Published in final edited form as:

Curr Protoc Immunol. 2020 December ; 131(1): e107. doi:10.1002/cpim.107.

Inflammasome assays in vitro and in mouse models

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Abstract

This unit presents assays that allow induction and measurement of different inflammasomes activation in the mouse macrophages and human PBMC (peripheral blood mononuclear cell) cultures and mouse peritonitis and endotoxic shock models. Basic protocol 1 describes how to prime the inflammasome in mouse macrophages with different Toll-like receptor agonists and TNF-α, followed by the induction of NLRP1, NLRP3, NLRC4, and AIM2 inflammasomes activation by their corresponding stimuli, and subsequent measurement of inflammasome activation-mediated maturation of IL-1β and IL-18 and pyroptosis. Since the well-established agonists for NLRP1 are inconsistent between mice and human, basic protocol 2 describes how to activate NLRP1 inflammasome in human PBMCs. Basic protocol 3 describes how to purify, crosslink, and detect ASC pyroptosome. Formation of ASC pyroptosome is a signature of inflammasome activation. A limitation of ASC pyroptosome detection is requiring a relative large cell number. An alternative protocol is provided to stain ASC pyroptosome by the anti-ASC antibody and measure ASC speck by fluorescence microscopy in the single cell. Intraperitoneal injection of LPS plus inflammasome agonists will induce peritonitis that is featured by an elevation of IL-1β in addition to other proinflammatory cytokines and an infiltration of neutrophils and inflammatory monocytes. Basic protocol 4 describes how to induce NLRP3 inflammasome activation and peritonitis by priming mice with LPS and subsequent challenging with MSU The method for measuring cytokines in serum and peritoneal lavage is also described. Finally, an alternative protocol describes how to induce noncanonical NLRP3 inflammasome activation by high-dose LPS challenging in a sepsis model.

Introduction

Inflammasomes are supramolecular complexes that play the pivotal role in mounting inflammation. It is composed of the receptor or sensor molecule NOD-like receptors (NLR) or Absent in melanoma (AIM2)-like receptors, adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and pro-caspase-1, the inflammasome platform

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regulates caspase-1 activation and subsequent interleukin-1β (IL-1β) and IL-18 maturation and inflammatory cell death, pyroptosis (Lamkanfi & Dixit, 2014). While adequate activation of the inflammasome is critical to protect the host from pathogens infection and heal tissue wounds, excessive inflammasome activation causes severe autoinflammatory and autoimmune diseases such as multiple sclerosis and arthritis (Guo, Callaway, & Ting, 2015). Gain-of-function mutations of NLRs lead to constitutive inflammasomes activation and the corresponding devastating inflammatory outcomes (Cordero, Alcocer-Gomez, & Ryffel, 2018; Drutman et al., 2019; Romberg, Vogel, & Canna, 2017; Zhong et al., 2016). Therefore, a comprehensive understanding of how the inflammasome activation is achieved and regulated is critical for developing inflammasomes-based therapeutics for inflammatory diseases.

The most well-characterized inflammasomes include NLRP1, NLRP3, NLRC4/NAIP (neuronal apoptosis inhibitory protein), and AIM2 inflammasomes, named based on their different sensor or receptor proteins (Broz & Dixit, 2016). Due to the significant role in inflammation, the activation of inflammasomes is a fine-tuned process requiring two-step signals. Signal one is the priming step which induces the precursor proteins of IL-1β and IL-18, elevates inflammasome components expression levels and precisely regulates their protein modification, providing the prerequisites for inflammasome complex formation. Signal two induces the activation of inflammasomes, which is specific to each corresponding inflammasome (Broz & Dixit, 2016). NLRP3 is the most extensively studied inflammasome, which can sense a variety of exogenous pathogen associated molecular patterns (PAMP) and endogenous damage associated molecular patterns (DAMP) (Swanson, Deng, & Ting, 2019). A noncanonical NLRP3 inflammasome activation is also documented. Mouse caspase-11 or human caspase-4/5 recognize cytosolic LPS and leads to pyroptosis. The resulted potassium efflux activates NLRP3 inflammasome (Hagar, Powell, Aachoui, Ernst, & Miao, 2013; Kayagaki et al., 2013; Shi et al., 2014; Yang, Wang, Kouadir, Song, & Shi, 2019). NLRC4 itself is not a receptor but associates with the receptors, NIAP, which respond to bacterial flagellin and type III secretion system components (T3SS) (Vance, 2015). AIM2 is a DNA sensing inflammasome, which is responsive to cytosolic bacteria and DNA viruses (V. A. Rathinam et al., 2010). Different from other inflammasomes, NLRP1 requires motif-dependent ubiquitination and subsequent degradation of its N-terminal subunit by proteasome for its activation (Sandstrom et al., 2019; Xu et al., 2019). Due to the sequence difference between human and mouse NLRP1 orthologues, the agonists triggering NLRP1 activation are variable among species (Levinsohn et al., 2012; Mitchell, Sandstrom, & Vance, 2019; Okondo et al., 2017; Zhong et al., 2018).

Activation of inflammasomes leads to self-cleavage of caspase-1 into its active subunit p10 and p20. Active caspase-1 not only cleaves pro-IL-1β and pro-IL-18 to their bioactive form but also cleave gasdermin D (GSDMD) into two parts, within which the N-terminus oligomerizes to form the pore on the cell plasma membrane and cause pyroptosis. Mature IL-1β, IL-18 and many other cellular contents are released into the extracellular spaces via GSDMD-formed pore (Evavold et al., 2018; He et al., 2015; Shi et al., 2015). Therefore, detection of the released mature IL-1β and IL-18 by ELISA and more definitively by immunoblot is a common surrogate measurement for determining inflammasome activation (Mariathasan et al., 2006). A common complementary method for this is the detection of

pyroptosis by measuring released lactate dehydrogenase (LDH) activity (Rayamajhi, Zhang, & Miao, 2013). During the inflammasome activation, the adaptor protein ASC will oligomerize into a filament structure, named pyroptosome (Fernandes-Alnemri et al., 2007). Two elegant approaches have been developed to observe the formation of ASC pyroptosome: (1) purify and crosslink ASC pyroptosome followed by a detection of ASC oligomers by immunoblot; and (2) immunofluorescence stain ASC pyroptosome (ASC speck) and observe ASC foci via fluorescence microscopy (Fernandes-Alnemri et al., 2007). Many animal models for studying inflammasomes activation and their functions have been established, among which peritonitis and endotoxic shock are simple procedures that have straightforward readouts. In the mouse peritonitis model, the inflammasome activation induces IL-1β and IL-18 and many other inflammatory cytokines and chemokines and inflammatory cells infiltration (Deng et al., 2019). In the endotoxic shock model, high-dose LPS activate caspase-11-mediated noncanonical inflammasome activation and associated lethality (Kayagaki et al., 2013).

In this report, we provided detailed protocols for priming and activating different inflammasomes with a variety of PAMPs and DAMPs in mouse macrophages (Basic protocol 1). As aforementioned, agonists for NLRP1 are inconsistent between mice and human. Therefore, we provide a separate protocol for activating NLRP1 inflammasome in human PBMC (Basic protocol 2). In Basic protocol 3, we provide a detailed protocol for purifying and detecting ASC pyroptosome, an important marker for inflammasome activation. In addition, we describe an alternative protocol to detect ASC pyroptosome by indirect fluorescence staining (Alternate protocol 1), which allows a single cell resolution for detecting inflammasome activation. Finally, we provide the protocol for inducing canonical NLRP3 inflammasome activation and associated peritonitis in the mouse model (Basic protocol 4). Alternatively, a method for inducing noncanonical NLRP3 inflammasome activation in a high-dose LPS mediated sepsis model is also provided (Alternate protocol 2). The protocol setup works well for us, but we also provided notes and comments whenever further optimizations and testing are recommended.

Basic protocol 1: Prime and activate inflammasomes in mouse macrophages

This protocol describes the method for priming mouse bone marrow derived macrophage (BMDM) and inducing NLRP3, NLRC4, NLRP1, and AIM2 inflammasome activation. BMDMs are first primed by the agonists for a variety of Toll-like receptors and induced activation of canonical NLRP3 inflammasome by ATP, Nigericin, Silica, monosodium urate (MSU), Alum, and phospholipid platelet activating factor receptor (PAF) and lysophosphatidylcholine (LPC), activation of noncanonical NLRP3 inflammasome by LPS transfection, activation of NLRC4 inflammasome by flagellin transfection, activation of NLRP1 inflammasome by lethal toxin (LT), and activation of AIM2 inflammasome by poly (dA:dT) transfection. The inflammasomes activation are assessed via detecting IL-1β and IL-18 by ELISA, cleaved IL-1β, IL-18, caspase-1, and gasdermin-D by immunoblot, and pyroptosis by LDH assay. The caspase-11 activation in the LPS-transfection condition is

assessed by measuring fluorescent substrate intensity and immunoblot of active caspase-11 subunit p26 (Lee et al., 2018; Shi et al., 2014).

Materials

Mouse bone marrow-derived macrophages (BMDM)

Complete Dulbecco's Modified Eagle Medium (DMEM): DMEM (11995065, Gibco) containing 10% (v/v) fetal bovine serum (FBS) (F2442, Sigma-Aldrich) and 1% (v/v) penicillin/streptomycin (15140122, Gibco)

Opti-MEM™ I Reduced Serum Medium (31985070, Gibco) and Opti-MEM™ Reduced Serum Medium containing GlutaMAX™ Supplement (51985034, Gibco)

Stimuli: Ultrapure LPS, *E. coli* 0111:B4 (tlrl-3pelps, InvivoGen), Pam3CSK4, HKLM, FLA-ST, FSL-1, ssRNA40, ODN1826 (Mouse TLR1–9 Agonist kit, tlrl-kit1mw, InvivoGen), recombinant murine TNF-α (315–01A, Peprotech), ATP (tlrl-atpl, InvivoGen), Nigericin (tlrl-nig, InvivoGen), MSU crystals (tlrl-msu, InvivoGen), silica (MIN-U-SIL, U. S. Silica), Imject™ Alum (77161, Thermo Fisher Scientific), PAF C16 (2940, Tocris Bioscience), LPC (16:0) (L5254, Sigma-Aldrich), *S. typhimurium* flagellin (tlrl-stfla, InvivoGen) *Baillus anthracis* anthrax lethal factor (172B, List Biological Laboratories), and poly (dA:dT) (tlrl-patn, InvivoGen)

Transfection reagent: FuGENE HD (E2311, Promega), DOTAP (11202375001, Sigma-Aldrich), and Lipofectamine™ 2000 (11668027, Thermo Fisher Scientific)

HEPES-buffered saline (HBS): 20 mM HEPES, cell culture grade, 150 mM NaCl, pH 7.4

Mouse IL-1β ELISA Kit (432604, BioLegend) and mouse IL-18 ELISA kit (7625, R&D Systems)

Cytotoxicity Detection Kit (LDH) (11644793001, Sigma-Aldrich)

Ac-Leu-Glu-Val-Asp-7-Amino-4-methylcoumarin (Ac-LEVD-AMC) (ALX-260–083, Enzo Life Sciences)

RIPA buffer: 50 mM Tris-HCl, pH7.4, 150 mM NaCl, 0.5% (g/ml) Sodium Deoxycholate, 0.1% (g/ml) Sodium Dodecyl Sulfate (SDS), 1% (v/v) Nonidet P-40 Substitute (NP-40), containing 1 × cOmplete™ Protease Inhibitor Cocktail (11697498001, Sigma-Aldrich)

Caspase assay buffer: 50 mM HEPES pH7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA and 10% glycerol

Antibodies: anti-IL-1β (AF401-NA, R&D Systems), anti-caspase-1 p10 (AG-20B-0044-C100, Adipogen), anti-NLRP3 (AG-20B-0014-C100, Adipogen), anti-ASC (AG-25B-0006-C100, Adipogen), anti-GSDMD (ab209845, Abcam), and anti-β-actin-HRP (sc-1615, Santa Cruz Biotechnology), anti-caspase-11 (EPR18628, Abcam)

Centrifuge

96-well black plate (Costar)

PerkinElmer EnSpire Multimode Plate Reader

NuPAGE™ 4 to 12% protein gel (Thermo Fisher Scientific), XCell SureLock Mini-Cell Electrophoresis System, (Thermo Fisher Scientific), and Wet/Tank Blotting Systems (Bio-Rad)

Priming the inflammasomes in BMDM

1a. Wash differentiated mouse BMDM in 10 cm tissue culture dishes with 10 ml PBS. Repeat washing, add 5 ml complete DMEM into the culture dish, scrape cells off gently with the cell scraper, then transfer the cells into the 50 ml conical tube. Centrifuge the tube 5 minutes (min) at 500 × g, 4°C, discard the supernatant, and resuspend the cells in complete DMEM to a final concentration of 2×10^6 cells/ml.

Scrape BMDMs gently in one direction, but do not scrape back and forth to avoid significant cell damage. Do not use trypsin, which potentially activates BMDMs.

2a. Add 0.5 ml BMDM suspension (1×10^6 cells) into one well of 24-well plate and allow cells to attach to the plate for overnight.

2 hours is usually enough for cells attachment, so use 2 hours attachment for the one-day experimental design.

3a. Replace the medium with 0.5 ml complete DMEM containing 500 ng/ml LPS to prime the cells for 3 hours (h).

Alternatively, prime BMDM by other PAMPs for different pattern recognition receptors (PRR) shown in Table 1 for 3 hours (Deng et al., 2019).

In addition to PAMPs, cytokines such as TNF-α (100ng/ml for 6 h) can also prime NLRP3 inflammasome in macrophages very well (Franchi, Eigenbrod, & Nunez, 2009; McGeough et al., 2017).

Activate canonical NLRP3 inflammasome by well-established stimuli

1b. During BMDM priming, supplement complete DMEM containing 500 ng/ ml LPS with different stimulus listed in Table 2.

2b. After 3 h priming, replace cell medium with 0.5 ml NLRP3 stimuli-containing medium prepared in 1b. Stimulate BMDM with ATP for 30 min, nigericin for 45 min, MSU, silica, and Alum for 6 hours.

It is noted that ATP and nigericin induces a strong inflammasome-dependent pyroptosis in a very short time. So keep the stimulation with ATP and nigericin no longer than 45 min to keep enough intact cells for immunoblot assays.

Always prepare silica stock solution in a fume hood, as silica powder will cause severe lung inflammation through inhalation.

3b. After stimulation, harvest cell-free supernatant for assessing mature IL-1β by immunoblot and ELISA and assessing cleaved caspase-1 (p10) and cleaved GSDMD by immunoblot.

It is difficult to detect caspase-1 p10 in the supernatant by immunoblot due to its low level. Therefore, it is important to seed cells in a high density (2×10^6 cells/ml) at the beginning. Also, use the 1.5 mm but not 1.0 mm thick NuPAGE gel to load more proteins. Use 0.2 μM pore-size nitrocellulose membrane for protein transfer and control transfer time at 45 min (100 V).

Antibody dilution: anti-IL-1β: 1:2000, anti-caspase-1 p10: 1:500, anti-GSDMD: 1:1000

4b. Wash the cells with 0.5 ml PBS, add 150 μl RIPA buffer, and lyse the cells on a shaker for 30 min at 4°C. Transfer the cell lysate into the 1.5 ml Eppendorf tube and centrifuge the tube 15 min at 16,000 × g, 4°C to remove the cell debris. Use the clear cell lysate for immunoblot of pro-IL-1β, pro-caspase-1, NLRP3, ASC, GSDMD, and β-actin.

Antibody dilution: anti-IL-1β: 1:2000, anti-caspase-1: 1:1000, anti-NLRP3: 1:1000, anti-ASC: 1:1000, anti-GSDMD: 1:1000, anti-β-actin: 1:5000

5b. To assess pyroptosis, prepare another 24-well plate of BMDM culture and replicate the LPS priming (1a to 3a) and NLRP3 activation (1b and 2b) described above, followed by the LDH assay according to the manufacturer's instructions.

Activate canonical NLRP3 inflammasome by phospholipid

1c. During BMDM priming, supplement plain DMEM containing 500 ng/LPS with 20 μM PAF or 20 μM LPC (Deng et al., 2019; Freeman et al., 2017).

It is critical to use plain DMEM to prepare PAF and LPC, as FBS will suppress PAF and LPC activating NLRP3 inflammasome.

2c. After 3 h priming, remove the cell medium, and wash the cells twice with 0.5 ml PBS to remove any serum.

Wash the cells with PBS extensively, as the residual FBS will suppress PAF- and LPC- triggered NLRP3 inflammasome activation.

3c. Add 0.5 ml PAF- or LPC-containing medium prepared in 1c into BMDM. Stimulate BMDM with PAF for 3 hours and LPC for 1 hour, respectively.

Prepare fresh PAF stock every time for the new experiment, as long time stored PAF cannot activate NLRP3 inflammasome. LPC stock solution keeps its ability to activate NLRP3 inflammaosme even after long-term storage at −20°C. The activation of NLRP3 inflammasome by PAF and LPC varies among different batches of reagents. Therefore, optimization of the time period of stimulation is recommended.

4c. After PAF- or LPC-stimulation, assess the inflammasome activation as described in 4b and 5b.

Activate noncanonical NLRP3 inflammasome by transfected LPS

1d. Twenty min before the end of 3 h priming of BMDM, prepare LPS/FuGENE HD complex by mixing 100 μl Opti-MEM with 600 ng LPS (final concentration at 1 μg/ml) and 1.5 μl FuGENE HD (finally 0.25% v/v) for each well. Use the same volume of solvent and FuGENE as the vehicle control. Vortex the transfection mixture for 10 seconds (sec) and incubate it at room temperature for 15 min. Then add the LPS/FuGENE HD complex dropwise to the BMDM.

FBS may affect transfection efficiency. Therefore, use LPS-containing Opti-MEM to prime the BMDM if the transfection of LPS is not efficient enough.

2d. Stimulate the BMDM for 3 h, and then assess the inflammasome activation as described in 4b and 5b.

3d. To determine LPS induced caspase-11 activation, prepare another 24-well plate of BMDM culture and replicate the LPS priming (1a to 3a) and LPS transfection (1d) described above.

4d. After 3 h, lyse the cells with caspase assay buffer for 30 min on ice, centrifuge the lysate at 21,000 × g for 2 min at 4 ℃, mix 100 μl clear cell lysate with caspase-11 specific substrate Ac-LEVD-AMC (at final concentration at 75 μM), transfer the reaction mixture to a 96-well black plate and incubated at 37°C for 1 h, and monitor substrate cleavage by measuring the emission at 450 nm on excitation at 365 nm on a fluorescent multi-well reader.

LPS-triggered activation of caspase-11 accompanied with an auto-cleavage of itself into p26 and p10 subunits. An alternative approach to determine LPS induced caspase-11 activation is to measure caspase-11 p26 in the supernatant from LPS-transfected BMDMs by immunoblot using anti-caspase-11 antibody (1:1000 dilution) (Lee et al., 2018). If the signal of cleaved caspase-11 is too weak for detection, electroporation of LPS into BMDM may be applied to increase the transfection efficiency. In addition, combine cell supernatant and cell lysate for immunoblot will increase the signal of caspase-11 p26 (Lee et al., 2018).

Human cells does not express caspase-11 but the orthologues caspase-4 and caspase-5. Similar to caspase-11, caspase-4 and caspase-5 bind to cytosolic LPS and results their activation (Shi et al., 2014). Ac-LEVD-AMC is also a substrate of human caspase-4.

Activate NLRC4 inflammasome by transfected Flagellin

1e. Twenty min before the end of 3 h priming of BMDM, prepare Flagellin/DOTAP complex by mixing 25 μl HBS buffer with 500 ng Flagellin (final concentration at 1 μg/ml) and 3 μl DOTAP for each well. Use the same volume of solvent and DOTAP as the vehicle control. Vortex the transfection mixture for 10 sec and

incubate it at room temperature for 15 min. Add 475 μl plain DMEM into the Flagellin/DOTAP complex and pipette gently to mix.

The transfection efficiency will vary with different cell confluences and different protocols forlab- generated BMDMs. Therefore, there is a need to optimize the amount of Flagellin and DOTAP for each new experimental setting.

DOTAP can achieve good transfection efficiency in both serum-free and serum-containing medium.

2e. Completely remove the priming medium and add 0.5 ml medium containing Flagellin/DOTAP complex to the cell.

3e. Stimulate the BMDM for 1 hour or longer, and then assess the inflammasome activation as described in 4b and 5b.

One hour usually gives a robust signal. Increase the stimulation time if a stronger activation is required.

Activate NLRP1 inflammasome by lethal toxin

1f. After 3 h LPS-priming, wash the BMDM in each well with 0.5 ml PBS once, and incubate with 0.5 ml Opti-MEM supplemented with GlutaMax containing 1 μg/ml *Bacillus anthracis* lethal factor for 3 −6 hours. Use the same volume of Opti-MEM supplemented with GlutaMax only as the vehicle control.

BMDM from C57BL/6J does not contain a NLRP1 responsive to lethal toxin. Therefore, for mouse NLRP1 activation, use BMDM derived from 129S6 or BALB/cJ strain (Okondo et al., 2018). Lethal toxin does not activate human NLRP1 inflammasome. Basic protocol 2 describes the method for activating human NLRP1 inflammasome.

Three hour usually gives a robust activation of mouse NLRP1 inflammasome. If not, extend the incubation time up to 6 hours.

2f. Assess the inflammasome activation as described in 4b and 5b.

Activate AIM2 inflammasome by transfected poly(dA:dT)

1g. Ten min before the end of 3 h LPS-priming of BMDM, prepare poly(dA:dT)/Lipofectamine complex by mixing 50 μl Opti-MEM containing 1 μg poly(dA:dT) (final concentration at 2 μg/ml) with 50 μl Opti-MEM containing 2.5 μl Lipofectamine 2000 for each well. Use the same volume of solvent for poly(dA:dT) and Lipofectamine 2000 as the vehicle control. Vortex the transfection mixture for 10 sec and incubate it at room temperature for 10 min.

The transfection efficiency may vary; therefore, optimize the ratio of DNA to lipofectamine from 1:2.5 to 1:5 (μg/μl).

2g. Add 100 μl poly(dA:dT)/Lipofectamine complex into each well of the BMDM culture. Stimulate for 3 h and assess the inflammasome activation as described in 4b and 5b.

Three hours usually give a strong activation of AIM2 inflammasome. If not, extend the stimulation time or increase the amount of transfected DNA.

Basic protocol 2: Activate human NLRP1 inflammasome by DPP8/9 inhibitor Talabostat

Bacterial lethal toxin activates mouse NLRP1 inflammasome but not human NLRP1 inflammasome. Recent studies show that inhibition of two serine proteases, DPP8 and DPP9, triggers NLRP1 inflammasome activation in both human and mouse cells (Gai et al., 2019; Zhong et al., 2018). This protocol describes the method for activating human NLRP1 inflammasome in LPS-primed human peripheral blood mononuclear cells (PBMC) by inhibiting DPP8/DPP9 with Talabostat, a nonselective inhibitor of post-proline cleaving serine proteases (Okondo et al., 2017).

Materials

PBMC: isolated from Buffy coat blood bag (Gulf Coast Regional Blood Center) using Ficoll-Paque PLUS (GE17-1440-02, Sigma-Aldrich)

Complete RPMI-1640 medium: RPMI-1640 (11875093) containing 10% (v/v) fetal bovine serum (F2442, Sigma-Aldrich) and 1% (v/v) penicillin/streptomycin (15140122, Gibco)

Stimuli: LPS-EB ultrapure (tlrl-3pelps, InvivoGen) and Talabostat (HY-13233, MedChemExpress)

Human IL-1β ELISA kit (557953, BD Biosciences) and human IL-18 ELISA kit (7620, R&D Systems)

Cytotoxicity Detection Kit (LDH) (11644793001, Sigma-Aldrich)

RIPA buffer: 50 mM Tris-HCl, pH7.4, 150 mM NaCl, 0.5% (g/ml) Sodium Deoxycholate, 0.1% (g/ml) SDS, and 1% (v/v) NP-40, containing 1 × cOmplete™ Protease Inhibitor Cocktail (11697498001, Sigma-Aldrich)

Antibodies: anti-IL-1β (AF-201-NA, R&D Systems), anti-caspase-1 p20 (2225, Cell Signaling), anti-NLRP3 (AG-20B-0014-C100, Adipogen), anti-ASC (AG-25B-0006-C100, Adipogen), anti-N-terminal GSDMD (ab215203, Abcam), anti-GSDMD (ab210070, Abcam) and anti-β-actin-HRP (sc-1615, Santa Cruz Biotechnology)

Centrifuge

NuPAGE™ 4 to 12% protein gel (Thermo Fisher Scientific), XCell SureLock Mini-Cell Electrophoresis System, (Thermo Fisher Scientific), and Wet/Tank Blotting Systems (Bio-Rad)

1. Isolate human PBMC from the Buffy coat by centrifugation with Ficoll according to the manufacturer's guidelines.
2. Seed 2 × 10^6 PBMC per well with 500 μl complete RPMI-1640 medium in 24-well plate.

3. Prime PBMC in each well by adding LPS (final concentration at 1 μg/ml) for 1 h.

4. Add Talabostat (final concentration at 10 μM/ml) into each well to activate NLRP1 inflammasome for 18 h. Use DMSO as the vehicle control.

   Prepare 10 mM Talabostat stock solution using DMSO.

5. After stimulation, collect the supernatant and spin down the suspension cells (500 × g, 5 min), and harvest cell-free supernatant for assessing mature IL-1β and IL-18 by ELISA and assessing mature IL-1β, cleaved caspase-1 p10 and cleaved GSDMD by immunoblot.

6. Wash the attached cells with 0.5 ml PBS once, add 150 μl RIPA buffer, and lyse the cells on a shaker for 30 min at 4°C. Transfer the cell lysate into the 1.5 ml Eppendorf tube and centrifuge the tube 15 min at 16,000 × g, 4°C to remove the cell debris. Use the clear cell lysate for immunoblot of pro-IL-1β, pro-caspase-1, NLRP3, ASC, GSDMD, and β-actin.

7. To assess pyroptosis, prepare another 24-well plate of PBMC culture and replicate the LPS priming and NLRP1 activation as described above, followed by the LDH assay according to the manufacturer's instructions.

Basic protocol 3: Purification and detection of ASC pyroptosome

Upon NLRP3, NLRC4, and AIM2 inflammasome activation, adaptor protein ASC binds to caspase-1 and is critical for caspase-1 activation and subsequent pyroptosis. During this event, ASC oligomerizes into a supramolecular filament structure, named the pyroptosome (Lu et al., 2014). This protocol describes the method for purifying the pyroptosome by a low speed centrifugation and detecting by DSS cross-linking and subsequent immunoblotting for ASC oligomers.

Materials

Mouse BMDM [*Copy Editor – please ask author for more information about this material]

Complete DMEM: DMEM (11995065, Gibco) containing 10% (v/v) FBS (F2442, Sigma-Aldrich) and 1% (v/v) penicillin/streptomycin (15140122, Gibco)

Stimuli: Ultrapure LPS, *E. coli* 0111:B4 (tlrl-3pelps, InvivoGen), Nigericin (tlrl-nig, InvivoGen)

Cell lysis buffer: PBS containing 1% NP40, 0.1 mM PMSF, complete protease inhibitor cocktail without EDTA (11873580001, Sigma-Aldrich)

Bicinchoninic Acid (BCA) Protein Assay Kit (23227, Thermo Fischer Scientific)

Disuccinimidyl suberate (DSS) (A39267, Thermo Fischer Scientific)

Antibodies: anti-ASC (AG-25B-0006-C100, Adipogen), anti-β-actin-HRP (sc-1615, Santa Cruz Biotechnology)

Cell scraper (Fisher Scientific)

Centrifuge

NuPAGE™ 4 to 12% protein gel (Thermo Fisher Scientific), XCell SureLock Mini-Cell Electrophoresis System, (Thermo Fisher Scientific), and Wet/Tank Blotting Systems (Bio-Rad)

1. Prime 4×10^6 BMDM in one well of 6-well plate with 3 ml complete DMEM containing 500 ng/ml LPS for 3 h followed by stimulating with 40 μM Nigericin for 45 min, as described in Basic protocol 1. Use untreated BMDM and LPS-priming only BMDM as the negative controls.

   Use minimum 4×10^6 BMDM for detecting ASC pryptosome. If using more cells, adjust the reagent usage in proportion in the following procedures.

   Besides nigericin, other inflammasome stimuli described in Basic protocol 1 can also be used here, just stick to the previously indicated stimulation time.

2. After stimulation, wash cells with 3 ml ice-cold PBS once and add 400 μl ice-cold lysis buffer onto the cell.

3. Scrape cells off the plate by the cell scraper into the lysis buffer, transfer the cell lysate into a 1.5 ml Eppendorf tube and pass the cell lysate through a 21-gauge needle 10 times to totally disrupted the cell membrane. Keep the cell lysate on ice all the time.

4. Remove nucleus and cell debris by centrifuging the tube at 250 × g for 5 min at 4°C.

   Avoid a high-speed centrifugation in this step to keep the ASC pyroptosome in the supernatant fraction.

5. Transfer the clear cell lysate into a new tube on ice and quantify the protein concentration by BCA Protein Assay. Use the same amount of total proteins for the following procedures.

6. Pellet the ASC pyroptosome from the cell lysate by centrifuging the tube at 5,000 × g for 10 min at 4°C. Transfer the supernatant into a new tube and keep it on ice for immunoblot.

7. Add 500 μl PBS into the tube and centrifuge at 5,000 × g for 10 min at 4°C to wash the pellet. Repeat this step.

   This wash step removes the residual ASC protein not in the pyroptosome.

8. During the PBS-washing, prepare 100 mM disuccinimidyl suberate (DSS) stock solution by adding 54 μl DMSO into 2 mg DSS and pipetting up and down to mix it.

9. After washing with PBS, resuspend the pellet with 300 μl lysis buffer used in step 2, and add 6 μl freshly made DSS stock solution to each tube (final concentration at 2 mM). Cross-link the resuspend pellet with DSS for 30 min at room temperature.

Do not resuspend the pellet with PBS alone, because precipitation will form after adding DSS into PBS.

10. Then add 6 μl 1 M Tris-HCl buffer (pH7.4) to quench the reaction for 15 min at room temperature.

11. Spin down the cross-linked pellet by centrifuging the tube at 6,000 × rpm for 5 min at 4°C.

12. Remove the supernatant and resuspend the pellet in 30 μl 2 × SDS-PAGE protein loading buffer.

    Remove the supernatant as much as possible to make sure the final concentration of protein loading buffer is ≥ 1 ×.

13. Boil the sample at 95°C for 10 min, centrifuge the tube at 16,000 × g for 2 min, and use the supernatant for SDS-PAGE. Use antibody against ASC to detect ASC monomer, dimer, and oligomer by immunoblot assay.

    Use cell lysate from step 6 to detect ASC monomer and loading controls such as β-actin.

Alternate protocol 1: Detection of ASC speck by immunofluorescence staining

In addition to cross-link and immunoblot, ASC proptosome can be observed as cytosolic foci (named ASC speck) by immunofluorescence microscopy (Fernandes-Alnemri et al., 2007). This protocol uses NLRP3 inflammasome activation by LPS plus nigericin as the example to describe the method for immunofluorescence staining of ASC speck in BMDM.

Additional Materials (Also see Basic protocol 3)

4% Paraformaldehyde (PFA): diluted from 16% Paraformaldehyde Aqueous Solution (Electron Microscopy Sciences) with PBS

Cell permeabilization buffer: PBS containing 0.2% (v/v) Triton X-100

Cell blocking buffer: PBS containing 5% (v/v) normal goat serum (31872, Thermo Fisher Scientific) and 0.1% (v/v) Tween-20

Antibodies: anti-ASC (AG-25B-0006-C100, Adipogen), Alexa Fluor 488 conjugated Goat anti-Rabbit secondary antibodies (Thermo Fisher Scientific)

Fluoro-Gel II mounting medium (17985–50, Electron Microscopy Sciences)

Cell scraper (Fisher Scientific)

Coverslip and slide (Electron Microscopy Sciences)

ZEISS LSM710 confocal microscope

1. Put an autoclaved coverslip into the well of 24-well plate and seed 1×10^6 BMDM for culturing with 500 μl complete DMEM overnight.

2. Stimulate BMDM with 500 ng/ml LPS for 3 h and followed by stimulation with 40 μM nigericin for 45 min as described in Basic protocol 1.

   Besides nigericin, other inflammasome stimuli described in Basic protocol 1 can also be used here, just stick to the previously indicated stimulation time.

3. After stimulation, remove the medium and wash the cells with 500 μl PBS once.

4. Remove PBS and add 300 μl fixation buffer (4% PFA/PBS) to fix cells for 20 min at 4°C.

5. Remove fixation buffer and add 500 μl permeabilization buffer (0.2% Triton X-100/PBS) to permeabilize cells for 5 min on ice.

6. Remove permeabilization buffer and wash cells with 500 μl PBS by gently shaking for 5 min. Repeat the washing step.

7. Remove the washing buffer and add 200 μl blocking buffer (5% v/v goat serum and 0.1% v/v Tween-20 in PBS) into each well to block for 30 min at room temperature.

8. Remove blocking buffer and incubate with 200 μl primary antibody for ASC (0.5 μg/ml in blocking buffer) at room temperature for 1 h.

   The primary antibody concentration can be optimized based on experience.

9. Remove primary antibody solution and wash with 500 μl PBS by gently shaking for 10 min. Repeat the washing for three times.

10. Remove the washing buffer and incubate with 200 μl Alexa Fluor 488 conjugated secondary antibodies (4 μg/ml in blocking buffer) for 1 h at room temperature.

11. Remove the secondary antibody solution and wash with 500 μl PBS supplemented with 0.1% (v/v) Tween-20 by gently shaking for 10 min. Repeat the washing for three times.

12. Remove washing buffer, rinse twice with 500 μl double distilled water, and mount the coverslip onto the slide using Fluoro-Gel II mounting medium. Image the cells with a ZEISS LSM 710 microscope. [*Copy editor – please query the author to describe what what they're looking for in the images. What visuals would indicate a positive result?]

    Fluoro-Gel II mounting medium contains DAPI (4, 6-diamino-2-phenylindole) for nuclear staining and can also preserve the fluorescence for a long term storage in the dark at 4°C.

Basic protocol 4: Activation of canonical NLRP3 inflammasome in mice by intraperitoneal delivery of MSU crystals

Intraperitoneal (i.p.) delivery of agonists will activate inflammasome *in vivo*. This activation will induce excessive IL-1β and IL-18 and other inflammatory cytokines, subsequently accompanied with infiltration of neutrophils and inflammatory monocytes, named peritonitis

(Deng et al., 2019; Spalinger et al., 2016; Zhao, Gillette, Li, Zhang, & Wen, 2014). Based on the literature and our own lab experiences, this protocol use i.p. injection of MSU to activate NLRP3 inflammasome in LPS-primed mice and determine inflammasome activation by assessing IL-1β and IL-18 levels in peritoneal lavage and serum by ELISA and peritoneal infiltration of neutrophils and inflammatory monocytes by flow cytometry (Deng et al., 2019; Martinon, Petrilli, Mayor, Tardivel, & Tschopp, 2006).

Materials

C57BL/6J mice: 6 to 8 weeks old

Stimuli: Ultrapure LPS, *E. coli* 0111:B4 (tlrl-3pelps, InvivoGen), MSU crystals (tlrl-msu, InvivoGen)

1 ml Insulin syringe (324826, BD)

5 ml syringe (302187, BD) and 25 G needle (GS-351, Terumo)

Fluorescence-activated cell sorting (FACS) buffer: PBS containing 2% (v/v) FBS

Antibodies: anti-CD16/CD32 (101302, BioLegend), anti-CD11b-FITC (101205, BioLegend), anti-Ly-6G-PE (127607, BioLegend), anti-Ly-6C-APC (128015, Biolegend)

Mouse IL-1β ELISA Kit (432604, BioLegend) and mouse IL-18 ELISA kit (7625, R&D Systems)

BD LSR II flow cytometer

1. Intraperitoneally (i.p.) inject each mouse with 1 mg/kg LPS (diluted in sterile PBS) or vehicle control (PBS) and keep mice in the mouse cage for 3 h priming. [*Copy Editor – please insert this citation here https://currentprotocols.onlinelibrary.wiley.com/doi/10.1002/0471142735.im0106s73]
2. During priming, prepare MSU suspension by add 0.5 ml of sterile PBS to one vial of MSU crystals (5 mg) and vortex thoroughly for 5 min to make a stock suspension of 10 mg/ml MSU.

   MSU crystals are not soluble in PBS and used as the suspension. Do not centrifuge.
3. After 3 h priming, vortex MSU suspension for 1 min and inject 200 μl MSU suspension in sterile PBS into each mouse via i.p. route by 1 ml insulin syringe.
4. 6 h later, collect the blood via cardiac puncture to prepare the serum. Then, i.p. inject 3 ml PBS into each mouse by 5 ml syringe with a 25 G needle, shake the mouse for 1 min, and collect the peritoneal lavage. [*Copy editor – please query author to provide citations for both puncture and lavage. A possible puncture citation is: https://currentprotocols.onlinelibrary.wiley.com/doi/10.1002/0471142735.im0106s73]

Be very careful when collecting the peritoneal lavage, avoiding damaging the blood vessel or organs. Damaged blood vessels and organs will release cells into the peritoneal cavity, which will affect the infiltrated cell numbers.

5. Measure IL-1β and IL-18 levels in the serum and peritoneal lavage by ELISA. [*Copy editor – please query author to provide a citation on how to do this.]

6. To analyze the peritoneal infiltrates, count the absolute number of peritoneal exudate cells. Then, spin down the cells at 500 × g for 5 min at 4°C, resuspend the cells with 200 ul FACS buffer, transfer cells into the U-bottom 96 well plate, centrifuge the plate at 1, 500 rpm for 3 min at 4°C, and discard the supernatant. Add 50 μl anti-mouse CD16/32 antibody (1:50 in FACS buffer) into cells to block Fc receptors on ice for 10 min and then stain the cells with 100 μl Ly-6G antibody, Ly-6C antibody and CD11b antibody (all antibodies are diluted 1:330 in FACS buffer) on ice for 30 min in the dark.

7. Centrifuge the plate at 1, 500 rpm for 3 min at 4°C. Discard the supernatant and wash the stained cells with 180 μl FACS buffer, centrifuge the plate at 1, 500 rpm for 3 min at 4°C and discard the supernatant. Repeat washing for a total of three times and then fix the cells with 200 μl 4% Paraformaldehyde solution in the dark at room temperature for 15 min.

   Alternatively, stain cells in the 1.5 ml Eppendorf tubes if there are not many samples. Wash cells with 1 ml FACS buffer once after staining.

8. Run the samples on the BD LSR II flow cytometer and analyze the ratio of neutrophils (CD11b+ and Ly-6G+) and inflammatory monocytes (CD11b+ and Ly-6C+). Calculate the cell number of infiltrating neutrophil and inflammatory monocyte by multiplying the absolute number of peritoneal exudate cells with their percentage respectively.

Alternate protocol 2: Activation of noncanonical NLRP3 inflammasome in mice by intraperitoneal delivery of LPS

Delivery of high dose LPS alone via i.p. can activate caspase-11-dependent noncanonical NLRP3 inflammasome (Hagar et al., 2013; Kayagaki et al., 2013). Activation of caspase-1 by cytosolic LPS leads to GSDMD cleavage and subsequent membrane pore formation and potassium efflux, which activates NLRP3 inflammasome (Yang et al., 2019). This protocol describes the method for activating caspase-11 and noncanonical NLRP3 inflammasome in mice and the relevant readout.

Additional Materials

Caspase assay buffer: 50 mM HEPES pH7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA and 10% glycerol

Ac-LEVD-AMC (ALX-260–083, Enzo Life Sciences)

96-well black plate (Costar)

PerkinElmer EnSpire Multimode Plate Reader

1. Intraperitoneally (i.p.) inject each mouse with 20 mg/kg LPS (diluted in sterile PBS) or vehicle control (PBS). Use *Casp11*$^{-/-}$ mice as the negative control.
2. 12 h later, collect the blood via cardiac puncture to prepare the serum. Then, intraperitoneally inject 3 ml PBS into each mouse and collect the peritoneal lavage.
3. Measure IL-1β and IL-18 levels in the serum and peritoneal lavage by ELISA.
4. Count, stain and analyze peritoneal exudate cells as described in Basic protocol 3.
5. To determine LPS-induced caspase-11 activation, lyse partial peritoneal exudate cells with caspase assay buffer and measure caspase-11 activity by Ac-LEVD-AMC as described in Basic protocol 1 step 4d.

   If monitoring mice survival, challenge mice with 54 mg/kg LPS via i.p. and monitor up to 96 h.

COMMENTARY

Background Information

The classic working model for inflammasomes activation requires two steps: priming (signal one) and activation (signal two) (Broz & Dixit, 2016). Priming offers the elevation of expression and switch of protein modification of inflammasome relevant gene products, as well as induction of pro-proteins of inflammasome-processed cytokines. [*Copy editor – please query author to clarify language of above sentence “and switch of…” is confusing.] In the activation step, a stimulus specific for each inflammasome triggers the formation of supramolecular inflammasome complex, which subsequently activates the caspase-1, processes pro-IL-1β and pro-IL-18, and induces pyroptosis (Sharma & Kanneganti, 2016). Inflammasome genes are highly expressed in myeloid cells either at the basal level or in an induced condition. Therefore, we use mouse macrophage and human PBMC as examples of target cells in this article. For the priming of murine macrophage and human PBMC, LPS is a well-established and efficient agonist, as the evident expression of LPS receptors on the surface of myeloid cells (Mariathasan et al., 2006; Okondo et al., 2018; Park & Lee, 2013). We choose to use LPS as signal one throughout all the protocols described in this article. However, we also suggested other PAMPs and TNF-α for priming based on our own experiences and the literature (Deng et al., 2019; Franchi et al., 2009). Each inflammasome has its own specific agonist and distinct mechanism for the activation. NLRP3 is the most extensively investigated inflammasome and has the most established agonists with various characteristics, but with unknown molecular mechanism for its activation. Nonetheless, most of NLRP3 stimuli go through a potassium efflux-dependent mechanism to trigger inflammasome activation (Swanson et al., 2019). In the Basic protocol 1, we detailed described protocols of how to activate canonical NLRP3 inflammasome with the most well established agonists ATP, nigericin, MSU, silica, and alum, which all converge onto potassium efflux to activate NLRP3 inflammasome (Swanson et al., 2019). In addition, we

also described how to activate caspase-11/4/5-dependent noncanical NLRP3 inflammasome activation by cytosolic LPS (Hagar et al., 2013; Kayagaki et al., 2013; Shi et al., 2014). NLRC4 inflammasome is critical for defending bacterial infection and is activated via the NAIP family of receptors which recognize bacterial flagellin and T3SS rod/needle proteins (Vance, 2015). We described how to induce NLRC4 inflammasome activation by cytosolic delivery of flagellin. AIM2 inflammasome is a DNA responsive inflammasome and can be activated by murine cytomegalovirus and poly (dA:dT) which we have described in Basic protocol 1 (V. A. Rathinam et al., 2010). Among all the known inflammasomes, NLRP1 is uniquely composed with an N-terminal domain and C-terminal domain which are resulted from an autoproteolytic cleavage of its unique FIIND domain. The degradation of N-terminal of NLRP1 by anthrax lethal toxin cleavage and a Shigella flexneri ubiquitin ligase induced ubiquitination can activate mouse NLRP1 (Sandstrom et al., 2019). We used lethal toxin as an example to describe mouse NLRP1 activation in the Basic protocol 1. Human NLRP1 cannot be activated by lethal toxin as it does not contain the corresponding motif for lethal toxin cleavage. However, a recent discovered mechanism that DPP8/9 represses NLRP1 activation by binding to NLRP1 FIIND domain offers DPP8/9 inhibitor Talabostat as a NLRP1 activator in both mouse and human (Okondo et al., 2017; Okondo et al., 2018; Zhong et al., 2018). In Basic protocol 2, we described how to activate human NLRP1 by Talabostat. IL-1β and IL-18 maturation and release and cell pyroptosis are typical readouts to determine inflammasomes activation (Guo et al., 2015; Lamkanfi & Dixit, 2014). In addition, ASC pyroptosome formation is a more straightforward evidence for inflammasome platform assembly (Fernandes-Alnemri et al., 2007). In Basic protocol 3, we detailed described a method for purifying and detecting ASC pyroptosome. This protocol is based on the literature but optimized based on our own experiences (Zhao et al., 2014). ASC pyroptosome is a supramolecular complex and can be observed as foci by antibody staining and fluorescence microscopy (Fernandes-Alnemri et al., 2007). In the alternative protocol 1, we described a method for indirect immunofluorescence staining of ASC pyroptosome that is well established by our lab and others (Swanson et al., 2017).

Aberrant activation of inflammasomes is associated with multiple autoinflammatory and autoimmune diseases and can lead to undesirable outcomes (Guo et al., 2015). Hence, animal models are important and valuable for investigating inflammasome activation. In the basic protocol 4, we described a simple method to induce NLRP3 inflammasome activation by intraperitoneal delivery of MSU crystals. NLRP3 activation leads to an elevation of IL-1β and IL-18 in both peritoneal lavage and serum and a subsequent neutrophils and proinflammatory monocytes infiltration, collectively causes peritonitis (Zhao et al., 2014). Once LPS enter into the cytosol, it binds and activates mouse caspase-11 and human caspase-4 and 5 to cleave GSDMD. N-terminal GSDMD form pores on cell membrane and leads to potassium efflux, which also drives NLRP3 inflammasome activation, termed noncanonical NLRP3 inflammasome activation (V. A. K. Rathinam, Zhao, & Shao, 2019; Yang et al., 2019). Lethal dose LPS injection of mice caused mortality has been associated with caspase-11 dependent noncanonical inflammasome activation. In the alternative protocol 2, we referred to Vishva M Dixit's paper for describing how to activate noncanonical inflammasome activation by LPS in an endotoxic mouse model (Kayagaki et al., 2013).

Critical Parameters and Troubleshooting

The most common problem for detecting mature IL-1β and IL-18 by immunoblot is their low levels in certain stimulation (Basic protocol 1). Usually if the cytokine level is below 200 pg/ml, it is difficult to get a clean blot for IL-1β, IL-18 and caspase-1 p10. There are a few solutions to improve the signal: (1) Increase the BMDM density to 2×10^6/ml medium (1×10^6/well in 24 well plate) to reach a higher level of cytokines in the supernatant. The 2×10^6/ml BMDM usually give more than 500 pg/ml of IL-1 β and IL-18 under the stimulation described in Basic protocol 1 in our hands. If a high level of cytokines is not achieved, extension of the stimulation time is another solution. (2) Mature IL-1β and IL-18 and active caspase-1 p10 are all small proteins. When performing an immunoblot, it is critical to use 0.25 μm pore-size nitrocellulose membrane for protein transfer and supplement the transfer buffer with 20% methanol, as the higher percentage of methanol helps retain small proteins on the membrane. If using a voltage of 100 V for transfer, do not transfer longer than 45 min to prevent the small proteins from passing through the membrane. (3) Use the vendor and clone of primary antibodies for IL-1β and caspase-1 p10 as listed in the material section of Basic protocol 1, as we and others have been successful in detecting processed, mature IL-1β or cleaved caspase-1 using these exact reagents.

Another potential problem in Basic protocol 1 is the transfection of flagellin by DOTAP. Although it is not necessary to use serum free medium for cell culture when performing transfection with DOTAP, according to the manufacturer's instruction, we always use serum reduced Opti-MEM to increase the transfection efficiency. For all other stimulations in Basic protocol 1, it is quite straightforward and may only need to optimize the dose of stimulus and stimulation time period accordingly.

In Basic protocol 2, LPS alone will cause an obvious induction of mature IL-1β, which is a result of NLRP3 activation in monocytes. To obtain the best difference between LPS only and LPS plus NLRP1 agonist induced IL-1β level, the dose and duration of LPS priming may need an optimization.

There are a few key points for purifying and detecting ASC pyroptosomes by immunoblot in Basic protocol 3. (1) Starting with a minimum 4×10^6 BMDM will generate sufficient ASC pyroptosomes in this protocol. (2) When disrupting the cell membrane, do not use a syringe needle smaller than 21G to make sure that the cell membrane is fully disrupted. (3) It is critical to use a low speed (250 × g) centrifugation to remove the cell debris and avoid spinning down any ASC pyroptosome. (4) When cross-linking ASC pyroptosomes by DSS, resuspend the pellet with cell lysis buffer but not PBS. We found precipitation forming when adding DSS into PBS-resuspended ASC pyroptosomes, although PBS was used in some literature. Once the precipitation is formed, the ASC pyroptosome was not detectable by immunoblot. It is quite straightforward to detect an ASC speck by immunofluorescence staining as described in Alternative protocol 1. The concentration of both primary antibody and secondary antibody may require optimization to get the best resolution. One-hour incubation of the primary antibody at room temperature usually generates decent results. However, overnight incubation at 4°C usually gives a better signal. One-hour incubation of the secondary antibody at room temperature is sufficient and the longer incubation time will generate higher background staining.

In the Basic protocol 4 for MSU-induced peritonitis mouse model, the induction of proinflammatory cytokines in peritoneal lavage is typically faster than that in the peripheral blood. The recruitment of neutrophil and inflammatory monocytes will only happen after cytokines like IL-1β induction. Although the elevation of cytokines in both peritoneal lavage and serum and the infiltration of inflammatory cells are all detectable at 6 h after MSU-challenge, to reach the maximum level of cytokines in the serum, it is optimal to harvest blood samples a few hours later than peritoneal lavage samples based on the optimization results. To get a more profound recruitment of inflammatory cells, harvest the peritoneal lavage at 12 h after MSU-challenge. Although the LPS-induced sepsis model in Alternate protocol 2 is straightforward, the lethal dose of LPS may need optimization in individual labs to obtain 80–100% mortality in 96 h after the LPS-challenge.

Understanding the Results

The agonists for priming and activating inflammasomes *in vitro* in this article are all well established. We expect to observe a ≥ 500 pg/ml IL-1β and IL-18 induction from NLRP3, NLRC4, NLRP1, and AIM2 inflammasome stimuli challenged LPS-primed BMDM by ELISA and 60 to 100% pyroptotic cells by LDH assay. At this activation level, the cleaved IL-1β (17 kD) and IL-18 (18 kD) and caspase-1 (10 kD) can be easily detected by immunoblot. For LPS plus Talabostat treated human PBMCs, LPS alone will induce some background of cleaved IL-1β and caspase-1 due to NLRP3 inflammasome activation, but LPS plus Talabostat will induce NLRP1 inflammasome activation and a more robust increase of cleaved IL-1β and caspase-1. After LPS plus nigericin stimulation, DSS-crosslinked ASC pyroptosome can be detected as a ladder of ASC monomer, dimer, tetramer, and oligomers by SDS-PAGE and immunoblot. Under a similar stimulation, an ASC speck can be observed as cytosolic foci by confocal microscope after immunofluorescence staining of ASC. In the LPS plus MSU challenged mice, we expect 200 to 400 pg/ml IL-1β and IL-18 are induced in the peritoneal lavage and serum and up to 2×10^6 infiltrated neutrophil and 1×10^5 inflammatory monocytes in the peritoneal cavity. In the LPS induced sepsis mouse model, we expected an induction of ≥ 500 pg/ml IL-1β and ≥ 20 ng/ml IL-18 in the serum at 12 h after 20mg/kg LPS challenge. We also expect 80 to 100% lethality of animals in the first 20 h after 54mg/kg LPS challenge and a significant protection from death in *caspase-11* $^{-/-}$ mice.

Time Considerations

The stimulation steps in Basic protocol 1 will take less than 10 h and the stimulation in Basic protocol 2 will take 21 h. ELISA of IL-1β and IL-18 will take a day and immunoblot of inflammasome components, cleaved IL-1β and IL-18, and caspase-1 will take 2 days. Induce, purify, and cross-link ASC pyroptosome will take less than a day. Detection of ASC pyroptosome by immunoblot will take 2 days. Induce and stain ASC speck will usually take a day but will take 2 days if incubating primary antibody overnight. Induction of peritonitis and harvest peritoneal lavage and serum will take a whole day. For the LPS induced sepsis model, induce IL-1β and IL-18 and harvest serum and peritoneal lavage will take 20 h while monitor mice survival will take 4 days. ELISA of cytokines and stain and analyze peritoneal exudate cells by flow cytometry will take another day.

Acknowledgement

Assays presented in this paper were developed in part with support by grants from the National Institutes of Health AI029564 (J.T.), CA232109 (J.T.), DK094779 (J.T.), and UNC CFAR Award P30 AI50410 (H.G.).

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Basic Protocol 1:

Prime and activate inflammasomes in mouse macrophages

Basic Protocol 2:

Activate human NLRP1 inflammasome by DPP8/9 inhibitor Talabostat

Basic Protocol 3:

Purification and detection of ASC pyroptosome

Alternate Protocol 1:

Detection of ASC speck by immunofluorescence staining

Basic Protocol 4:

Activate canonical NLRP3 inflammasome in mice by intraperitoneal delivery of MSU crystals

Alternate Protocol 2:

Activate noncanonical NLRP3 inflammasome in mice by intraperitoneal delivery of LPS

Table 1.

PAMPs used for priming BMDM

PAMP	Target PRR	Final concentration
Pam3CSK4	TLR2/TLR1	1 μg/ml
HKLM	TLR2	10^7 cells/ml
FLA-ST	TLR5	1 μg/ml
FSL-1	TLR2/TLR6	1 μg/ml
ssRNA40	TLR7	1 μg/ml
ODN1826	TLR9	200 ng/ml

Table 2.

NLRP3 stimuli

Stimulus	Final concentration
ATP	5 mM
Nigericin	40 μM
MSU	100 μg/ml
Silica	200 μg/ml
Alum	200 μg/ml