Chapter 6

Measuring Inflammasome Activation in Response to Bacterial Infection

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Abstract

Inflammasomes are multi-protein signaling platforms assembled in response to viral and bacterial pathogens as well as endogenous danger signals. Inflammasomes serve as activation platforms for the mammalian cysteine protease caspase-1, a central mediator of innate immunity. The hallmarks of inflammasome activation are the processing of caspase-1, the maturation and release of interleukin-1β (IL-1β) and the induction of pyroptosis, a lytic inflammatory cell death. This protocol describes methods for studying inflammasome activation in response to bacterial pathogens in bone-marrow derived murine macrophages (BMDMs). In particular, we outline the protocols to measure cytokine maturation by ELISA and pyroptosis by the release of Lactate Dehydrogenase (LDH). In addition, we describe methods to visualize endogenous ASC specks or foci in infected cells and to study the release of processed caspase-1, caspase-11 and mature cytokines into the cell supernatant by Western blotting. General considerations are discussed to design and optimize the infection protocol for the study of inflammasome activation by other bacterial pathogens.

Key words Inflammasome, Caspase-1, Caspase-11, Pyroptosis, Interleukin-1, LDH release, NLRs, Bacterial infections, ASC speck

1 Introduction

The innate immune system is the first line of defense against microbial infection and is activated by the engagement of germline-encoded pattern-recognition receptors (PRRs). These receptors recognize a variety of unique bacterial molecules as well as endogenous danger signals, and initiate appropriate inflammatory responses. A key event in the inflammatory pathway is the activation of the inflammasome, a multiprotein platform that activates caspase-1 [1]. Activated caspase-1 promotes the maturation and secretion of pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18 [2, 3] and the release of proteins involved in tissue repair and cytoprotection [4]. In addition, active caspase-1 induces a pro-inflammatory form of cell death called pyroptosis [3, 5].

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Inflammasomes typically consist of three main components: (1) a cytoplasmic sensor/receptor, (2) caspase-1, and (3) in most cases, the adaptor protein ASC. Several types of inflammasomes have been identified and are named after the sensor that initiates the assembly of the complex. Thus far, only members of the NLR and the PYHIN protein families were shown to initiate inflammasome activation. These proteins function as specific sensors for various pathogens and endogenous danger signals. For example, Nlrp1b is required for caspase-1 activation in response to anthrax lethal toxin [6]. NLRP3 responds to a large variety of structurally and chemically different molecules, but the molecular mechanism linking these molecules to NLRP3 activation remains poorly understood [7]. Several viral and bacterial pathogens are known to activate the NLRP3 inflammasome [8]. NLRC4 activates caspase-1 after infection with bacteria expressing flagella and/or Type 3 secretion systems (T3SS) (such as *Salmonella* spp., *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Legionella pneumophila* and others) [9–13]. In mice Nlrc4 appears to specifically detect flagellin with the help of Naip5/6 and the T3SS rod subunits through Naip2, while human NAIP detects the T3SS needle subunit [14–16]. Finally, AIM2 recognizes the presence of double stranded DNA in the cytoplasm and is activated during infections with certain DNA viruses and the cytosolic bacterial pathogens *Francisella novicida* and *L. monocytogenes* [17–24].

An important component of most inflammasomes is the bipartite adaptor protein ASC, which is required by many NLRs and AIM2 to recruit pro-caspase-1 into the inflammasome complex. ASC stands for "apoptosis-associated speck-like protein containing a CARD," which describes its ability to form a large, macromolecular speck in the cytoplasm [25]. ASC specks, also known as ASC foci or pyroptosomes, form rapidly in response to pathogens [19, 26] or when ASC is over-expressed in cells [27, 28]. Visualization of endogenous ASC foci showed that the structures are 1–2 μm in diameter, serve as an activation platform for pro-caspase-1 [26], and are necessary for efficient pro-IL-1β maturation [29]. In addition, it has been shown that ASC foci co-localize with DNA and AIM2 during *F. novicida* infections (*see* Fig. 1) [19]. Finally, since ASC oligomerization does not require caspase-1 activity, the formation of endogenous ASC foci can be used as a measure for inflammasome complex formation, even in the absence of caspase-1 [26].

Following its activation by dimerization and proteolytic cleavage in the inflammasome, the active caspase-1 initiates several effector mechanisms [3]. The most prominent is the proteolytic maturation and release of the proinflammatory cytokine IL-1β. The release of mature IL-1β is closely controlled by a two-step activation mechanism. The first step (Signal 1) is the induction of pro-IL-1β expression by priming signals, such as the activation of

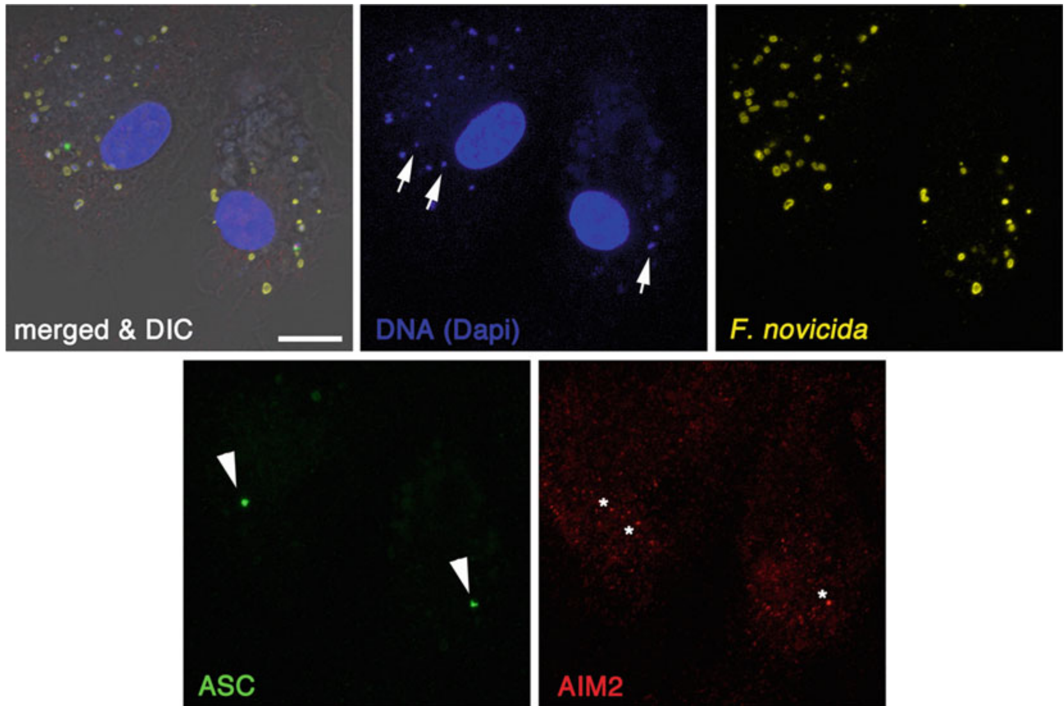

Fig. 1 ASC oligomers (ASC foci or specks) are formed in infected cells. Immunofluorescence staining of wild-type BMDMs infected with *F. novicida*. Cells were fixed and labeled with DAPI (DNA, *blue*), anti-*Francisella* antibodies (*yellow*), anti-ASC antibodies (*green*), and anti-AIM2 antibodies (*red*). *Arrowheads* point out ASC foci, *stars* point to AIM2 speckles, and *arrows* indicate DNA released by lysed bacteria. Scale bar is 10 μm

membrane bound PRRs and the subsequent transcriptional response. The second step (Signal 2) is the activation of the inflammasome itself. However, caspase-1 has also been shown to process and release other cytokines that do not need priming (e.g., IL-18). The exact mechanism by which caspase-1 mediates cytokine release is unknown, but seems to involve a yet uncharacterized secretion pathway, that results in the simultaneous release of processed caspase-1 itself. The second caspase-1 effector mechanism is the induction of a lytic cell death, termed pyroptosis [5], which is thought to remove the replicative niche of intracellular pathogens and reexpose them to extracellular immune responses [30].

Recently, a noncanonical inflammasome was described that activates caspase-11 instead of caspase-1 in murine macrophages [31]. Activation of the noncanonical inflammasome has been linked to TRIF-dependent production of type-I-interferon, and is initiated in response to a variety of Gram-negative bacteria [32, 33]. Similarly to caspase-1 activation, activation of caspase-11 initiates cell lysis and cytokine maturation (in conjunction with NLRP3 and caspase-1), and can thus be analyzed using the same methods that are used to study canonical inflammasomes.

Although some inflammasomes (e.g., NLRP3) detect danger signals, most are dedicated to the recognition of viral or bacterial pathogens. Microbial infections have therefore become an important tool in inflammasome study, since they represent relevant model systems for inflammasome activation, which cannot be achieved using artificial stimuli, such as transfection of DNA. In addition, most pathogens activate several pattern recognition receptors during the course of the infection, thus providing the possibility to study the complex network of innate immune signaling pathways. Here we describe protocols to study caspase-1 and caspase-11 processing, cytokine maturation, pyroptosis, and formation of ASC foci in primary murine BMDMs in response to infections with *F. novicida*, which activates the AIM2 inflammasome. We further discuss considerations to adapt these protocols to study inflammasome activation in response to other bacterial pathogens.

2 Materials

2.1 Differentiation and Long-Term Storage of Murine BMDMs

1. Mice.
2. Autoclaved scissors and forceps.
3. Flushing medium: DMEM high-glucose, unsupplemented.
4. 10 mL syringes.
5. 26Gx1/2 needles.
6. Sterile, non-tissue culture treated 10 cm petri dishes.
7. 70 % Ethanol.
8. Paper towels.
9. Ice.
10. 2 beakers (200–300 mL).
11. Sterile 50 mL tubes.
12. Sterile 5, 10, and 25 mL pipettes.
13. Hemocytometer.
14. Macrophage medium: DMEM supplemented with 10 % FCS, 100 U/mL Pen/Strep, 10 mM HEPES, and 10 % L929-conditioned medium.
15. Centrifuge with rotor fitting 50 mL tubes.
16. Cell culture facility and equipment including 37 °C, 5 % CO_2, water-jacketed cell culture incubator and laminar flow hood.
17. Phosphate buffered Saline (PBS), tissue culture grade.
18. Ice-cold Fetal Calf Serum (FCS).
19. Ice-cold FCS with 20 % DMSO (Dimethyl sulfoxide, tissue culture grade).
20. Cryo vials (1–1.5 mL volume).
21. Isopropanol freezing chamber.

2.2 Thawing and Propagating BMDMs

1. Cryo vials containing 1×10^8 BMDMs/mL.
2. Dry Ice.
3. 37 °C water bath.
4. 70 % Ethanol.
5. Sterile 50 mL tubes.
6. Sterile 5, 10, and 25 mL pipettes.
7. Sterile, non-tissue culture treated 10 cm petri dishes.
8. Macrophage medium.
9. PBS, tissue culture grade.
10. Ice.
11. Centrifuge with rotor fitting 50 mL tubes.
12. Cell culture facility and equipment including 37 °C, 5 % CO_2, water-jacketed cell culture incubator and laminar flow hood.

2.3 Harvesting and Pre-stimulating of BMDMs for an Infection

1. Sterile 50 mL tubes.
2. Sterile 5, 10 and 25 mL pipettes.
3. Sterile, non-tissue culture treated 10 cm petri dishes.
4. Macrophage medium.
5. PBS, tissue culture grade.
6. Ice.
7. Centrifuge with rotor fitting 50 mL tubes.
8. Cell culture facility and equipment including 37 °C, 5 % CO_2, water-jacketed cell culture incubator and laminar flow hood.
9. 1 mg/mL of ultrapure *E. coli* LPS in PBS. Store at −20 °C.

2.4 Measurement of Pyroptosis and Cytokine Maturation

1. Tissue-culture treated flat bottom 96-well plates.
2. Macrophage medium.
3. 12-Tip multichannel pipette, 50–200 μL volume per tip.
4. Frozen stock of *F. novicida*.
5. Tryptic Soy Broth (TSB).
6. Spectrophotometer.
7. Bacterial incubator at 37 °C.
8. PBS.
9. 10 % sterile cysteine in PBS.
10. Centrifuge with swing-out buckets that holds 96-well plates.
11. Gentamicin stock solution at 100 mg/mL in PBS. Store at −20 °C.
12. Cell culture facility and equipment including 37 °C, 5 % CO_2, water-jacketed cell culture incubator and laminar flow hood.
13. Standard LDH release assay.

14. Lysis solution: 10 % Triton X-100.
15. ELISA Kits for IL-1α, IL-1β or IL-18.
16. 96-Well ELISA plates.
17. Plate sealers or Parafilm.
18. 96-Well plate reader (absorbance) and analysis software.

2.5 Analysis of Caspase-1 Processing by Western Blotting

1. Tissue-culture treated 6-well plates.
2. Macrophage medium.
3. Frozen stock of *F. novicida*.
4. Tryptic Soy Broth (TSB).
5. Spectrophotometer.
6. Bacterial incubator at 37 °C.
7. PBS.
8. 10 % sterile cysteine in PBS.
9. Centrifuge with swing-out buckets that holds 6-well plates.
10. Gentamicin stock solution at 100 mg/mL in PBS. Store at −20 °C.
11. Cell culture facility and equipment including 37 °C, 5 % CO_2, water-jacketed cell culture incubator and laminar flow hood.
12. Safe-Lock 2 mL tubes (Eppendorf).
13. 100 % Trichloroacetic acid.
14. Ice.
15. Tabletop centrifuge at 4 °C.
16. Heat block at 95 °C.
17. RIPA buffer: 50 mM Tris–HCl, 150 mM NaCl, 0.1 % SDS, 0.5 % Sodium-Deoxycholate and 1 % NP-40.
18. Ice-cold acetone.
19. SDS-PAGE sample buffer.
20. 1 M Tris–HCl, pH 12.
21. SDS-PAGE and Western blot equipment
22. 14 % Tris/Glycine Gels (1.5 mm thick gels, large wells) and Tris/Glycine running buffer.
23. 0.2 μm PVDF membranes.
24. Tris-buffered Saline with 0.1 % Tween-20 (TBS-T).
25. Blocking Buffer: TBS-T with 2.5 % Skim Milk powder, 2.5 % BSA.
26. Primary and secondary-HRP coupled antibodies.
27. ECL Western blotting reagent.
28. Film, Western blot cassette, dark room, and developer.
29. Membrane stripping solution.

2.6 Visualization of Endogenous ASC Oligomers

1. Tissue-culture treated 24-well plates.
2. Sterile glass coverslips (12 mm diameter).
3. Macrophage medium.
4. Frozen stock of *F. novicida*.
5. Tryptic Soy Broth (TSB).
6. Spectrophotometer.
7. Bacterial incubator at 37 °C.
8. PBS.
9. 10 % sterile cysteine in PBS.
10. Centrifuge with swing-out buckets that holds 96-well plates.
11. Gentamicin stock solution at 100 mg/mL in PBS. Store at –20 °C.
12. Cell culture facility and equipment including 37 °C, 5 % CO_2, water-jacketed cell culture incubator and laminar flow hood.
13. Fixative: 4 % Paraformaldehyde in PBS.
14. Blocking solution: PBS with 3 % BSA, 0.1 % Saponin.
15. Primary antibodies for ASC (rat anti-ASC, clone 8E4.1, Genentech Inc. or rabbit anti-ASC, AL177, Enzo Life Sciences), bacteria, and other inflammasome components (e.g., pro-caspase-1, sc-514, Santa Cruz Biotechnology Inc.).
16. Secondary fluorophore coupled antibodies.
17. Mounting medium with DAPI.
18. Microscopy slides.
19. Hydration chamber.
20. Parafilm.

3 Methods

Ensure adequately trained persons carry out all animal procedures and that all experiments have received prior ethics approval according to your institutions requirements.

3.1 Differentiation and Long-Term Storage of Murine BMDMs

1. Fill two beakers with 100–200 mL 70 % Ethanol and move to laminar flow hood.
2. Put two forceps and one scissor in each beaker. One beaker contains “outer” tools (for touching the exterior of the mouse); the other contains “inner” tools (only for touching the mouse once fur has been removed).
3. Fill syringes with 10 mL flushing medium and attach the 26Gx1/2 needles. Usually one syringe with 10 mL medium is sufficient to flush all four bones of one mouse.

4. Euthanize a mouse by CO_2 inhalation and/or cervical dislocation.
5. Lay the mouse down on its back on a paper towel and spray the mouse with 70 % Ethanol.
6. Transfer the mouse on a dry paper towel and transfer it to the laminar flow hood.
7. *Start with outer tools*: Using the forceps, hold one leg up by its skin at the ankle. Using the scissors make a small incision into the skin and starting at this incision cut the skin open from the ankle to the belly, taking care not to cut into the peritoneum.
8. Hold the foot up with the forceps and extend the small starting incision fully around the ankle. Next, pull the skin upward over the foot and downward over the leg until the whole leg and the lower part of the body is exposed.
9. Using scissors cut the hip bone and remove the whole leg. Be careful not to cut the femur at this step. Remove the foot and place the whole leg into a sterile 10 cm petri dish.
10. Repeat the procedure on the other leg.
11. *Switch to inner tools*: Using scissors and forceps remove the muscle tissue until the bones and the knee joint are exposed.
12. Cut the leg at the knee joint to separate femur and tibia. Put the bones into a clean 10 cm petri dish.
13. To flush out the bone marrow, hold one bone in the middle with forceps and cut open both sides of the bone. The bone marrow cavity with the bright red bone marrow should now be accessible.
14. Carefully insert a needle into the bone-marrow cavity and flush out the marrow into a 50 mL tube with 2–3 mL of flushing medium per bone. If necessary turn the bone around and flush out any remaining bone marrow from the other side.
15. Repeat this process with the remaining three bones of the same mouse.
16. Centrifuge the bone marrow for 5 min at $300 \times g$, remove the supernatant and resuspend in 1 mL macrophage medium using a 1 mL pipet.
17. Add an additional 4 mL of macrophage medium and mix by gently pipetting.
18. Count the cells using a hemocytometer and determine the total number of progenitor cells. Plate 5×10^6 progenitor cells into a non-tissue culture treated 10 cm petri dish in 10 mL macrophage medium and incubate at 37 °C for 3 days.
19. After 3 days check the cells under a microscope. Part of the progenitor cells should already have settled down and are starting to form colonies, while others will still be free floating.

20. Add 4 mL of fresh pre-warmed macrophage medium directly into each petri dish without removing the medium already in the dishes, and incubate at 37 °C for 3 days (*see* **Note 1**).
21. Remove the medium containing non-adherent cells and add 5 mL of ice-cold PBS per petri dish. Leave the petri dish at 4 °C for 15–30 min.
22. Gently scrape the BMDMs with a cell scraper and transfer to a 50 mL tube. More PBS can be added to make the collection easier.
23. Centrifuge the cells for 5 min at 4 °C at $300 \times g$, remove the supernatant and resuspend the cell pellet in ice-cold FCS. Count the macrophages to determine the concentration of cells/mL.
24. Prepare a dilution of BMDMs at 2×10^8/mL in ice-cold FCS. Gently mix this dilution with ice-cold FCS containing 20 % tissue culture grade DMSO to make a suspension at 1×10^8 BMDMs/mL in FCS with 10 % DMSO.
25. Aliquot 1 mL of the final suspension into cryo vials and freeze overnight in an −80 °C freezer using an isopropanol freezing chamber.
26. Transfer to liquid nitrogen for long-term storage.

3.2 Thawing and Propagating BMDMs

1. Get the required amount of vials of frozen macrophages from liquid nitrogen long-term storage and keep them on dry ice. One vial per BMDM genotype is usually sufficient and the procedure for one vial is outlined below.
2. Quickly thaw the BMDMs in a 37 °C water bath just until the moment the cells are thawed. Spray the vials with 70 % Ethanol, dry and move to a laminar flow hood.
3. Transfer the BMDMs into a 50 mL tube.
4. Slowly add 9 mL of cold macrophage medium drop-wise, while swirling.
5. Pellet the BMDMs by centrifugation at $300 \times g$ for 5 min at 4 °C, remove the supernatant and resuspend them in 10 mL macrophage medium. This step removes most of the DMSO.
6. Transfer the BMDMs into 1 petri dish and incubate them overnight at 37 °C.
7. Check the macrophages under a microscope. Most of the BMDMs should be adherent at this time.
8. Remove the medium containing any non-adherent cells and add 5 mL of ice-cold PBS per petri dish. Leave the petri dish at 4 °C for 15–30 min.
9. Gently scrape the BMDMs with a cell scraper and transfer to a 50 mL tube. More PBS can be added to make the collection easier.

10. Centrifuge the cells for 5 min at 4 °C at $300 \times g$, remove the supernatant and resuspend the cell pellet in 50 mL macrophage medium.
11. Transfer the BMDMs into 5 petri dishes and incubate at 37 °C for 2 days. After that the BMDMs are ready to be seeded for an infection as outlined below. The BMDMs can be kept up to 10 days after being thawed.

3.3 Harvesting and Pre-stimulating of BMDMs for an Infection

1. Move the petri dishes containing BMDMs to a laminar flow hood. You can expect $3–4 \times 10^8$ BMDMs per plate at this time, i.e., a total of approximately 2×10^9 BMDMs.
2. Remove the medium containing any non-adherent cells and add 5 mL of ice-cold PBS per petri dish. Leave the petri dish at 4 °C for 15–30 min.
3. Gently scrape the BMDMs with a cell scraper and transfer to 50 mL tube. More PBS can be added to make the collection easier.
4. Centrifuge the cells for 5 min at 4 °C at $300 \times g$, remove the supernatant and resuspend the cell pellet in 30 mL macrophage medium.
5. Count the BMDMs twice using a hemocytometer to determine the concentration of cells/mL.
6. Prepare the desired amount of a cell suspension at 2.5×10^5 BMDMs/mL. Replate remaining BMDMs at $2–3 \times 10^8$ BMDMs/dish into petri dishes for later use. The BMDMs can be kept up to 10 days after being thawed.
7. To pre-stimulate the macrophages and induce the expression of the pro-forms of IL-1α and IL-1β, add LPS to a final concentration of 0.1 μg/mL to the cell suspensions. However, depending on the type of experiment prestimulation might not be necessary or desirable (*see* **Note 2**).
8. Depending on whether you want to analyze pyroptosis and the release of mature cytokines or the processing of caspase-1 into its p10 and p20 subunits, seed the cells according to the protocols outlined in Subheadings 3.4 and 3.5 below.

3.4 Measurement of Pyroptosis and Cytokine Maturation

1. Seed BMDMs into 96-well plates at 5×10^4 cells/well in 0.2 mL macrophage medium and incubate overnight at 37 °C. For a typical experimental setup (*see* **Note 3** and Fig. 1).
2. Start overnight cultures of *F. novicida* directly from frozen stock into TSB supplemented with 0.1 % cysteine. Grow overnight at 37 °C with constant shaking. Other pathogens might require different growth conditions to maximize inflammasome activation (*see* **Note 4**).
3. *F. novicida* do not require subculturing prior to infection. However, if your pathogen of choice requires growth to

logarithmic phase to induce expression of virulence factors (*see* **Note 5**), subculture your bacterial overnight culture 1:50–1:100 for 3–4 h before proceeding with the protocol.

4. Measure the OD600 of your bacterial culture in a spectrophotometer. Using an appropriate conversion factor determine the CFU/mL of your bacterial culture. For *F. novicida* use the following conversion: OD600 1.0 = 3×10^9 CFU/mL (*see* **Note 6**).
5. Prepare a dilution of your bacteria in macrophage medium at the desired Multiplicity of Infection (MOI), i.e., the final number of bacteria per cell in your infection assay.
6. Remove the medium from the 96-well plate containing your BMDMs and add 0.2 mL/well of the bacterial suspension or plain macrophage medium as required by the experimental setup.
7. Centrifuge the plate for 15 min at 700 × *g* to synchronize the infection and then incubate for 30 min at 37 °C to allow the bacteria to invade the cells.
8. Add 1/10 of well volume (20 μL) of macrophage medium containing 1 mg/mL gentamicin to reach a final concentration of 100 μg/mL in order to kill all extracellular bacteria, and incubate for 90 min at 37 °C (*see* **Note 7**).
9. Wash the infected BMDMs twice with pre-warmed macrophage medium. Add fresh pre-warmed macrophage medium containing 10 μg/mL of gentamicin.
10. Move the plates back into the incubator for the optimal incubation time. With *F. novicida* at an MOI of 100, inflammasome activation can be detected as early as 4 h post-infection (*see* **Note 6**).
11. Remove the plates from the incubator 10 min prior to the desired time-point and remove 1/10 of volume from the uninfected wells that serve as the 100 % lysis control. Add back 1/10 of volume of lysis solution and move back to the incubator for 10 min.
12. Mix the culture medium in the wells of the 100 % lysis control with a multichannel pipette.
13. For pyroptosis measurements (LDH release assay) transfer 50 μL of supernatant from each well to a new 96-well plate. Measure LDH release immediately on fresh supernatant samples according to the manufacturer's protocol.
14. Transfer the remaining supernatant from the infection plate to a new 96-well plate excluding the 100 % lysis control samples. The samples on this plate can be used immediately for IL-1α, IL-1β, or IL-18 ELISAs according to the manufacturer's instruction or frozen at −20 °C for later use.

3.5 Analysis of Caspase-1 Processing by Western Blotting

1. Seed BMDMs into 6-well plates at 1.25×10^6 cells per well in 5 mL of macrophage medium and incubate overnight at 37 °C (*see* **Note 8**).
2. Follow **steps 2–8** of Subheading 3.4 to infect the macrophages.
3. Remove the medium containing 100 μg/mL gentamicin from the macrophages. Wash the infected BMDMs twice with pre-warmed plain DMEM to remove all traces of FCS. Add 1.8 mL fresh pre-warmed, unsupplemented DMEM containing 10 μg/mL of gentamicin (*see* **Note 8**).
4. Move the plates back into the incubator for the remainder of the infection.
5. At the desired time-point, remove the plate from the incubator and transfer the supernatant (1.8 mL) to a 2 mL Safe-Lock tube. Add 0.2 mL of 100 % Trichloroacetic acid to reach a final concentration of 10 %.
6. Vortex and precipitate the proteins for at least 1 h on ice. Lysate samples can be prepared simultaneously.
7. Centrifuge the tubes for 30 min at 4 °C at full speed in a tabletop centrifuge.
8. Remove the supernatant and wash the pellet with 1 mL of ice-cold acetone. Centrifuge for 10 min at 4 °C at full speed in a tabletop centrifuge.
9. Remove the supernatant and air-dry the pellet for 10–20 min at room temperature (RT).
10. Resuspend the pellet in 20–40 μL of 1× SDS-PAGE sample buffer. If the sample buffer turns yellow, re-adjust the pH by adding 1 μL of 1 M Tris–HCl pH 12.
11. Incubate the samples for 5–10 min at 95 °C. Let cool and spin down.
12. Following protein precipitation of supernatants, prepare cell lysates in RIPA buffer as follows: Transfer plates on ice after removing the supernatants for analysis.
13. Wash 2× with PBS.
14. Add RIPA buffer containing protease inhibitors according to plate format (for volumes, *see* **Note 9**) and incubate for 10 min on ice.
15. Scrape the cells and transfer to 1.5 mL tubes.
16. Incubate on ice for 30 min, with occasional vortexing.
17. Centrifuge for 10 min at 4 °C at full speed in a tabletop centrifuge.
18. Transfer the supernatant to a new tube without aspirating the pellet and add 2× SDS-PAGE sample buffer.

19. Heat the samples for 5–10 min at 95 °C, spin down and use for SDS-PAGE. The pro-forms of caspase-1, caspase-11 and IL-1β can be readily detected in cell lysates.
20. Proceed to Western blotting of precipitated supernatants and cell lysates as follows: Load the total sample of one well of a 6-well plate per lane of a 14 % SDS-PAGE and subject to electrophoresis.
21. Transfer the gel on a PVDF membrane (45 min at 100 V for BioRad MiniProtean gels).
22. Block the membrane for 1 h in blocking buffer at RT with constant shaking.
23. Add your primary antibody at an appropriate concentration in blocking buffer (*see* **Note 8**). Incubate overnight at 4 °C with constant shaking.
24. Remove the primary antibody and store at −20 °C for repeated use. Wash membranes with three quick washes and three additional 10 min washes with TBS-T.
25. Add the secondary HRP-coupled antibody at an appropriate dilution in blocking buffer and incubate for 45 min at RT, with constant shaking.
26. Remove secondary antibody. Wash membranes with three quick washes and at least four additional 10 min washes with TBS-T.
27. Tap-dry the membrane and transfer on saran wrap or parafilm. Add 1 mL ECL Western blot reagent, spread on the membrane and incubate for 5 min at RT.
28. Tap dry membrane, transfer to a Western blotting cassette and develop the blot using standard techniques and equipment.
29. To re-probe your blot with additional antibodies (*see* **Note 8**), strip the membrane with a mild stripping solution according to the manufacturer's instructions.
30. Repeat **steps 22–28** of Subheading 3.5 with different antibodies.

3.6 Visualization of Endogenous ASC Oligomers

1. Seed BMDMs into 24-well plates containing coverslips at 2.5×10^5 cells per well in 1 mL of macrophage medium and incubate overnight at 37 °C.
2. Follow **steps 2–10** of Subheading 3.4 to infect the macrophages.
3. Remove plate from incubator and wash each well 3× with PBS.
4. Remove the final wash and add 1 mL of fixative to each well and fix the cells for 15 min at 37 °C.
5. Wash each well 3× with PBS.
6. Prepare a hydration chamber, cover bottom with parafilm.

7. Transfer coverslips to hydration chamber, cover with PBS to prevent drying.
8. Remove PBS and add primary antibodies in blocking buffer (0.1 mL per coverslip) at the appropriate concentration (*see* **Note 10**). Incubate for 30 min.
9. Remove primary antibodies and wash coverslips 3× with PBS.
10. Add secondary fluorophore-coupled antibodies in blocking buffer. To outline the actin cytoskeleton, fluorophore-coupled Phalloidin (such as Alexa Fluor® 594 Phalloidin, Invitrogen) can be added to the secondary antibodies. Incubate for 30 min.
11. Remove secondary antibodies and wash coverslips 3× with PBS.
12. Mount coverslips on Microscopy slides using mounting medium with DAPI and visualize ASC specks by fluorescence microcopy or confocal microscopy.

4 Notes

1. This step ensures that the cells have enough MCSF to fully differentiate. After 6 days most of the bone marrow-derived macrophages (BMDMs) should have differentiated and are adherent to the plate.
2. Analysis of inflammasome-mediated cytokine maturation and release requires pretreatment with an appropriate TLR-ligand such as LPS, PAM3CSK or others. Pre-stimulation provides the Signal 1 of the inflammasome (*see* Subheading 1) and induces the expression of pro-IL-1α or pro-IL-1β, as well as certain inflammasome receptors (NLRP3, AIM2). In contrast, NLRC4, Nlrp1b, ASC and pro-caspase-1 are constitutively expressed. Pre-stimulation might not be necessary or desirable, since the bacterial infection itself can provide the Signal 1. For example, *F. novicida* activates TLR2 during infection, and *S. typhimurium* LPS is an excellent activator of TLR4 [32]. Thus, infection of unstimulated BMDMs with *F. novicida* will lead to the induction of AIM2 and pro-IL-1, and initiate inflammasome activation. Although inflammasome activation will be significantly delayed in unstimulated macrophages compared to pre-stimulated macrophages, this setup might be nevertheless desirable, if the goal is to study how different innate immune signaling pathways interact over the course of a pathogen infection, for example how TLR4/TRIF-signaling induces pro-caspase-11 expression [32, 33]. Unlike IL-1β maturation, pyroptosis does not require any Signal 1, if initiated by a constitutively expressed NLR, such as NLRC4 or Nlrp1b. Therefore, we recommend to pre-stimulate if analyzing inflammasome

activation by these receptors, since pyroptosis can lyse the infected cell before detectable levels of pro-IL-1 are expressed.

3. Both, pyroptosis and the release of mature cytokines can be easily detected with commercially available kits. In general, 50 μL of supernatant are required to measure the release of LDH and 100 μL are necessary for cytokine ELISA. Thus, both assays can be done from the supernatant collected from 96-well plates. IL-1β ELISAs are most commonly used, but ELISAs for IL-1α and IL-18 are available as well. The measurement of pyroptosis is based on the detection of the cytosolic Lactate Dehydrogenase (LDH), which is released during necrosis and pyroptosis, or by cell lysis with a detergent. The percentage of cell death is calculated as follows: (LDH infected − LDH uninfected/LDH total lysis − LDH uninfected) × 100. Therefore, the experimental setup requires additional uninfected wells that serve as uninfected and total lysis controls (Fig. 2). Total lysis is achieved by the addition of lysis solution (10 % Triton X-100). Use the following outline as an example on how to set up your 96-well plate experiment: For inflammasome studies, we recommend to use BMDMs from wild-type C57/BL6 mice and C57/BL6 *Casp-1-deficient* mice (aka *Casp-1/Casp-11* dKO) [31]. If your pathogen activates inflammasomes you expect to see a significant difference in LDH release or cytokine maturation between these two genotypes. Additional BMDMs of different genotypes can be added, such as *AIM2*$^{-/-}$ BMDMs for *F. novicida* infections, since *F. novicida* activates the AIM2-inflammasome in murine BMDMs [17–19]. We recommend testing different MOIs of your pathogen and unstimulated versus pre-stimulated cells to find the optimal infection conditions. All sample conditions should be done in triplicate or quadruplicate, as outlined in Fig. 2.
4. The protocol outline above describes the infection with *F. novicida,* an intracellular pathogen that activates AIM2 in murine BMDMs, a cytoplasmic DNA sensor [17–19]. However the basic infection protocol is similar for intracellular as well as extracellular pathogens.
5. The first point to consider, when planning your infection, is to find the optimal growth or induction conditions that your pathogen requires to express its virulence factors and/or successfully invade cells. The particular growth phase your bacterial culture is in can significantly change the outcome of an infection. For example, logarithmic phase *S. typhimurium* will express its SPI-1 Type 3 secretion system and inject flagellin into BMDMs, thus activating the NLRC4 inflammasome within 1–2 h [9, 29]. In contrast, stationary phase *S. typhimurium* downregulates SPI-1 expression and activates NLRP3, NLRC4 and the noncanonical caspase-11 inflammasome through other

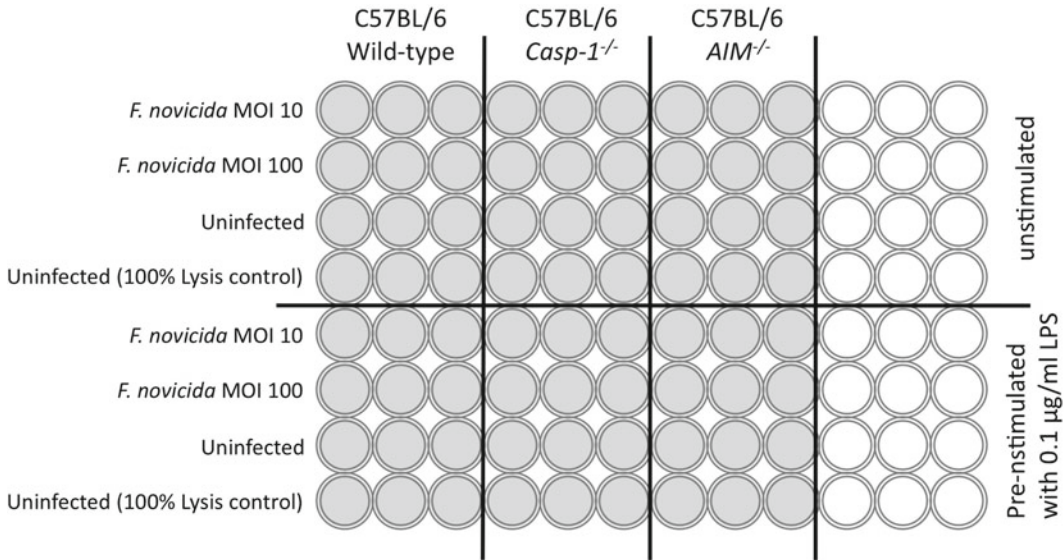

Fig. 2 Overview of an infection setup in 96-well plate format. BMDMs of the indicated genotypes are seeded at 5×10^4/well, and are either left unstimulated or pre-stimulated with 0.1 µg/mL LPS. The next day, BMDMs are infected with *F. novicida* at the indicated Multiplicity of Infection (MOI) or left uninfected. For LDH release assays, lysis solution is added to uninfected wells labeled 100 % lysis control before collecting the supernatants for analysis

virulence factors [26, 32]. Thus, understanding the particular pathogen and its virulence factors is of crucial importance to successfully study inflammasome activation.

6. Another point to consider is the MOI, i.e., the average number of bacteria per cell and the length of the infection before supernatant samples are collected. We recommend starting with a range of different MOIs and a short time-course to determine the optimal condition to measure maximal inflammasome activation. Be aware that the optimal length of infection can vary between 30 min and 24 h or more.

7. Finally, depending on the length of the infection, it is important to determine at what time point to stop bacterial uptake by killing the extracellular bacteria by the addition of gentamicin. This step is important, since many pathogens will start replicating extracellularly in the macrophage medium and would otherwise overgrow the BMDM culture. For most bacteria incubation times in the range of 30–60 min are sufficient. Opsonizing the bacteria could be used to force this process. Importantly, some bacteria activate the inflammasome so fast that the infection can be stopped before gentamicin needs to be added. An example for this is the activation of NLRC4 by logarithmic phase *S. typhimurium*, which takes only 1–2 h [9].

Importantly, long exposure to high concentrations of the membrane impermeable antibiotic gentamicin can nevertheless result in the leakage of the antibiotic into the cells, where it can affect bacterial viability. Therefore, we recommend replacing the medium containing 100 μg/mL of gentamicin (a concentration sufficient to kills extracellular bacteria) with medium containing 10 μg/mL (merely preventing bacterial growth) for longer infections.

8. The protocol outlined in Subheading 3.5 describes how to collect and concentrate supernatant samples from cells infected with *F. novicida*. Although Western blots can also be done on samples from 96-well plates, this is difficult as the amount of processed caspase-1 p10 and p20 or caspase-11 p30 in the cell supernatants is very low. Our protocol allows for a 100-fold concentration of supernatant samples, thus making the detection of processed caspase subunits significantly easier. The key to this concentration step is to precipitate the supernatant of $1–1.25 \times 10^6$ BMDMs. As mentioned in the protocol, it is important to collect the secreted proteins into plain, unsupplemented DMEM, since serum albumin in the medium will overload the gel after precipitation. The same protocol with minor modifications can be used for different pathogens or other inflammasome stimuli. For pathogens/stimuli that lead to a rapid activation of the inflammasome, such as logarithmic phase *S. typhimurium*, we recommend to change the protocol as follows: Wash the BMDMs twice with plain pre-warmed DMEM before the infection. Then, infect the cells with 1.8 mL of plain, unsupplemented medium containing your bacteria. After 1–2 h, collect the 1.8 mL of supernatant, still containing extracellular bacteria, and process the supernatant as outlined in Subheading 3.5. We do not recommend leaving the BMDMs too long in medium without FBS and MCSF. For very long infections (20–24 h), such the activation of the noncanonical caspase-11 inflammasome [31–33], it might be therefore advisable to start collecting the supernatant later, for example only after 8–10 h of infection. In that case, the wash steps outlined in **step 3** of Subheading 3.5 are done in macrophage medium with supplement. Then, at 8–10 h post infection, the cells are washed twice with plain pre-warmed DMEM and 1.8 mL of plain, unsupplemented medium is added to collect secreted proteins. Besides blotting for caspase-1 p10 and p20 subunits or caspase-11 p30 (clone 17D9), we routinely reprobe the membranes for IL-1α (Biovision), IL-1β (R&D Systems), and IL-18 (Abcam) in addition or as an alternative to the ELISAs described in Subheading 3.4. Additionally, the release of cytoplasmic danger signals such as HMGB1 can also be detected by Western blotting.

9. Lysate samples from 96-well and 6-well plates can be prepared in RIPA buffer using standard protocols. For addition of RIPA buffer containing protease inhibitors, use 20 μL/well in 96-well plates, 300 μL/well in 6-well plates. The pro-forms of caspase-1, caspase-11, and IL-1β can be readily detected in cell lysates.
10. The optimal concentration of primary antibodies needs to be determined empirically. We use rat anti-ASC (clone 8E4.1, Genentech Inc.) at 1:1,000, rabbit anti-ASC (AL177; Enzo Life Sciences) at 1:1,000, and rabbit anti-caspase-1 (sc-514, Santa Cruz Biotechnology Inc.) at 1:100.

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