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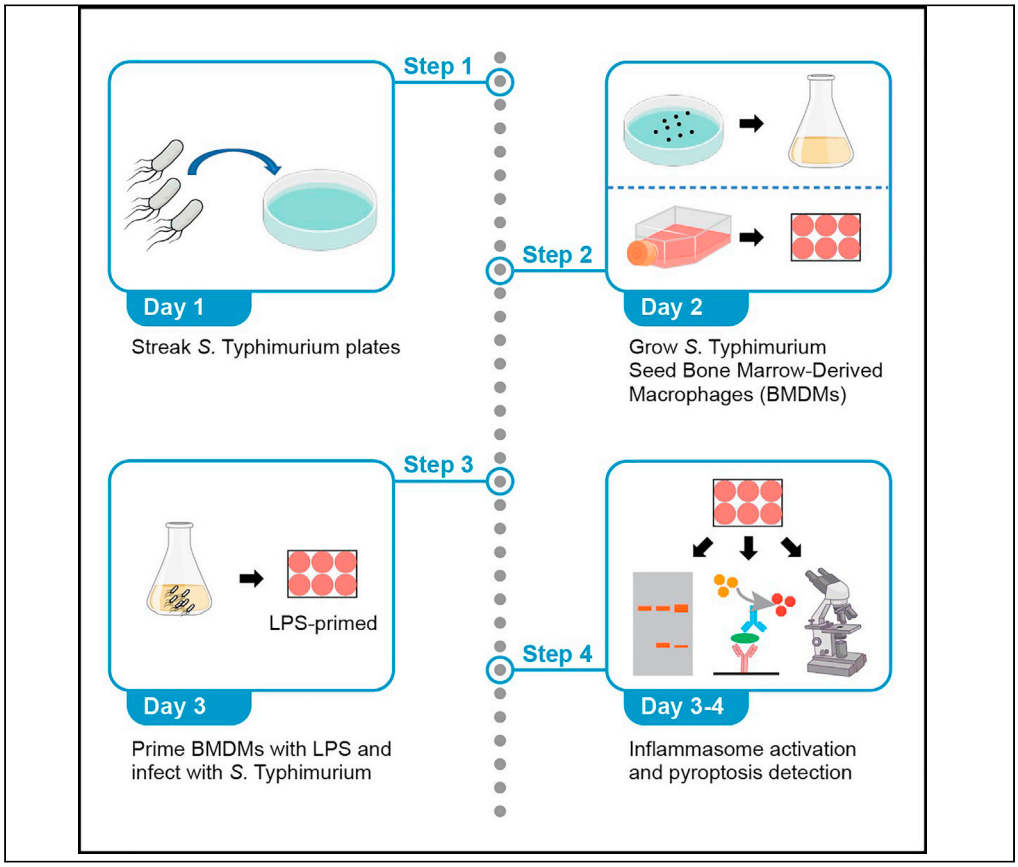
Protocol for measuring NLRC4 inflammasome activation and pyroptosis in murine bone-marrow-derived macrophages



NLR family CARD domain containing protein 4 (NLRC4) inflammasome activation and the associated pyroptosis are critical for protection against infection by bacterial pathogens. This protocol presents a detailed procedure to activate and measure NLRC4 inflammasome activation and pyroptosis upon *Salmonella Typhimurium* infection. The techniques can be adapted to monitoring the activation of other types of inflammasomes and pathogenic stimuli.

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Highlights

Detailed steps to obtain log-phase *S. Typhimurium* for NLRC4 inflammasome activation

Reproducible procedures for detection of Caspase-1 and IL-1β from culture supernatant

Procedures to identify the oligomerization of ASC

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Protocol for measuring NLRC4 inflammasome activation and pyroptosis in murine bone-marrow-derived macrophages

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SUMMARY

NLR family CARD domain containing protein 4 (NLRC4) inflammasome activation and the associated pyroptosis are critical for protection against infection by bacterial pathogens. This protocol presents a detailed procedure to activate and measure NLRC4 inflammasome activation and pyroptosis upon *Salmonella Typhimurium* infection. The techniques can be adapted to monitoring the activation of other types of inflammasomes and pathogenic stimuli.

For comprehensive details on the use and execution of this protocol, please refer to Dong et al. (2021).

BEFORE YOU BEGIN

This protocol describes specific steps to monitor the activation of NLRC4 inflammasome and the associated pyroptosis in bone marrow-derived macrophages (BMDMs) upon *Salmonella Typhimurium* (*S. Typhimurium*) infection. Therefore, BMDMs and *S. Typhimurium* strain should be ready before you begin.

BMDMs isolation and culture

Timing: 7 days

1. To differentiate the murine bone marrow cells into BMDMs, we cultured bone marrow cells in DMEM/F12 with 10% FBS, L-Glutamine (2 mM), Penicillin/Streptomycin (1:100), HEPES buffer (10 mM), and 20% L929 conditional medium in sterile plastic Petri dishes. Please refer to *Curr. Protoc. Immunol.* (Zhang et al., 2008) for the protocol of isolation and culture of murine BMDMs.

   Note: We consistently isolated around 50–60 million bone marrow cells from one 8–12 weeks old mouse after red blood cells lysis. We cultured bone marrow cells in 10 cm Petri dishes with 5 million cells per dish. While bone marrow cells or BMDMs can be cryopreserved in liquid nitrogen with 90% FBS and 5% DMSO, we used freshly prepared cells for our experiments.

   Note: If L929 conditional medium is not available, 20 ng/ml macrophage colony-stimulating factor (M-CSF) can be used for BMDMs differentiation. However, the yield of macrophages is less when recombinant M-CSF is used.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse Polyclonal anti-IL1β	R&D system	AF-401-NA
Mouse Monoclonal anti-Caspase-1 (p20)	Adipogen	AG-20B-0042
Rabbit Polyclonal anti-ASC	Adipogen	AG-25B- 0006-C100
Rabbit Monoclonal anti-GSDMD	Abcam	ab209845
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, FITC	Thermo Fisher Scientific	F-2765
Bacterial and virus strains		
Salmonella Typhimurium	ATCC	14028
Chemicals, peptides, and recombinant proteins		
Lipopolysaccharides from Escherichia coli O111:B4	Millipore Sigma	L2630
Disuccinimidyl suberate (DSS)	Thermo Fisher Scientific	21655
M-CSF	PeproTech	315-02
Pam3csk4	InvivoGen	tlrl-pms
Critical commercial assays		
IL-1β Mouse Uncoated ELISA Kit	Invitrogen	88-7013-88
Experimental models: Cell lines		
Bone marrow-derived macrophages (BMDMs)	Isolated from C57BL/6J mice	N/A
L929 cell line	ATCC	CCL-1
Software and algorithms		
GraphPad prism 9	GraphPad	https://www.graphpad.com/
Image J	(Schneider et al., 2012)	https://imagej.nih.gov/ij/
Other		
DMEM/F12	Cell Medium Facility, UIUC	N/A
FBS	Sigma	F2442-500ML
Penicillin-Streptomycin	Fisher Scientific	MT30002CI
HEPES	Fisher Scientific	MT25060CI
L-Glutamine	Fisher Scientific	MT25005CI
Petri Dishes with Clear Lid	Fisher Scientific	FB0875712
LB broth	Fisher Scientific	BP97235
LB Agar, powder	Thermo Fisher Scientific	22700025
2× Laemmli Sample Buffer	Bio-Rad	1610737
Methanol	Fisher Scientific	A412-500
Chloroform	Millipore Sigma	C2432-500ML
Pierce Protease Inhibitor Mini Tablets	Thermo Fisher Scientific	A32953
DAPI Solution	Thermo Fisher Scientific	62248
Mowiol 4-88	Millipore Sigma	475904-100GM-M
1,4-Diazabicyclo[2.2.2]octane	Millipore Sigma	D27802-25G
Glycerol	Millipore Sigma	G5516-500ML
Tween-20	Millipore Sigma	P1379-500ML
Cover glass	Fisher Scientific	12-541A
Western blot running and transfer system	Bio-Rad Laboratories	1658001FC
Light microscope	Thermo Fisher Scientific	AMF5000
Confocal microscope	Carl Zeiss AG	ZEISS LSM 510
Microcentrifuge (22°C and 4°C)	Thermo Fisher Scientific	75002432
Cell/bacteria culture incubator	Fisher Scientific	11-676-600
Water bath	Fisher Scientific	Isotemp 215
Hemocytometer	Fisher Scientific	0267151B
Biosafety cabinet	Fisher Scientific	1300 Series A2

MATERIALS AND EQUIPMENT

TBS Buffer		
Reagent	Final concentration	Amount
Tris-HCL, pH 7.4 (1 M)	50 mM	2.5 mL
NaCl (2.5 M)	150 mM	3 mL
Triton X-100	0.5% (v/v)	0.25 mL
Protease Inhibitor Mini Tablets	n/a	5 tablets
ddH_2O	n/a	44.75 mL
Total	n/a	**50 mL**
Store at 4°C. Add one tablet to 10 mL buffer right before use.		

Mounting Medium		
Reagent	Final concentration	Amount
Tris, pH 8.5 (1 M)	100 mM	5 mL
DAPI Solution (1 mg/mL)	0.3 μg/mL	15 μL
Mowiol 4-88	10% (w/v)	5 g
1,4-Diazabicyclo [2.2.2] octane	1% (w/v)	0.5 g
Glycerol	25% (v/v)	12.5 mL
ddH_2O	n/a	32.5 mL
Total	n/a	**50 mL**
Store at −20°C for 12 months. The medium remains stable for 1 month when stored at 4°C.		

Other buffers:	
Name	Composition
PBS (1×), 1 L	137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.47 mM KH2PO4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH adjusted to 7.2 with HCl
PBST	PBS with 0.02% Tween-20 (v/v)
Immunofluorescence Block buffer	PBST with 5% BSA (w/v)

STEP-BY-STEP METHOD DETAILS

Timing: 3 days for step 1

Timing: 24 h for step 2

Timing: 1 h for step 3

Timing: 15 min for step 4

Timing: 24 h for step 5

Timing: 24 h for step 6

1. Prepare *S. Typhimurium* for infection
   This step describes the details to prepare the log-phase *S. Typhimurium* for activating NLRC4 inflammasome in BMDMs.
   a. On day one, streak *S. Typhimurium* on a Lennox Broth (LB) plate with aseptic technique and place it in an incubator at 37°C 16 h.

b. On day two, seal the LB plate with parafilm in the morning and store it at 4°C to avoid the over-growth of *S. Typhimurium* growth on the plate. In the evening, pick one colony into 5 mL sterile LB broth and grow the *S. Typhimurium* in the incubator at 37°C for 16 h with shaking (200 rpm).
c. On day three, sub-culture *S. Typhimurium* at 1:100 (add 20 μL bacterial culture into 2 mL fresh LB broth) for an additional 4–6 h with shaking (200 rpm) at 37°C.
d. Collect *S. Typhimurium* by centrifuging at 200 g for 3 min at 22°C.
e. Remove supernatant and wash the *S. Typhimurium* pellet twice with sterile PBS.
f. Resuspend the *S. Typhimurium* pellet with 1 mL sterile PBS and measure the OD600.

⚠ CRITICAL: The sub-culture of *S. Typhimurium* at 37°C for an additional 4–6 h (Step c) is essential for inflammasome activation. During this period, *S. Typhimurium* reaches the log-phase of growth for the maximum expression of *Salmonella* pathogenicity island 1 (SPI-1), which activates NLRC4 inflammasome.

2. Seed BMDMs and LPS priming

This step describes how to seed and prime BMDMs with LPS before *S. Typhimurium* infection.

a. On day two, after 7 days of BMDMs differentiation, aspirate off the BMDM culture medium and add 5 mL sterile PBS to the Petri dish.
b. Gently detach the BMDMs from the Petri dish by pipetting with a 1 mL pipette.

Note: BMDMs can be easily detached from the Petri dishes by pipetting. If tissue culture dishes are used for BMDM differentiation, cell can be collected with cell scraper by pre-treating with EDTA disassociation buffer (10 mM EDTA in PBS) for 10–15 min, followed by trypsin (0.25%) treatment for 2 min. A detailed comparison between these collecting methods is described in *J. Immunol Methods* (Chen et al., 2015).

c. Count the cell numbers with a hemocytometer and seed 1.5×10^6 BMDMs per well in a 6-well plate in DMEM/F12 with 10% FBS, L-Glutamine (2 mM), Penicillin/Streptomycin (1:100), HEPES buffer (10 mM). Culture BMDMs in a cell culture incubator for 16 h.

Note: Adjust the cell numbers accordingly if other cell culture plates are used. We seed 1.0×10^6 BMDMs per well in a 12-well plate, and 0.5×10^6 BMDMs per well in a 24-well plate.

d. On day three, prime BMDMs with 0.5 μg/mL LPS (dissolved in endotoxin-free water) for 4 h before *S. Typhimurium* infection.

Note: LPS priming for NLRC4 inflammasome activation is not always necessary. *S. Typhimurium* infection could significantly increase the protein level of IL-1β as early as 30 min, making LPS priming less critical in activating the NLRC4 inflammasome. Nevertheless, we recommend priming BMDMs, especially when your gene of interest could affect *Il1b* and *Il18* expression independent of NLRC4 inflammasome activation during *S. Typhimurium* infection.

e. After LPS priming for 4 h, wash the BMDMs 3 times with sterile PBS. Change to the FBS-free medium before infection.

Note: Intracellular LPS can activate non-canonical NLRP3 inflammasome; therefore, PBS washes after LPS priming is necessary. Alternatively, you can prime BMDMs with a TLR2 ligand, such as Pam3csk4.

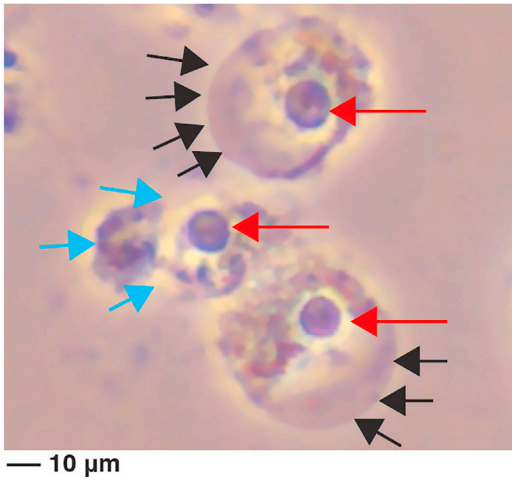

Figure 1. Morphological characteristics of pyroptotic cells under a light microscope

LPS-primed BMDMs were infected with *S. Typhimurium* for 1 h (MOI, 10). The pyroptotic cells can be identified with protruding nuclei (red arrow), bubble-like protrusion (black arrow), and cell membrane rupture (cyan arrow).). Scale bar, 10 μm

⚠ CRITICAL: Changing to FBS-free media before the infection is important since it helps reduce the number of non-specific proteins from FBS when precipitating proteins from cell culture supernatants.

3. Infection of BMDMs with *S. Typhimurium*

This step describes the final step of infection and how to identify pyroptotic cells after inflammasome activation.

a. Infect the primed BMDMs with *S. Typhimurium* at a multiplicity of infection (MOI) of 10. The simple conversion to determine the volume of *S. Typhimurium* added to one well at a certain Multiplicity of Infection (MOI) is described as:
MOI × Cell number/well = OD600 × (1.5×10^9) × Volume (ml)

Note: Log-phase *S. Typhimurium* expressing SPI-I activates NLRC4 inflammasomes after 30 min of infection at an MOI of 10, and about 80% BMDMs undergo inflammasome-induced pyroptosis within 1 h (Figure 1).

4. Cell culture supernatant protein precipitation

This step describes the detailed methanol-chloroform protein precipitation method to detect cleaved caspase-1 and cleaved IL-1β in the cell culture supernatant.

Inflammasome activation features the cleavage of pro-caspase-1, pro-IL-1β, and gasdermin-D (GSDMD) into their functional forms and can be detected by western blot. Western blot is used to detect cleaved caspase-1 (20 kDa) and GSDMD (35 kDa) in the cells, as well as secreted caspase-1 (20 kDa) and IL-1β (17 kDa) in the cell culture supernatant. We provide a reproducible cell culture supernatant protein precipitation method for the detection of secreted cleaved caspase-1 and cleaved IL-1β by western blot. Secreted IL-1β in the supernatant can also be used by ELISA.

a. After the infection, collect 1 volume of supernatant (e.g., 600 μL) into a 1.5 mL Eppendorf tube. Add $^1/_4$ volume (150 μL) of chloroform and 1 volume of methanol (600 μL) to the supernatant and flip the tubes to mix.
b. Centrifuge at 13,800 *g* for 5 min at 22°C.
c. Aspirate off the top layer without disrupting the protein layer in the middle and add another 1 volume of methanol (600 μL).
d. Centrifuge at 13,800 *g* for 5 min at 22°C. The protein pellet should be visible at the bottom of the tube.
e. Remove the supernatant and air dry the pellet for 5 min.
f. Boil the pellet in 1× laemmli buffer before subject to western blot.

Note: Since the cleaved caspase-1 and IL-1β have low molecular weights, we recommend using at least 12% SDS-PAGE to make sure that these small proteins don't run out of the gel. We usually ran SDS-PAGE at a constant 100 V for 60 mins and transfer at a constant 400 A for 60 min.

5. Detect the oligomerization of apoptosis-associated speck-like protein containing a CARD (ASC).
   a. After the infection, lyse the cells with 200 ul TBS buffer for 30 min on a rocker at 4°C.
   b. Collect the cells with cell scrapers and centrifuge at 6,000×*g* at 4°C for 15 min. Collect the Triton X-100 soluble fraction (lysate) as ASC western blot loading control.
   c. Wash the Triton X-100 insoluble fraction (pellet) twice with PBS (6,000×*g* at 4°C for 5 min) and re-suspend the pellet with 300 ul TBS buffer.
   d. Break the pellet by vigorous pipetting and vortexing.
   e. Add disuccinimidyl suberate (DSS) to a final concentration of 4 mM and incubate in a water bath at 37°C for 30 min. You will observe precipitation during the crosslinking. Gently vortex the tube periodically.
   f. After crosslinking, centrifuge at 6,000×*g* for 15 min at 4°C and dissolve the pellet in 1× laemmli buffer before subject to western blot.

6. Detect ASC specks by immunofluorescence staining.
   a. Before seeding BMDMs, autoclave square-shape cover glasses and put them into wells of a 6-well plate. Seed BMDMs at a lower density (5×10^5/well). Infect the cells with *S. Typhimurium* as described above.
   b. After infection, gently move the cover glasses to the wells of a new 6-well plate. Fix and permeabilize cells with 100% methanol (prechilled at −20°C) at 22°C for 5 min.

   Note: No additional permeabilization step is required when methanol is used to fix the cells since 100% methanol fixes and permeabilizes cells at the same time. Alternatively, cells can be fixed and permeabilized with 4% paraformaldehyde (in PBS) and 0.5% TritonX-100 (in PBS), respectively, for 10 min at 22°C.

   c. Wash the cells with PBS 3 times and incubate the cells with block buffer for 30 min at 22°C.
   d. Dilute ASC antibody (2 μg/mL) in PBST and incubate at 4°C 10–16 h without shaking.
   e. Wash the cells with PBS 3 times and incubate the cells with goat anti-rabbit FITC secondary antibody (2 μg/mL) for 1 h at 22°C in the dark.
   f. Mount the cells with the mounting medium containing DAPI (0.3 ug/mL) and capture the images with a fluorescent or confocal microscope. We used the Zeiss LSM 510 Meta confocal microscope.

   Note: Besides FITC, any appropriate fluorophore conjugated goat anti-rabbit antibody can be used for the staining.

EXPECTED OUTCOMES

Western blot is the most widely used method to detect inflammasome activation with the cleaved Caspase-1 and IL-1β in cell culture supernatant (Figure 2A). Cleaved form of Caspase-1 could be detected in both cell extracts and supernatant when inflammasome is activated with small amount of pro-caspase-1 in the supernatant (Figure 2A). Cleaved IL-1β could be readily detected in the supernatant with small amount of pro- IL-1β (Figure 2A). Cleaved GSDMD forms pores on cell membrane and is recognized as the executor of pyroptosis. Therefore, cleaved GSDMD in western blot directly indicates pyroptosis and should be readily detected in the cell extracts (Figure 2B).

ASC oligomers function as platforms for recruiting pro-caspase-1 during inflammasome activation. Therefore, ASC oligomerization is considered as one of the hallmarks of inflammasome activation and can be detected by western blot (Figure 3A) and immunofluorescence (Figure 3B).

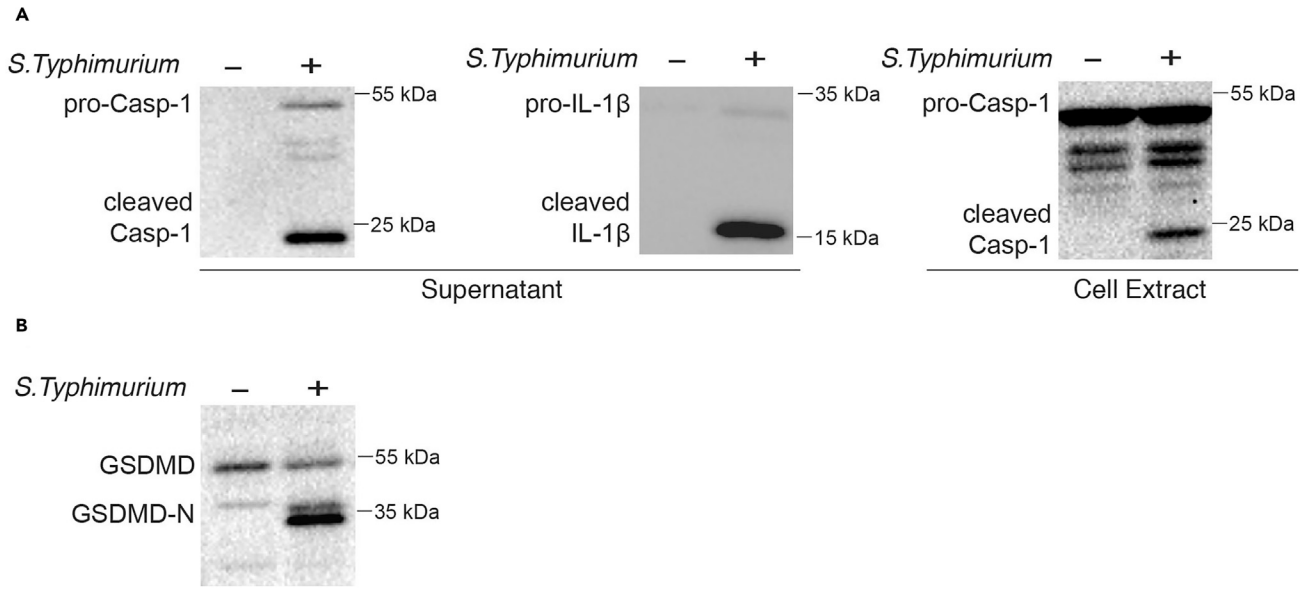

Figure 2. Inflammasome activation detected by western blots

(A and B) LPS-primed BMDMs were infected with *S. Typhimurium* for 1 h (MOI, 10). Cleaved caspase-1, IL-1β in the cell culture supernatant, and cleaved caspase-1 in the cell extract indicates inflammasome activation (A). Cleaved GSDMD in the cell extract implies pyroptosis occurrence (B). Scale bar, 20 μm.

LIMITATIONS

Multiple inflammasomes have been identified thus far, including NLRP3, Aim2, Pyrin, and others (Karki and Kanneganti, 2019). Although they share a similar mechanism of activation, they respond differently to distinct stimuli. Therefore, this protocol is limited to NLRC4 inflammasome activation by *S. Typhimurium*. Other bacteria, such as *Pseudomonas aeruginosa* or *Shigella flexneri*, can also activate NLRC4 inflammasome; however, the protocol for bacteria culture and infection may differ from that presented here for *S. Typhimurium*. Different *S. Typhimurium* strains are also known to activate NLRC4 inflammasome at different time points via distinct mechanisms. Despite the diversity of stimuli, the preparation of BMDMs and the detection of inflammasome activation described in this protocol can be adapted to detect all other types of inflammasomes.

TROUBLESHOOTING

Problem

Failure to observe the characteristics of pyroptotic cells under the microscope (Figure 1).

Potential solution

Failure to activate the NLRC4 inflammasome by the stationary *S. Typhimurium*, which SPI-1 expression is inhibited, could be one of the reasons. Therefore, it is essential to use the log-phase *S. Typhimurium* in order to successfully activate NLRC4 inflammasome.

In addition, in the late stage of pyroptosis, the bubble-like protrusions burst eventually. This might lead to the failure of detecting the bubble-like structure for some pyroptotic cells. However, you can still check cleaved Caspase-1, IL-1β, and GSDMD signal by western blot to ensure a successfully inflammasome activation and pyroptosis.

Problem

Weak signals of cleaved Caspase-1 and cleaved IL-1β (Step 4).

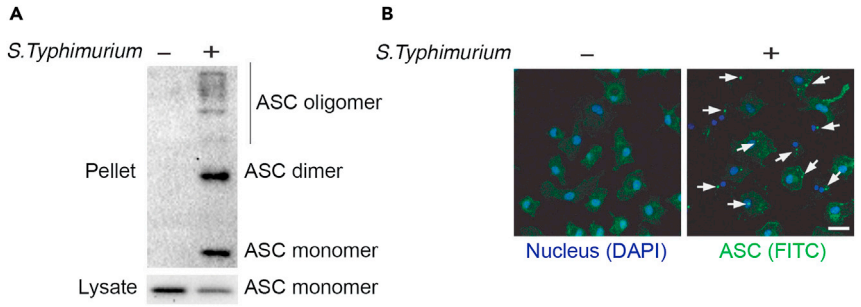

Figure 3. ASC oligomer formation during inflammasome activation

(A and B) LPS-primed BMDMs were infected with *S. Typhimurium* for 1 h (MOI, 10). Triton X-100 soluble (lysate) and insoluble (pellet) fractions were immunoblotted with ASC antibody (A). Confocal microscopy of BMDMs infected with *S. typhimurium* for 1 h (MOI, 10) staining for ASC (FITC) and nucleus (DAPI). ASC specks are marked with arrows (B).

Potential solution

During inflammasome activation, BMDMs undergo a hyperactivation state before pyroptosis, where most of the IL-1β secreted through the pores formed by GSDMD-N fragments on the cell membrane. Therefore, the IL-1β level in the cell extract is always low and hard to detect. To our experience, the cleaved caspase-1 levels are relatively even between cell extract and culture supernatant. Therefore, we recommend doing western blots against cleaved caspase-1 and cleaved IL-1β from the cell culture supernatant.

Problem

Non-specific inflammasome activation signal in uninfected control (Step 4).

Potential solution

Intracellular LPS can activate Caspase 11-dependent non-canonical NLRP3 inflammasome. If BMDMs are primed with LPS before infection, it is necessary to wash the cells sufficiently to remove LPS. Otherwise, the signals could be mixed signals from both non-canonical NLRP3 and NLRC4 inflammasomes. This can be evidenced by inflammasome activation in uninfected control cells. To completely eradicate the possibility of non-canonical NLRP3 inflammasome activation, the TLR2 ligand Pam3csk can be used to prime the cells.

Problem

Failure to detect inflammasome activation when using other stimuli.

Potential solution

The time needed to induce the activation of inflammasomes varies significantly with different stimuli. To determine the best time point for inflammasome activation, we recommend checking BMDMs visually for pyroptotic cells every 30 min to ensure that the optimal time points for the detection of inflammasome activation won't be missed.

LPS priming is another way to secure a successful detection of cleaved caspase-1 and cleaved IL-1β. LPS priming increase pro-caspase-1 and pro-IL-1β drastically, and this provides cells with sufficient pro-caspase-1 and its substrate pro-IL-1β before inflammasome activation.

Problem

No ASC oligomer in western blot (Step 5e).

Potential solution

Failure to detect ASC oligomer is mainly due to the inefficient DSS crosslinking. If DSS crosslinking fails, all the oligomers would break down to monomer during the SDS-PAGE.

The critical step that ensures the detection of ASC oligomers by western blot is to break the Triton X-100 insoluble (pellet) fraction as much as possible before crosslinking. In addition, it is necessary to gently vertex the tube periodically during crosslinking.

Failure to activate the NLRC4 inflammasome could be another reason. We recommend doing a test with immunofluorescence first (Step 6). This will provide a clear visualization of ASC oligomers, an indication for the successful inflammasome activation.

RESOURCE AVAILABILITY

Lead contact

Lin-Feng Chen (lfchen@illinois.edu)

Materials availability

Requests for resources and reagents should be directed to and will be fulfilled by the lead contact.

Data and code availability

This study did not generate any data sets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Conceptualization and writing – review & editing, X.D. and L.-F.C.; writing – original draft, X.D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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