

COMMUNICATION

Cellular localization of NLRP3 inflammasome

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ABSTRACT

Inflammasome is a large protein complex activated upon cellular stress or microbial infection, which triggers maturation of pro-inflammatory cytokines interleukin-1 β and interleukin-18 through caspase-1 activation. Nod-like receptor family protein 3 (NLRP3) is the most characterized inflammasome activated by various stimuli. However, the mechanism of its activation is unclear and its exact cellular localization is still unknown. We examined the potential co-localization of NLRP3 inflammasome with mitochondria and seven other organelles under adenosine triphosphate, nigericin or monosodium urate stimulation in mouse peritoneal macrophages using confocal microscopy approach. Our results revealed that the activated endogenous apoptosis-associated speck-like protein containing a CARD (ASC) pyroptosome forms in the cytoplasm and co-localizes with NLRP3 and caspase-1, but not with any of the organelles screened. This study indicates that the ASC pyroptosome universally localizes within the cytoplasm rather than with any specific organelles.

KEYWORDS NLRP3, inflammasome, ASC pyroptosome, confocal microscopy, cytoplasmic

INTRODUCTION

Inflammasome is formed with the participation of certain pattern recognition receptors and senses various danger signals (Martinon et al., 2002; Agostini et al., 2004; Mariathasan et al., 2004; Poeck et al., 2010). As a large protein complex, inflammasome controls the activation of the proteolytic enzyme caspase-1 through the apoptosis-associated speck-like protein containing a CARD (ASC), which subsequently regulates the maturation of the pro-inflammatory cytokines interleukin-1 β

(IL-1 β) and IL-18 (Agostini et al., 2004). IL-1 β and IL-18 are secreted into the extracellular space and function as versatile cytokines (Dinarello, 2009) that recruit more immune cells, educate lymphocytes, and eventually induce inflammatory effects.

The Nod-like receptor family protein 3 (NLRP3) is the most common and best studied inflammasome. NLRP3 is activated by a wide range of signals that cover both endogenous and pathogenic origins. Endogenous danger signals, such as adenosine triphosphate (ATP), amyloid- β fibrils, and uric acid crystals, together with pathogens such as *Listeria monocytogenes*, *Candida albicans*, and influenza A virus, can activate the NLRP3 inflammasome (Martinon et al., 2006; Dostert et al., 2008; Eisenbarth et al., 2008; Allen et al., 2009; Gross et al., 2009; Thomas et al., 2009). However, the detailed mechanism of its activation is still unknown. Three distinct mechanisms have been reported, namely, reactive oxidative stress (ROS) (Schroder et al., 2010), lysosome damage (Homung and Latz, 2010), and potassium leakage (Arlehamn et al., 2010). However, none of them can explain all the observed phenomena, and the relationship between them need to be elucidated.

In addition to caspase-1 activation and IL-1 β secretion, NLRP3 inflammasome activation is also characterized by ASC pyroptosome formation, which is the aggregation of ASC as a pre-step to activate caspase-1 (Fernandes-Alnemri et al., 2007). Interestingly, only one pyroptosome is found to form per cell (Fernandes-Alnemri et al., 2007). Furthermore, to our knowledge, the ASC pyroptosome represents the assembled NLRP3 inflammasome. Several studies have investigated the localization of ASC in THP-1 human cell line and M1/M2 polarized macrophages with different stimuli (Bryan et al., 2009; Pelegrin and Surprenant, 2009; Bryan et al., 2010). However, no exact localization of the pyroptosome in primary peritoneal macrophages has been reported. Detailed localization study of the pyroptosome may help us understand the activation

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process and provide evidence on the mechanism of NLRP3 inflammasome activation. The objective of this study was to determine the potential localization of the NLRP3 inflammasome under the activated state in peritoneal macrophages using confocal microscopy approach. The immunofluorescence results revealed the cytoplasmic localization of endogenous pyroptosome, which co-localized with NLRP3 and caspase-1 rather than to any detected organelles, indicating the organelle-free cytoplasmic localization of the NLRP3 inflammasome in macrophages upon activation.

RESULTS

ASC pyroptosome was cytoplasmic but not localized to the mitochondria upon ATP stimulation in mouse macrophages

Mitochondria are the main sources of inflammasome-activating ROS. NLRP3 inflammasome activation is largely impaired when the mitochondrial activity is inhibited (Nakahira et al., 2010; Zhou et al., 2011). Therefore, the mitochondria may be signal-integrating organelles, and are probably the organelles for NLRP3 inflammasome activation. We stimulated low-dose lipopolysaccharide (LPS)-primed peritoneal macrophages with ATP, an NLRP3 inflammasome activator, and stained the mitochondria with MitoTracker to investigate the hypothesis. The confocal results showed that the ASC dispersed over the nucleus and cytoplasm in the LPS-primed cells. The ASC aggregated across the whole cell and formed in the cytoplasm (green foci) upon further stimulation with ATP, but did not localize to the mitochondria (Fig. 1A). The ASC pyroptosome co-localized with NLRP3 and caspase-1, although most NLRP3 were not aggregated in the foci (Fig. 1B and 1C), consistent with previous studies. The activation state was also confirmed by abundant IL-1 β secretion from the same set of cells (Fig. 1D). These results suggested that the ASC pyroptosome is normally formed in ATP-activated macrophages together with NLRP3 inflammasome activation, but localizes in the cytoplasm and not in the mitochondria.

ASC pyroptosome did not co-localize with other detected organelles upon ATP stimulation in mouse macrophages

Seven other organelles were detected upon ATP stimulation to determine the exact localization of ASC pyroptosome. The peritoneal macrophages were primed and stimulated as described in MATERIALS AND METHODS. GM-130, calnexin, α -tubulin, early endosome antigen 1 (EEA1), vimentin, and γ -tubulin were used as markers of Golgi apparatus, endoplasmic reticulum, microtubule, endosome, phagosome, and centromere, respectively (Webb et al., 2001; Latz et al., 2004; Eng et al., 2007; David et al., 2010; Wolff et al., 2011; Yuan et al., 2012). LysoTracker was applied for lysosome detection. The confocal results suggested that the ASC pyroptosome not co-localize with any of the organelles (Fig. 2A–G), but co-localizes with NLRP3 and caspase-1 (Fig. 2H and 2I). NLRP3 inflammasome is normally activated (Fig. 2J).

ASC pyroptosome was not localized to the mitochondria upon nigericin or MSU stimulation in mouse macrophages

Besides ATP, NLRP3 inflammasome is activated by various stimuli, including nigericin and MSU (Martinon et al., 2006;

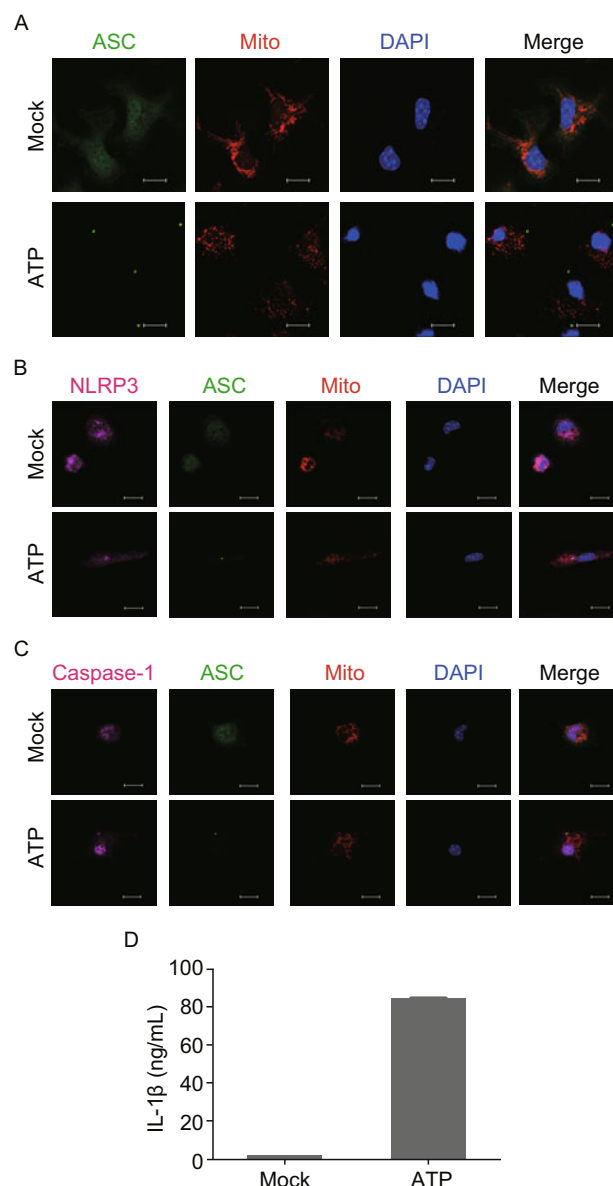
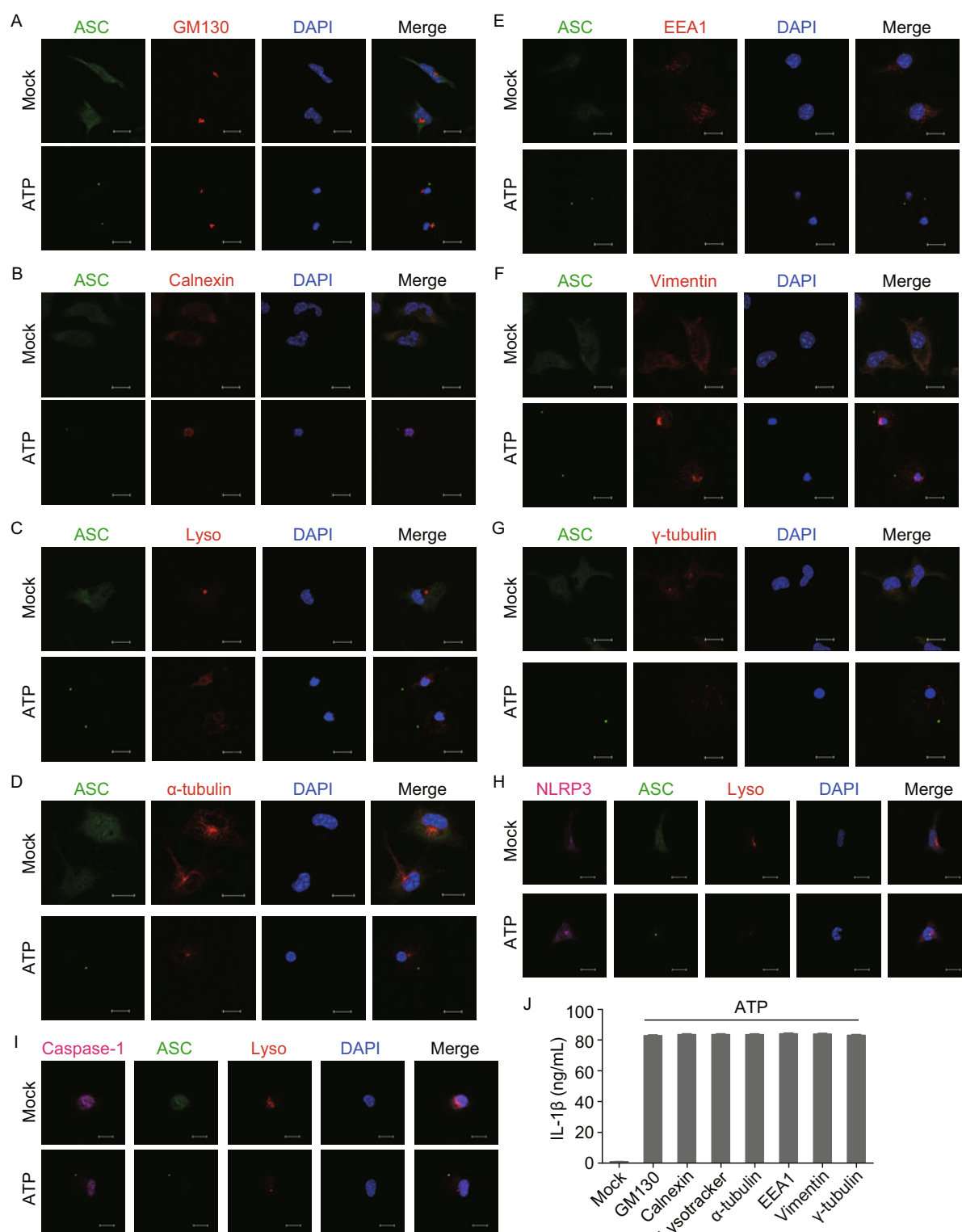


Figure 1. ASC pyroptosome localized in the cytoplasm, but not in the mitochondria, upon ATP stimulation. (A) Immunofluorescence microscopy of LPS-primed peritoneal macrophages stained with MitoTracker for 40 min and left unstimulated (upper panel) or stimulated with 5 mmol/L ATP for 30 min (lower panel). Scale bar, 10 μ m. (B and C) Immunofluorescence microscopy of LPS-primed peritoneal macrophages stained with MitoTracker and left unstimulated (upper panel) or stimulated with ATP (lower panel). NLRP3 (B) or caspase-1 (C), ASC, and DNA (with DAPI) were separately stained. Scale bar, 10 μ m. (D) ELISA results of IL-1 β production in peritoneal macrophages treated similar to A–C. Data represent at least three experiments (mean $\pm$ SD in D).



Hu et al., 2010). We used nigericin or MSU and detected ASC pyroptosome localization to investigate whether the ASC pyroptosome location varied upon different stimuli. The distribution of ASC was found to be similar as the ATP-stimulated macrophages. No co-localization of ASC pyroptosome with mitochondria was found upon nigericin or MSU stimulation (Fig. 3A), but IL-1 β was normally secreted (Fig. 3D). Both NLRP3 and caspase-1 showed co-localization with ASC pyroptosome upon stimulation (Fig. 3B and 3C).

ASC pyroptosome showed no co-localization with other screened organelles upon nigericin or MSU stimulation

Seven organelles were also screened in nigericin or MSU stimulation. The confocal results suggested that none of them is the organelle for ASC pyroptosome localization (Fig. 4A–G). Enzyme-linked immunosorbent assay (ELISA) results of IL-1 β proved the activation of NLRP3 inflammasome (Fig. 4J). These results again confirmed the co-localization of ASC pyroptosome with NLRP3 and caspase-1 (Fig. 4H and 4I).

DISCUSSION

The NLRP3 inflammasome is critical for protection against pathogens and induction of adaptive immune responses (Eisenbarth et al., 2008; Ichinohe et al., 2009). Deregulated NLRP3 inflammasome activation is associated with multiple diseases such as gout, Crohn's disease, atherosclerosis, and type II diabetes (Martinon et al., 2006; Duewell et al., 2010; Zaki et al., 2010; Wen et al., 2011). However, the mechanism of NLRP3 inflammasome activation and its exact localization in the cell remain unclear. The determination of the locus of the activated inflammasome will help identify the possible activation process and the precise activation mechanism.

Mitochondria are potential organelles for NLRP3 inflammasome activation because of their vital role in the process. Mitochondrial ROS production and mitochondria DNA (mtDNA) release are required for NLRP3 inflammasome activation. Meanwhile, mitochondrial dysfunctions lead to NLRP3 inflammasome activation (Nakahira et al., 2010; Zhou et al., 2011). Therefore, the mitochondria were first chosen for the localization exploration. ASC aggregation is believed to recruit procaspase-1 for its activation and is supposed to be the locus for NLRP3 inflammasome activation. Thus, the ASC focus was used as readout for the NLRP3 inflammasome localization study. Peritoneal macrophages were used as targeting cells because of their sensitivity to inflammasome induction (Mariathasan et al., 2004). We observed that the ASC dispersed across the cell, both in the nucleus and in the cytoplasm, in the unstimulated LPS-primed macrophages. All of the ASC were gathered in the cytoplasm and formed dots upon ATP stimulation, but were not localized to the mitochondria.

Several organelles are involved in the ASC activation process, such as the endoplasmic reticulum, which is believed to co-localize with exogenous NLRP3 (Zhou et al., 2011). Thus,

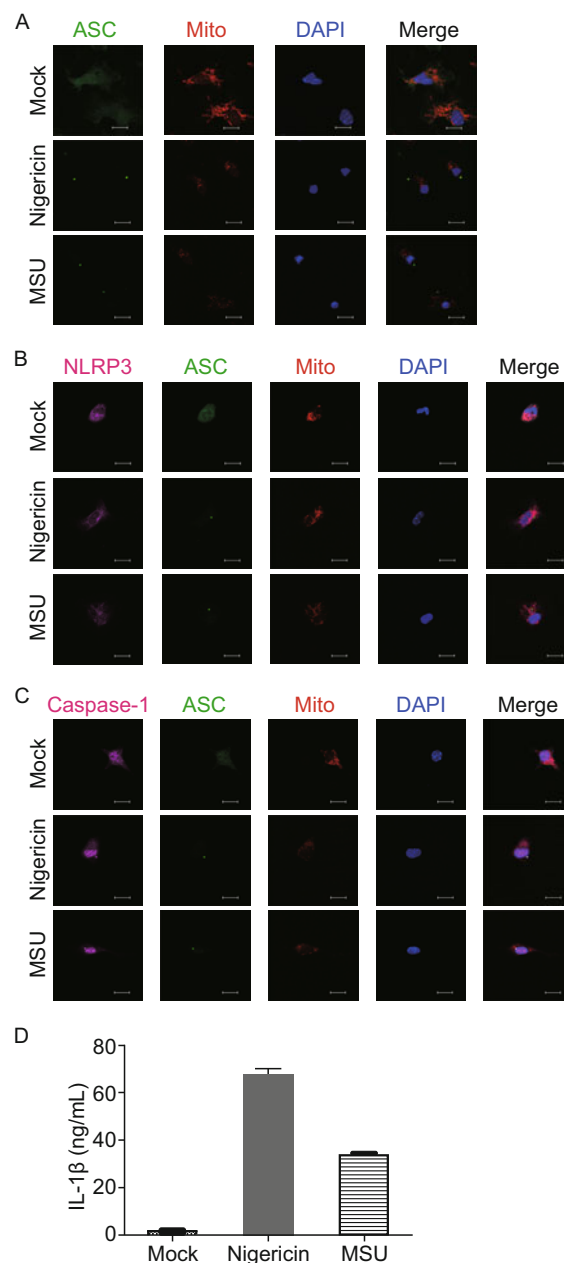


Figure 3. ASC pyroptosome did not localize in the mitochondria upon nigericin or MSU stimulation. (A) Immunofluorescence microscopy of LPS-primed peritoneal macrophages stained with MitoTracker for 40 min and left unstimulated (upper panel), stimulated with 20 μ mol/L nigericin for 30 min (middle panel) or with 500 μ g/mL MSU for 3 h (lower panel), followed by staining for ASC and DNA (with DAPI). (B and C) Immunofluorescence microscopy of LPS-primed peritoneal macrophages stained with MitoTracker, left unstimulated (upper panel), stimulated with nigericin (middle panel) or with MSU (lower panel), followed by staining for NLRP3 (B) or caspase-1 (C), ASC and DNA (with DAPI). Scale bar, 10 μ m. (D) ELISA results of IL-1 β production in peritoneal macrophages treated similar to A–C. Data represent at least three experiments (mean $\pm$ SD in D).

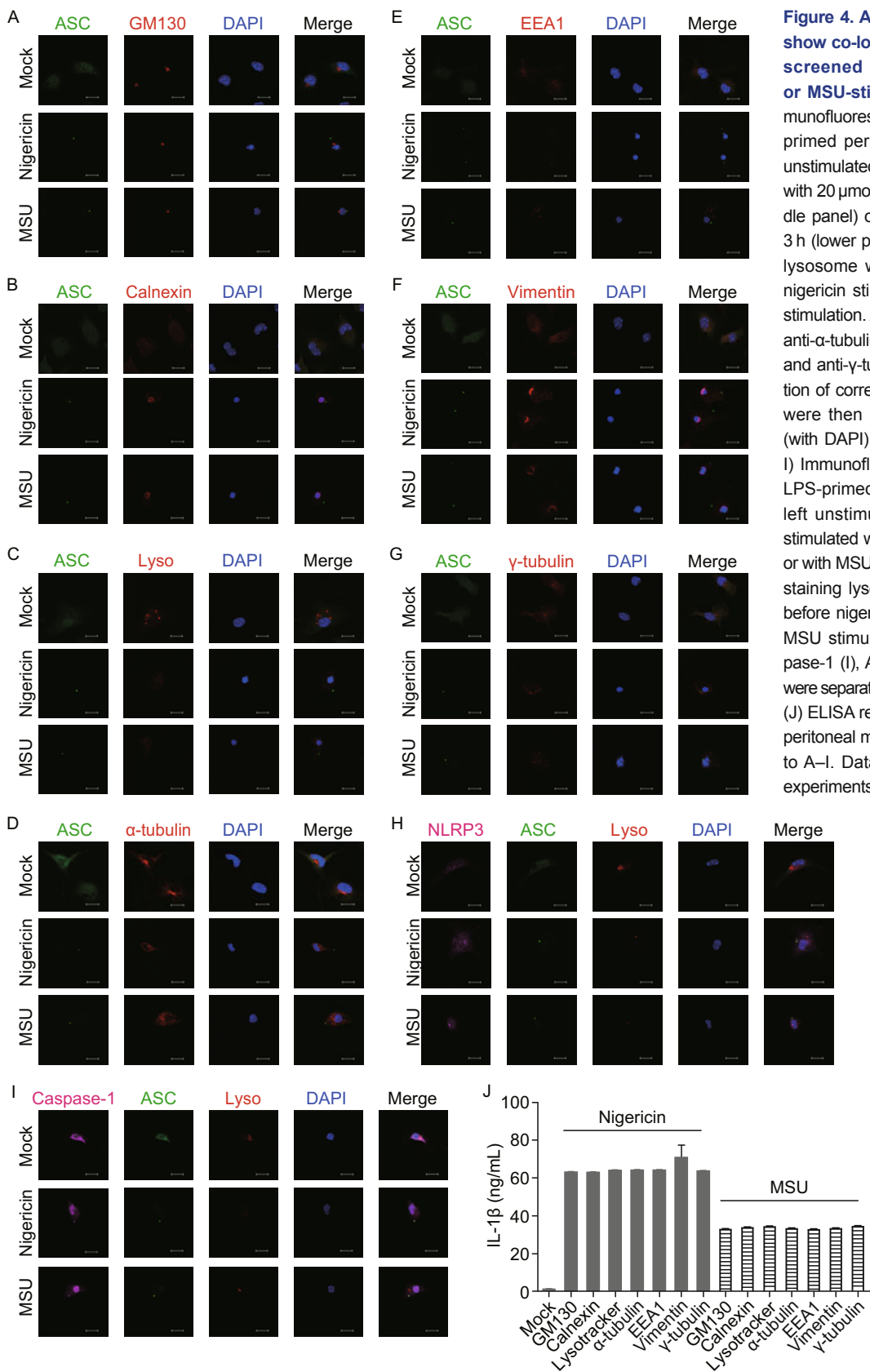


Figure 4. ASC pyroptosome did not show co-localization with any of the screened organelles in nigericin or MSU-stimulated cells. (A–G) Immunofluorescence microscopy of LPS-primed peritoneal macrophages, left unstimulated (upper panel), stimulated with 20 μ mol/L nigericin for 30 min (middle panel) or with 500 μ g/mL MSU for 3 h (lower panel). LysoTracker staining lysosome was added 40 min before nigericin stimulation or 2 h after MSU stimulation. Anti-GM-130, anti-calnexin, anti- α -tubulin, anti-EEA1, anti-vimentin, and anti- γ -tubulin were used for detection of corresponding organelles. Cells were then stained of ASC and DNA (with DAPI). Scale bar, 10 μ m. (H and I) Immunofluorescence microscopy of LPS-primed peritoneal macrophages left unstimulated (upper panel) and stimulated with nigericin (middle panel) or with MSU (lower panel). LysoTracker staining lysosome was added 40 min before nigericin stimulation or 2 h after MSU stimulation. NLRP3 (H) or caspase-1 (I), ASC, and DNA (with DAPI) were separately stained. Scale bar, 10 μ m. (J) ELISA results of IL-1 β production in peritoneal macrophages treated similar to A–I. Data represent at least three experiments (mean $\pm$ SD in J).

we tested seven other organelles including the Golgi apparatus, endoplasmic reticulum, microtubule, endosome, phagosome, centromere, and lysosome. However, none of them exhibited co-localization with ASC pyroptosome upon ATP stimulation.

We used two other activators of NLRP3 inflammasome, nigericin and MSU, to determine whether the cytoplasmic localization of ASC pyroptosome was universal for different stimuli. Similar results were observed among three different stimulations, that is, the ASC pyroptosome was not co-localized with any of the tested organelles. Although NLRP3 showed limited aggregation into the ASC speck, the ASC pyroptosome was co-localized with caspase-1 and NLRP3 upon different stimulations, consistent with previous studies.

Our study aimed to explore the endogenous localization of activated ASC pyroptosome in primary peritoneal macrophages and provide evidence on its localization in the cytoplasm, but not to specific organelles. The results were similar among ATP, nigericin, and MSU stimulations, indicating the universal localization of ASC with different activators. Although localization with other organelles is also possible, our results eliminate the most probable organelles. Further study needs to be conducted to detect the dynamic activity of NLRP3 inflammasome. The candidate proteins interacting with NLRP3 inflammasome should also be tested to determine the activation mechanism and possible regulation process.

MATERIALS AND METHODS

Cells and stimulation

Peritoneal macrophages from C57BL/6 were prepared as follows. Briefly, mice were intraperitoneally injected with 1 mL of 4% thioglycolate (Sigma). Peritoneal exudates at the fourth day post-infection were isolated from the peritoneal cavity. Subsequently, cells were incubated at 37°C for 6 h with Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (Gibco) and washed twice with DMEM. After additional overnight culture, the adherent cells were used as the peritoneal macrophages. The peritoneal macrophages were primed with 200 ng/mL LPS from *Escherichia coli* 0111:B4 (Sigma) for 5 h before stimulation with 5 mmol/L ATP (Sigma) for 30 min, 20 µmol/L nigericin (Sigma) for 30 min or 500 µg/mL MSU (Sigma) for 3 h.

Confocal microscopy

Peritoneal macrophages were plated overnight on coverslips and stimulated as described above. After stimulation, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with Triton X-100 in PBS for 5 min, and blocked with 1% bovine serum albumin in PBS for 30 min. Subsequently, cells were incubated with antibodies for various organelles, including anti-GM130 (Golgi apparatus, BD), anti-calnexin (endoplasmic reticulum, Sigma), anti-EEA1 (endosome, BD), anti-α-tubulin (microtubule, Sigma), anti-vimentin (phagosome), and anti-γ-tubulin (centromere). After incubation with antibodies for 2 h, cells were washed and incubated with Alexa 561 goat-anti-mouse antibody (BD) for 1 h, added with fluorescein isothiocyanate (FITC)-conjugated anti-

ASC antibody for 1.5 h, and rinsed with PBS.

Mitochondrial and lysosome detection was performed by staining cells with MitoTracker (Molecular Probes) or LysoTracker (Molecular Probes) for 40 min before ATP or nigericin stimulation and incubation with FITC-conjugated anti-ASC antibody for 1.5 h. NLRP3 and caspase-1 detection was performed by incubating cells with anti-NLRP3 (Enzo Life Sciences) or anti-caspase-1 (Santa Cruz) antibodies for 2 h before incubation with anti-ASC antibody. Finally, all cells were stained with 4',6-diamidino-2-phenylindole (DAPI). Confocal microscopic analyses were performed using Leica TCS SP2. Anti-vimentin and anti-γ-tubulin were provided by Xueliang Zhu's laboratory.

ELISA

Mouse IL-1β in culture supernatants were measured using an ELISA kit (R&D Systems) according to the manufacturer's protocol.

Statistical analysis

Data were presented as mean ± standard deviation of three independent experiments. Statistical comparisons between different treatments were performed using an unpaired Student's *t*-test. *P* < 0.01 was considered significant and *P* < 0.001 was highly significant.

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ABBREVIATIONS

ASC, apoptosis-associated speck-like protein containing a CARD; IL-1β, interleukin-1β; LPS, lipopolysaccharide; MSU, monosodium urate; NLRP3, Nod-like receptor family protein 3; ROS, reactive oxidative stress

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