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Microscopic Detection of ASC Inflammasomes in Bone Marrow Derived Macrophages Post Stimulation

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[Abstract] An inflammasome is an intracellular multiprotein complex that plays important roles in host defense and inflammatory responses. Inflammasomes are typically composed of the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), cytoplasmic sensor protein, and the effector protein pro-caspase-1. ASC assembly into a protein complex termed ASC speck is a readout for inflammasome activation. Here, we provide a step-by-step protocol for the detection of ASC speck by confocal microscopy in Bone marrow derived macrophages (BMBDs) triggered by chemical stimuli and bacterial pathogens. We also describe the detailed procedure for the generation of BMDMs, stimulating conditions for inflammasome activation, immunofluorescence cell staining of ASC protein, and microscopic examination. Thus far, this method is a simple and reliable manner to visualize and quantify the intracellular localization of ASC speck.

Graphic abstract:

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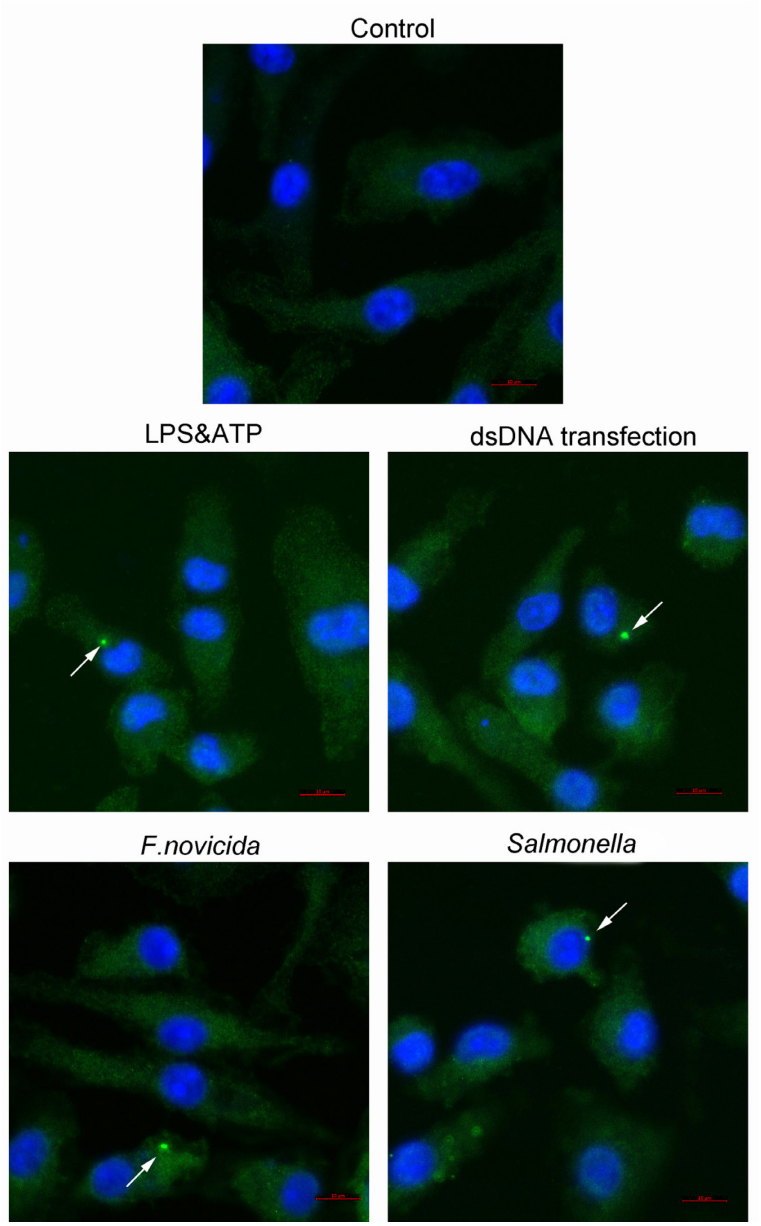

Figure 1. Confocal microscopy detection of ASC speck formation in untreated WT BMDMs and WT BMDMs stimulated with LPS and ATP, transfected with dsDNA, and infected with *F. novicida* or *Salmonella* as indicated. Arrow indicates the ASC speck. Scale bars: 10 μm.

Keywords: ASC, Inflammasome, Fluorescence staining, Confocal microscopy, NLRP3, AIM2, NLRC4

[Background] The innate immune system has a key role in initiating and orchestrating host defense by detecting invading pathogens through membrane-bound and cytosolic pattern recognition receptors (PRRs), which recognize pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs). Inflammasome activation is an essential innate immune event in response to pathogenic infection and sterile stimuli that causes the initiation of pyroptotic cell death and the release of the proinflammatory cytokines IL-1β and IL-18. Inflammasome assembly is triggered by the activation of

upstream sensors, such as NLRP1, NLRP3, AIM2, NLRC4, and PYRIN. Upon activation, sensor proteins form a complex in an ASC-dependent and -independent manner to mediate caspase-1 cleavage and activation. Cleaved caspase-1 in turn leads to the maturation of proinflammatory cytokines IL-1β and IL-18 and the process of GSDMD-mediated pyroptosis (Rathinam and Fitzgerald, 2016). The adaptor ASC protein is composed of a PYRIN domain (PYD) and caspase recruitment domain (CARD), which help ASC function as an adaptor to interact with upstream sensor and effector caspase-1 (Agrawal and Jha, 2020). ASC speck formation is a hallmark of inflammasome activation. Confocal microscopy and flow cytometry are two major methods to detect ASC speck formation (Stutz *et al.*, 2013; Sester *et al.*, 2015; Beilharz *et al.*, 2016; Hoss *et al.*, 2018).

We performed and published the determination of ASC speck formation in BMDMs after the activation of NLRP3, AIM2, and NLRC4 inflammasomes (Guo *et al.*, 2020). In comparison with the method using ASC-GFP fusion protein and flow cytometry, this procedure is able to detect endogenous ASC speck formation and can visualize and quantify subcellular localization of the inflammasome complex with the help of cellular organelle staining. This protocol can be utilized to evaluate any ASC speck formation in other cells after ASC-dependent inflammasome activation.

Materials and Reagents

1. Microscope Slides (Citotest, catalog number: 198105)
2. 12-well plate (Jet, catalog number: TCP011012)
3. 10 cm cell culture dish (Jet, catalog number: TCD010100)
4. 15/50 ml sterile centrifuge tube (Jet, catalog number: CFT011500)
5. Serological Pipet (JETBIOFIL, catalog number: GSP-010-005/GSP-010-010)
6. 25 cm Cell Scraper (BIOFIL, catalog number: CSC011025)
7. Microscope Cover Glass (NEST, catalog number: 801008, Φ15 mm)
8. 5 ml syringe with 26 G needle
9. 20 ml syringe with 18 G needle
10. Mice (C57/BLJ6, Beijing Vital River Laboratory Animal Technology Co., Ltd)
11. RPMI 1640 (Gibco, catalog number: 31800022, 4°C)
12. DMEM/F12 (Gibco, catalog number: 12500062, 4°C)
13. FBS (Hyclone, catalog number: SH30084.03, -20°C/4°C)
14. Nonessential amino acids (Gibco, catalog number: 111140-050, 4°C)
15. Penicillin-streptomycin (Gibco, catalog number: 15140-122, 4°C)
16. PBS (Gibco, catalog number: 21600-069, 4°C)
17. 4% paraformaldehyde (PFA) (Sangon Biotech, catalog number: E672002-0500, 15-25°C (RT))
18. BSA (Sangon Biotech, catalog number: A500023, 4°C)
19. Anti-ASC (AdipoGen, catalog number: AG-25B-0006, -20°C)
20. Saponin (Sigma, catalog number: 47036, 4°C)

21. Fluorescence conjugated secondary antibody (Alexa Fluor™ 488 goat anti-Rabbit) (Invivogen, catalog number: A11008, -4°C)
22. LPS (Invivogen, catalog number: tlrl-smlps, -20°C)
23. ATP (Sigma, catalog number: FLAAS, -20°C)
24. X-fect Transfection Reagent (X-fect polymer, X-fect buffer) (Clontech, catalog number: 631318, -20°C)
25. 4',6-diamidine-2-phenylindole dihydrochloride (DAPI) (CST, catalog number: 8961 S, -20°C)
26. Anti-fluorescence attenuation sealant (Solarbio, catalog number: S2100, 4°C)
27. TWEEN® 20 (Sigma-Aldrich, catalog number: P2287)
28. Cell-neubauer improved (LW Scientific, 0.0025 mm^2)
29. BBL™ Trypticase™ Soy Broth (BD, catalog number: 211768, RT)
30. NaCl (Sangon Biotech, catalog number: A501218-0001, RT)
31. Tryptone (OXOID, catalog number: LP0042, RT)
32. Yeast extract (OXOID, catalog number: LP0021, RT)
33. Agar (SbaseBio, catalog number: A010-1.1, RT)
34. TSB solid media (see Recipes)
35. TSB liquid media (see Recipes)
36. LB solid media (see Recipes)
37. LB liquid media (see Recipes)
38. 1% BSA (see Recipes)
39. 0.1% saponin (see Recipes)
40. 0.1% PBST (see Recipes)

Equipment

1. Opthalmic scissors (Beijing Bao Yuan Industrial Technology, catalog number: M-Y003)
2. Opthalmic forceps (Beijing Bao Yuan Industrial Technology, catalog number: M-Y005)
3. Confocal Microscope (ZEISS, model: LSM880)
4. Microscope (ZEISS, model: Primo vert iLED)
5. Biological Safety Cabinets Clean Benches (ThermoFisher Scientific, model: 1300)
6. CO_2 Incubator (ThermoFisher Scientific, model: 3111)
7. Portable Pipet-Aid (Drummond, catalog number: 4-000-201)
8. Diaphragm vacuum pump (Tianjin Jinteng Experiment Equipment, model: GM-0.33A)
9. Nanophotometer p-class (IMPLEN, model: NT-80)
10. -80°C freezer (Thermo, model: FDE30086FV)

Software

1. ZEN black_2-3SP1 (ZEISS, https://www.zeiss.com/corporate/us/home.html)

2. ZEN blue 2.6 (ZEISS, https://www.zeiss.com/corporate/us/home.html)

Procedure

A. Culture of *Francisella novicida* (U112 strain) and *Salmonella* Typhimurium (SL1344 strain)
   1. *Francisella novicida* (U112)
      a. Recovery of *Francisella novicida*: Streak the frozen *Francisella novicida* stock onto TSB solid media (with 1% L-acetylcysteine) and place in a 37°C incubator for 24 h.
      b. Culture of *Francisella novicida*: Place a single colony of *Francisella novicida* into 5 ml TSB liquid media (with 1% L-acetylcysteine) and grow in 37°C shaker with 200 rpm rotation overnight.
      c. Subculture of *Francisella novicida*: Transfer 3 ml of overnight cultured *Francisella novicida* into 6 ml fresh TSB liquid media (with 1% L-acetylcysteine) and incubate at 37°C while shaking at 200 rpm for 2 h.
      d. Measurement of the concentration of bacteria: Use 1 ml of subcultured *Francisella novicida* to measure the value of OD_{600} with the Nanophotometer p-class (IMPLEN, NT80) and calculate the concentration of bacteria according this equation "1 (OD_{600})=1 × 10^6 CFU/µl".
   2. *Salmonella* Typhimurium (SL1344)
      a. Recovery of *Salmonella* Typhimurium: Streak the frozen *Salmonella* Typhimurium stock onto LB solid media and place in a 37°C incubator for 24 h.
      b. Culture of *Salmonella* Typhimurium: Place a single colony of *Salmonella* Typhimurium into 3 ml LB liquid media and grow in 37°C shaker with 200 rpm rotation overnight.
      c. Subculture of *Salmonella* Typhimurium: Transfer 0.5 ml of overnight cultured *Salmonella* Typhimurium into 2.5 ml fresh LB liquid media and incubate at 37°C while shaking at 200 rpm for 2 h.
      d. Measurement of the concentration of bacteria: Use 1 ml of subcultured *Salmonella* Typhimurium to measure the value of OD_{600} with the Nanophotometer p-class (IMPLEN, NT80) and calculate the concentration of bacteria according to this equation "1 (OD_{600})=1 × 10^6 CFU/µl".

B. Preparation of L929 supernatant
   1. Seed 5 × 10^5 L929 cells in 10 cm Petri dish.
   2. Culture L929 cells in the incubator (37°C, 5% CO_2) for 7 days in RPMI 1640 media supplemented with 10% FBS.
   3. Collect the supernatant with a pipette into a 50 ml centrifuge tube.
   4. Centrifuge at 1,962 × *g* for 10 min and then transfer the supernatant with a pipette into a new 50 ml centrifuge tube. Freeze at -80°C.

C. Generation of BMDMS

1. Sacrifice the mice and spray 75% alcohol on their whole body. Remove the skin and fur from the hind legs and cut off the hind legs after the hip joint. Place in PBS.
2. Remove the muscle tissue from the legs and cut off both ends from the tibia and femur (Video 1).

Video 1. Generation of BMDMs. (This video was made at the Kunming Institute of Zoology according to guidelines from the Kunming Institute of Zoology on Animal Care and was approved by the Animal Research Ethics Board of the Kunming Institute of Zoology under protocol SMKX-20,6020.)

3. Flush the bone marrow (BM) cells with L929 supernatant-containing DMEM/F-12 media (1:3) supplemented with 10% FBS, 1% nonessential amino acids, and 1% penicillin-streptomycin using a 5 ml syringe with a 26 G needle. Aspirate the flushed bone marrow several times through a 20 ml syringe with an 18 G needle to obtain a single cell suspension.
4. Culture BM cells in the incubator (37°C, 5% CO_2) for 5 days. Use 10 ml media for each Petri dish and add 5 ml of fresh L929 supernatant-containing DMEM/F-12 media to the Petri dish on day 3.

D. Preparation of cell slides
1. Place autoclaved cover slips on a 12-well plate.
2. Wash three times with PBS, for 3 mins each time.
3. Scrape the cells from the culture dish with a cell scraper, resuspend in 10 ml DMEM/F-12 media with 10% FBS, and count cells using a cell counting chamber.
4. Seed 5 × 10^5 BMDMs into 12-well plates and incubate at 37°C and 5% CO_2 overnight. Make sure the cell confluency the next day is around 60%.

E. Cell stimulation and fixation
1. Change media with 500 µl of fresh DMEM/F-12 media without L929 supernatant before treatment.
2. Treat BMDMs with different conditions, as follows, and incubate at 37°C and 5% CO_2.

a. Stimulate BMDMs with 2 µl of LPS (500 ng/ml, 4 h) and 2 µl of ATP (5 mM, 20 min).
b. Transfect BMDMs with dsDNA (2 µl of 1.5 µg vector control plasmid) using the X-fect transfection reagent (X-fect polymer 0.45 µl and X-fect buffer 100 µl) for 15 min.
c. Infect BMDMs with10 µl of *Francisella novicida* U112 strain (100 MOI, 12 h).
d. Infect BMDMs with10 µl of *Salmonella* Typhimurium (3 MOI, 1 h).

3. Remove medium by suction from each well and wash three times by gently swirling with PBS.
4. Remove PBS by suction and fix with commercial 4% paraformaldehyde for 15 min at RT.
5. Remove paraformaldehyde by suction and wash the fixed BMDMs on the cover slip by gently swirling with 500 µl PBS three times.

F. Immunofluorescence staining

1. Block and permeabilize the cells in 250-300 µl 1% BSA with 0.1% saponin at 15-25°C (RT) for 1 h.
2. Remove block buffer by suction and gentle swirling of the cells with PBS three times for 3 min each time.
3. Incubate the cells in 300 µl primary antibody solution (anti-ASC in 1% BSA, 0.1% saponin, 1:150 dilution) at 4°C overnight.
4. Wash the cells three times with 500 µl 0.1% PBST on a rocking platform, for 3 min each time.
5. Stain the cells with fluorescence conjugated secondary antibody in PBS (1:300 dilution) at 15-25°C (RT) for 1 h.
6. Wash the cells three times with 500 µl 0.1% PBST on a rocking platform, for 3 min each time.
7. Stain the cells with 250-300 µl DAPI (5 µg/ml, prepared in PBS) at 15-25°C (RT) for 5-10 min.
8. Wash the cells three times with 0.1% PBST on a rocking platform, for 3 min each time.

G. Immobilization of cover slip

1. Remove the cover slip from the 12-well plate with tweezers and air dry.
2. Drop anti-fluorescence attenuation sealant into the cell-containing side.
3. Attach the cell-containing side of the cover slip to microscope slides (25 × 75 mm). Drop sealant onto the edge of the cover slip to bind the cover slip and microscope slide together and prevent sample drying. Avoid air bubbles and air dry for 10 min at 15-25°C (RT).

H. Confocal scanning and image acquisition

1. Scan cells on the ZEISS-LSM880 confocal microscope (100×, the oil-immersion lens). Perform the examination with the ZEISS-LSM880 confocal laser scanning microscope using the following settings: excitation 488 nm and emission 530 nm for ASC speck detection, normal scanning speed, and frame 1024 × 1024. The ASC speck formation was detected and illustrated in Figure 1. Image acquisition was performed with the software ZEN black_2-3SP1.
2. Analyze the data by using the software ZEN blue 2.6 (Protocol in supplementary material).

Recipes

1. TSB solid media (1 L)
   30 g BBL™ Trypticase™ Soy Broth
   15 g Agar
   Autoclave at 121°C for 15 min.
   Add L-acetylcysteine (1% volume ratio) to the autoclaved media when the temperature is around 60°C.
2. TSB liquid media (1 L)
   30 g BBL™ Trypticase™ Soy Broth
   Autoclave at 121°C for 15 min.
   Add L-acetylcysteine (1% volume ratio) to the autoclaved media when the temperature is around 60°C.
3. LB solid media (1 L)
   10 g NaCl
   10 g Tryptone
   5 g Yeast extract
   15 g Agar
   Autoclave at 121°C for 15 min
4. LB liquid media (1 L)
   10 g NaCl
   10 g Tryptone
   5 g Yeast extract
   Autoclave at 121°C for 15 min
5. 1% BSA
   1 g BSA
   100 ml PBS
6. 0.1% saponin
   0.1 g saponin
   100 ml PBS
7. 0.1% PBST
   1 ml TWEEN® 20
   1 L PBS

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Competing interests

The authors declare no conflicts of interests.

Ethics

The present study was approved by the institutional review board of the Kunming Institute of Zoology, Chinese Academy of Sciences. Animal experiment in this protocol: SMKX-2016020; validity period: January 2017 to January 2022.

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