. Therefore, we devised a BRET technique to fill this gap and measure NLRP3 oligomerization and association with ASC in real time ([15] and this study). However, by using different techniques it is difficult to define which of the events occur first during inflammasome activation: single ASC speck formation imaged by immunofluorescence or ASC oligomerization assessed by cross-linking and caspase-1 activation. Finally, we were unable to find IL-1β in the ASC speck, which is consistent with our recent findings whereby we used a specific biosensor to measure pro–IL-1β processing in real time and in situ to show that cytokine cleavage occurs throughout the cytosol (36). These observations suggest that ASC specks do not represent the main subcellular compartment in which the cytokine is processed to its mature form.

We also found in this study that the signaling pathways that activate the NLRP3 inflammasome in response to hypotonicity (15, 37) are the same as for ASC oligomerization, as detected by chemical cross-linking. Both were dependent on low intracellular potassium concentration, the RVD, TRPM7 and TRPV2 activation, intracellular Ca2+ increase, and activation of TAK1 kinase. This is not surprising because, in the current inflammasome assembly model, NLRP3 is required to induce ASC oligomerization; therefore, everything affecting the activation of NLRP3 is likely to affect downstream ASC oligomer formation. However, our data also show similar signaling pathways for the NLRP3-independent formation of ASC specks, which were dependent on TRPV2 channel activation during cellular RVD and TAK1 phosphorylation. These data indicate that TAK1 might regulate NLRP3 inflammasome activation through the control of ASC speck formation and suggest that both events—ASC speck formation and NLRP3 inflammasome activation—share the same intracellular signaling pathway. In fact, potassium efflux is a common denominator for NLRP3 activation (2, 29, 38, 39) and ASC speck formation (3). This observation could explain why inflammasomes other than NLRP3 also seem to require low intracellular potassium concentrations to be functional under certain conditions (40). In a recent publication it was argued that hypotonic stimulation of macrophages was inducing NLRP3 activation through the low K+ concentration of the extracellular hypotonic buffer used and not via cell swelling and RVD (29). However, our previous work demonstrates that increasing osmolarity of the hypotonic solution with mannitol or sorbitol, and keeping the same low K+ concentration of the extracellular buffer, abolished the activation of NLRP3, as well as the induction of cell swelling and RVD (15). Also, inhibitors of CI− and TRP channels abolished RVD and NLRP3-dependent caspase-1 activation, but they did not affect intracellular K+ depletion (15). Furthermore, a recent report found that macrophages deficient in aquaporin 1 (or blocking aquaporin function) resulted in impaired caspase-1 and IL-1β release in response to hypotonicity-induced cell swelling (41). All of these data suggest that K+ efflux from macrophages per se is required, but not sufficient, to induce inflammasome activation.

Another striking similarity that we found between ASC oligomerization and NLRP3 inflammasome activation is their sensitivity to the blocker glyburide. Glyburide is known to selectively block NLRP3 inflammasome formation by acting in an undefined way upstream of NLRP3 (30), and we found in this study that glyburide also affects ASC oligomerization after hypotonic stimulation by affecting the RVD process. This effect is also similar to the inhibition of the NLRP3 inflammasome by cytokine release inhibitory drug 3, which targets ASC oligomerization (18). Recently, it was found that ASC specks are released from macrophages after inflammasome stimulation and act extracellularly as a danger signal to propagate inflammation (27, 32). In this study, we found that, in contrast with IL-1β release, hypotonicity induced the release of ASC specks in the absence of NLRP3, suggesting that the release of ASC specks could be a potent inflammatory mediator that could be present in the absence of IL-1β cytokine release. Consistent with this, blockade of extracellular ASC specks was found to reduce traumatic brain injury (42). Thus, ASC is emerging as a potential target for which highly selective drugs would block the inflammasome and IL-1β signaling.

Altogether, our data demonstrate that, during macrophage swelling, ASC speck formation and oligomerization share the same signaling pathway as NLRP3 inflammasome activation, which includes TRPV2 activation, intracellular Ca2+ increase, and TAK1 signaling. However, speck formation identified by immunocytochemistry occurred independently of NLRP3 and did not lead to caspase-1 activation. Meanwhile, ASC oligomers detected by immunocytochemistry and chemical cross-linking only formed in the presence of NLRP3, leading to functional inflammasomes and the activation of caspase-1 in response to hypotonic stimulation.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


**Figure S1.** Cell swelling and RVD are independent on ASC or caspase-1 activity. (A) Relative cell area of immortalized bone marrow derived macrophages from wild type (WT) or ASC-deficient (Pycard−/−) mice incubated in hypotonic solution in the presence or absence of caspase-1 inhibitor (Ac-YVAD-AOM). Average and s.e.m. of n = 50 cells/condition and representative of 3 independent experiments. (B) Experimental design to study long-term effects of ASC oligomerization.
Figure S2. Regulatory volume decrease is blocked by high extracellular K\(^+\), NPPB and glyburide. (A) Regulatory volume decrease measured as the ratio of the initial cellular volume and the final cellular volume after 3 h treatment with different hypotonic solutions of THP-1 cells primed with LPS and IFN-γ (100 ng/ml each, 16 h); hypotonic solutions were composed with: 120 mM glycerol with 140 mM of NaCl (Na\(^+\)-Gly) or 140 mM of KCl (K\(^+\)-Gly). (B) Relative intracellular K\(^+\) concentration of THP-1 cells primed as in (A) but incubated for 1h in the presence of different solutions as indicated; data represent the average and s.e.m. of \(n=3\) independent experiments; ***\(p<0.001\). (C) Western blot analysis of the cleavage of pro-IL-1\(\beta\) to its active p17 form (IL-1\(\beta\)) and of ASC from THP-1 activated as in (A). Cells: cell lysates, Sups: supernatants; Western blot representative of 3 different experiments. (D) Regulatory volume decrease ratio of THP-1 cells primed with LPS and IFN\(\gamma\) (100 ng/ml each, 16 h) and incubated for 3 h with hypotonic solution in the presence or absence of NPPB (100 µM) or Glyburide (100 µM) as indicated. For (A) and (D), data represent average and s.e.m. of \(n=50\) cells/condition and representative of at least 3 independent experiments.
Figure S3. (A,B) Effective TRPV2 gene silencing in THP-1 cells. (A) Western blot for TRPV2, ASC and tubulin from THP-1 lysate of control non-infected cells (No-Lv), GFP-lentivirus infected cells (Lv-GFP) or cells infected with lentivirus coding for GFP and TRPV2 shRNA (Lv-shRNA). (B) TRPV2 immunostaining in THP-1 infected as in (A); scale bar 40 μm. (C) ASC and IL-1β Western blot from purified cross-linked ASC oligomer proteins (ASC Oligomer), total cell extracts (Cells) and cell supernatants (Sups) of THP-1 cells infected with lentivirus coding for scramble shRNA sequence primed with LPS and IFN-γ (100 ng/ml each, 16 h) and subsequently stimulated with 300 and 90 mOsml of extracellular osmolarity for 60 min. (D) Representative pictures of wild type murine peritoneal macrophages primed with LPS (1 μg/ml for 4 h) and stained for ASC (green), actin (Phalloidin-Texas red, red) and nuclei (DAPI, blue) after incubation for 40 min in isotonic or hypotonic solution. Scale bar 10 μm.