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A comprehensive guide for studying inflammasome activation and cell death

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Abstract

Inflammasomes are multimeric heterogenous mega-Dalton protein complexes that play key roles in the host innate immune response to infection and sterile insults. Assembly of the inflammasome complex following infection or injury begins with the oligomerization of the upstream inflammasome-forming sensor and proceeds through a multistep process of well-coordinated events and downstream effector functions. Together, these steps allow for elegant experimental readouts to reliably assess the successful activation of the inflammasome complex and cell death. Here, we describe a comprehensive protocol that details several *in vitro* (in bone-marrow derived macrophages) and *in vivo* (in mice) strategies for activating the inflammasome and explain how to subsequently assess multiple downstream effects in parallel to unequivocally establish the activation status of the inflammasome and the cell death pathways. Our workflow assesses inflammasome activation via the formation of the ASC speck, cleavage of caspase-1 and gasdermin D, release of IL-1 β , IL-18, caspase-1, and lactate dehydrogenase from the cell, and real-time analysis of cell death by imaging. Analyses take up to approximately 24 hours to complete. Overall, our multifaceted approach provides a comprehensive and consistent protocol for assessing inflammasome activation and cell death.

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Author contributions

R.E.T., R.K.S.M., and T.-D.K. conceptualized the manuscript. R.E.T. and R.K.S.M. wrote the first draft. All authors reviewed and approved the final draft of the manuscript.

TWEET A comprehensive Protocol for characterizing inflammasome activation and cell death from @KannegantiLab @StJudeResearch.

EDITORIAL SUMMARY This protocol describes a toolbox for comprehensive characterization of inflammasome activation and cell death in response to both *in vivo* (in mice) and *in vitro* (using bone-marrow derived macrophages) models of infection, sterile insults and cancer.

COVER TEASER Inflammasome activation and cell death methods

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files. No datasets were generated or analyzed during the current study. All source data behind the figures are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing financial interests.

Keywords

inflammasome; NLRP3; NLRC4; NLRP1; AIM2; pyrin; ASC; caspase-1; IL-1 β ; IL-18; gasdermin D; pyroptosis; cell death; infection; NLR; TLR; caspase-11; ZBP1; caspase-6; caspase-8; RIPK1; RIPK3; PANoptosis; PANoptosome

Introduction

Inflammasomes are multimeric protein complexes that assemble in response to a cytosolic innate immune receptor detecting pathogen- or damage-associated molecular patterns (PAMPs or DAMPs) and loss of cellular homeostasis (homeostasis-altering molecular processes, HAMPs)^{1–5}. These sensors are evolved to recognize a diverse array of ligands including proteins, RNA and DNA. While inflammasome activation is critical for the innate immune response to infection, aberrant activation of the inflammasome and subsequent cell death is associated with several autoinflammatory diseases^{6–8}. These include cryopyrin-associated periodic syndrome^{9–11}; familial Mediterranean fever^{12,13}; and purulent arthritis, pyoderma gangrenosum, and cystic acne (PAPA)^{14–16}, among several others. Additionally, inflammasomes have been associated with several forms of cancer¹⁷. Understanding the mechanisms of inflammasome activation is critical for establishing novel therapeutic targets for these diseases.

Inflammasomes contain cytosolic innate immune receptors, or sensors as they are often called. Most of the inflammasome-forming sensors are specific to unique triggers, allowing cells to respond and distinguish between stressors¹⁸. The well-studied inflammasome sensors include nucleotide-binding domain and leucine-rich repeat receptor (NLR) family pyrin domain (PYD)-containing 1 (NLRP1), NLRP3, NLR family caspase recruitment domain (CARD)-containing 4 (NLRC4), absent in melanoma 2 (AIM2), and pyrin^{1,3,7,19–35}. Once an inflammasome sensor detects its cognate trigger, it uses homotypic interactions to recruit other CARD- and/or PYD-containing proteins to the inflammasome complex. Many inflammasome sensors interact with an adapter protein known as apoptosis-associated speck-like protein containing a CARD (ASC) through PYD-PYD interactions^{1,7}; ASC in turn recruits the effector caspase-1 to the complex through CARD-CARD interactions. Alternatively, inflammasome sensors that contain a CARD domain can recruit caspase-1 directly through CARD-CARD interactions^{36–38}. Inflammasome complex formation enables the proximity-induced dimerization and self-processing of caspase-1 to cleave the full-length protein into its active form^{39–41}. Activated caspase-1 can now cleave its substrates, including gasdermin D and the proinflammatory cytokines pro-IL-1 β and pro-IL-18. Cleavage of gasdermin D releases its N-terminal fragment (GSDMD-N), which forms pores in the cell membrane to execute a form of cell death called pyroptosis^{42,43}. These pores also allow the release of matured IL-1 β and IL-18, which further amplifies the inflammatory signaling^{2,44,45}. Because inflammasome activation is a multistep process with several distinct effector functions, such as cell death and the release of DAMPs, it is important to use complimentary approaches to establish the functional relevance of the activation.

Consistent and reliable readouts are necessary to quantify inflammasome activation and monitor the activity of multiple downstream pathways. To this end, we have optimized

several inflammasome activation conditions that reliably provide robust induction of caspase-1 cleavage and subsequent pyroptosis. Similarly, we have developed essential protocols to allow for the monitoring of multiple inflammasome activation readouts in parallel to understand the full scope of the activity occurring on the cellular level. This Protocol can be used to study bacterial infection (such as *Escherichia coli*⁴⁶, *Citrobacter rodentium*^{46,47}; *Pseudomonas aeruginosa*⁴⁸, *Salmonella enterica* serovar Typhimurium^{35,46,49}, *Burkholderia thailandensis*^{35,50} and *Francisella novicida*^{46,51,52}), viral infection (such as influenza A virus^{53,54} and murine cytomegalovirus⁵⁵), fungal infection (such as *Aspergillus fumigatus*^{56,57} and *Candida albicans*⁵⁸), inflammatory diseases (such as sepsis⁵⁹, skin disease⁶⁰, bone disease^{61–63}, metabolic disorders⁶⁴, atherosclerosis⁶⁵ and neurodegenerative disease^{66,67}) and cancers (such as colitis-induced colorectal cancer^{68–71}). This protocol is widely applicable to many different research areas in health and disease in which the inflammasome plays a role.

Overview of the Procedure

The procedure starts with inflammasome activation (see Table 1), either *in vivo* (Step 1) or *in vitro* in bone marrow-derived macrophages (BMDMs) (Steps 2–31). *In vivo* inflammasome activation can be triggered by bacterial infection (Step 1 Options A–E), viral infection (Option F), fungal infection (Options G–H), inflammatory diseases (Options I–Q) and in response to cancer (Option R). For the *in vitro* inflammasome activation procedures, BMDMs are first isolated (Steps 2–18) and differentiated (Steps 19–30) before inflammasome activation is triggered by ligand-based triggers (Step 31 Options A–G), bacterial infection (Options H–J), viral infection (Options K–L) and fungal infection (Options M–N). The procedure is designed to monitor multiple molecules in the inflammasome activation cascade and to assess the functional outcome of inflammasome activation, i.e. pyroptosis, in parallel. This allows a complete picture of the cellular processes and mechanisms occurring. To achieve this, we have integrated several different molecular techniques and assays into a single streamlined protocol. Within the same experiment, we monitor cell death in real-time (Step 32 Option A), visualize ASC specks with microscopy (Step 33 Option A), perform LDH assays (Step 33 Option B), perform ELISA assays to determine the amount of caspase-1 and IL-18 (Step 33 Option C) and IL-1 β released from cells (Step 33 Option D), and perform western blots to determine caspase-1 and gasdermin D cleavage (Step 33 Option E) (Fig. 1). In our pipeline, four 12-well plates of cells are used, one for real-time cell death monitoring, one for separate protein lysate and supernatant collection, one for combined supernatant and protein lysate collection, and one to fix for microscopy, with 3 biological replicates per sample on each plate. This allows us to carry out all the necessary downstream analyses in a single experiment. Additionally, the 12-well format was specifically selected to ensure sufficient quantities of each sample are collected to allow for extensive biochemical analyses. However, this method can be adapted to use fewer cells in a 24- or 96-well format.

Applications of the method

Here we provide detailed procedures for numerous *in vivo* and *in vitro* inflammasome activation conditions in which our lab has expertise. Moreover, many inflammasome activators described by others^{22,24,72,73} can also be used in combination with the

downstream assays. Importantly, our method uses simple laboratory techniques to monitor caspase-1 activation, gasdermin D cleavage, IL-1 β and IL-18 release, and cell death in freshly isolated and differentiated murine bone marrow-derived macrophages (BMDMs). However, other murine cell types such as bone marrow-derived dendritic cells and even tissue samples can also be used^{57,74,75}. BMDMs derived from cryopreserved mouse bone marrow can also be employed⁷⁶. Furthermore, another potential application is that these methods could be extended for use in human cell types such as peripheral blood mononuclear cells⁷⁷. Inflammasome activation can also be monitored in a variety of other species, including rats, swine, cattle, dogs, bats, small ruminants, and birds⁷⁸. Primary macrophages from wild type and transgenic mice are ideal for these studies because they contain the necessary machinery for inflammasome activation, but immortalized cells such as the commonly used THP-1 human monocytic line and *in vitro* transformed BMDMs (iBMDMs) can be beneficial if a gene for which no gene-deficient mouse line is available is being studied. However, caution should be used when selecting immortalized cell lines, as many of these are deficient in critical components of inflammasome assembly and/or activation^{29,79,80}. For example, RAW264.7 cells, a common murine macrophage cell line, do not contain ASC, making them deficient in inflammasome activation⁷⁹. The inflammasomes can be reconstituted in cell lines lacking the corresponding components, but it is important to consider whether the reconstituted system will be physiologically relevant for the question of interest. HEK293 cells can be reconstituted with ASC and caspase-1 to induce inflammasome formation and caspase-1 activation, but this does not fully recapitulate the normal NF- κ B signaling cascade, calling into question the relevance of this model⁸⁰.

In addition to pyroptosis, this protocol allows the observation and assessment of other forms of cell death, apoptosis and necroptosis, which can also be induced by many of the inflammasome triggers^{49,53,54,59,61,62,81–84}. Combining several readouts allows for the differentiation of inflammasome activation and pyroptosis from these other forms of cell death. For example, necroptosis will also lead to cells appearing dead in real-time cell death analyses and will yield similar LDH assay results to pyroptosis, but will not have the characteristic ASC speck formation, caspase-1 and gasdermin D cleavage and IL-1 β and IL-18 release. Similarly, apoptosis will cause cells to appear dead in real-time cell death analyses, but the use of the alternative techniques allows pyroptosis to be differentiated. Recent studies from our group have also highlighted that there is extensive crosstalk between the inflammasome/pyroptosis and apoptosis and necroptosis^{49,53,54,59,61,62,81,82,85}, leading to the concept of PANoptosis to describe them in a unified mechanism⁸³. This process is driven through a PANoptosome complex that can contain the traditional inflammasome components NLRP3, ASC and caspase-1 along with ZBP1, caspase-6, caspase-8, RIPK1, RIPK3 and others^{54,59,86,87}. This protocol can also be applied to the evaluation of PANoptosis.

Comparison with other methods

There are several other methodologies that have been proposed to monitor inflammasome activation. However, most focus on only one of the molecules involved in inflammasome activation and do not provide a complete picture of the cellular events occurring. Monitoring caspase-1 cleavage and activation has historically been one of the primary methods to

determine whether inflammasome activation is occurring. Caspase-1 western blotting to visualize the pro- and cleaved forms of the protein is commonly used^{88,89}. Additional methods employing fluorescent reporters have also been developed. In these methods, caspase-1 substrates fused to fluorophores that become fluorescent following substrate cleavage can be used to measure caspase-1 activity in cell culture supernatants^{90,91} and cell and tissue lysates⁹². Fluorophores have also been developed to react with active caspase-1 and form a covalent bond, allowing visualization of the molecule in intact cells by microscopy⁹³. Recently, a transgenic mouse line was created that constitutively expresses a caspase-1 biosensor in the form of a bioluminescent luciferase reporter construct with a caspase-1 cleavage site that becomes fluorescent upon cleavage⁹⁴. While this mouse allows *in vivo* monitoring of caspase-1 activation, doing so requires the use of an IVIS imaging chamber, a very expensive piece of equipment. Additionally, all of the aforementioned reporter methods will only determine whether caspase-1 is active; they will not provide information about subsequent gasdermin D cleavage, inflammatory cytokine maturation and release or cell death. Furthermore, the reporter substrates often have low specificity and can be cleaved by other caspases as well⁹⁵, making results from these assays difficult to interpret without an accompanying caspase-1 western blot.

Another common methodology that has been used to monitor inflammasome activation is ASC oligomerization visualization. This can be achieved through microscopy; western blotting for ASC monomers, dimers, and oligomers⁹³; or flow cytometry⁹⁶. Flow cytometry allows for the most rapid quantification of the number of cells containing ASC oligomers, but cells are generally very fragile following inflammasome activation, and flow cytometry conditions can lead to cell rupture. Monitoring ASC oligomerization also has the downfall that it does not provide any evidence as to whether caspase-1 or its downstream substrates are activated.

When cell lines are used in place of primary cells, additional methodologies for monitoring inflammasome activation can be employed. For example, fluorescence resonance energy transfer (FRET)-based tracking can be performed to visualize the oligomerization of inflammasome filaments based on the FRET ratio⁹⁷. This method requires the expression of recombinant proteins with the FRET donor and FRET acceptor molecules, which is notoriously difficult to do endogenously in primary innate immune cells. A method for monitoring gasdermin pore formation *in vitro* has also been described⁹⁸. This method uses a liposomal system containing Tb³⁺-DPA fluorescent complexes to measure the amount of gasdermin D N-terminal molecules that are inserted into the liposomal membrane based on the release of the fluorescence. However, this requires the production of recombinant gasdermin D and uses an extracellular system for the readout. Additionally, using gasdermin D cleavage and pore formation alone as a readout for inflammasome activation is complicated by the fact that gasdermin D can be cleaved in an inflammasome-independent mechanism by caspase-8 under some circumstances^{99,100}.

The more comprehensive protocols that have been applied to date monitor cytokine, caspase-1, and LDH release and visualize ASC speck formation¹⁰¹, but do not incorporate gasdermin D cleavage or visualization of cell death as readouts. Overall, while several methods have been described to monitor inflammasome activation, each has limitations and

does not provide a comprehensive view of cellular events on its own. Combining several simple methodologies as we have done here has the advantage of following the entire process of inflammasome activation from initial complex assembly to cytokine release and cell death.

Limitations

The main limitation of this protocol is that it does not allow the monitoring of inflammasome activation *in vivo* in real time. There is also a technical limitation that careful cell isolation technique is required to allow assessment of inflammasome activation in the cell type(s) of interest. For these analyses, it is important that cells are not exposed to stress, such as nutrient, temperature, or mechanical stress, beforehand, as this can lead to cellular stress responses that will complicate interpretation. ... Additionally, our protocol is designed and optimized for use with mice and mouse BMDMs. However, the techniques are highly transferable to other species and cell types.

Expertise needed to implement the protocol

The inflammasome readout methods employed in our protocol are designed to be simple enough to be implemented by graduate students and principle investigators alike, as they do not require skills beyond a basic familiarity with typical molecular biology and aseptic techniques. The isolation of bone marrow precursor cells does require some practice to consistently obtain high-quality cells and optimal yields, but this can typically be achieved with 3–4 practice experiments. Depending upon the *in vitro* or *in vivo* inflammasome activation assay selected, researchers may need additional skill sets. General rodent-handling and injection experience will be necessary for most of the *in vivo* models. Additionally, bacterial, viral, or fungal culture techniques may be needed for *in vivo* and *in vitro* inflammasome activation studies. Although none of the pathogens included in this protocol require anything beyond BSL2 containment and all can be cultured with minimal difficulty, it is mandatory to obtain formal training and the required governmental and institutional approvals before proceeding with experiments.

Experimental design

Downstream inflammasome and cell death assays.—These particular readouts were chosen to profile each step of the inflammasome activation cascade. Visualization of ASC speck formation confirms that ASC oligomerization and the initialization of the inflammasome complex have occurred (Step 33 Option A). Caspase-1 cleavage, the next step in the inflammasome activation cascade, is confirmed by western blotting (Step 33 Option E). Following caspase-1 cleavage, caspase-1 will go on to cleave its downstream substrates, and this is monitored with gasdermin D western blotting (Step 33 Option E). Pyroptosis, the endpoint of inflammasome activation, is monitored using a real-time cell death assay (Step 32 Option A), LDH assay (Step 33 Option B), and by assaying for the release of the inflammasome-dependent cytokines IL-1 β and IL-18 and caspase-1 in the supernatant (Step 33 Options C and D). The use of multiple cell death assays allows for the distinction of pyroptosis from other forms of cell death, like apoptosis and necroptosis. At a

minimum, caspase-1 cleavage by western blot should be assessed to determine that inflammasome activation has successfully occurred.

Detecting secreted and intracellular targets.—In this protocol, IL-1 β , IL-18 and LDH are monitored in the supernatant, gasdermin D is monitored in the cell lysate, and caspase-1 is monitored in both the supernatant and in the combined supernatant and protein lysate. While secreted caspase-1 in the supernatant is a good marker of inflammasome activation, there is still a substantial amount of activated caspase-1 left in the cells undergoing pyroptosis. Therefore, caspase-1 is both intracellular and extracellular once inflammasome activation and pyroptosis have begun, and combining this protein lysate with the supernatant further enhances the detection of caspase-1 cleavage and improves the sensitivity of the assay. Additionally, because the supernatant dilutes the protein lysate from the cells, assaying intracellular proteins like gasdermin D from the combined lysate typically may not produce strong enough signal for analysis.

Imaging and image analysis.—This protocol utilizes the IncuCyte Zoom incubator system for real-time cell death analysis by live-cell fluorescence imaging. However, in instances where this equipment is not available, several alternative strategies can be used. Any microscope with live-cell fluorescence imaging functionality can be used. Images can also be manually acquired using an inverted fluorescence microscope. However, this will limit the number of wells that can be analyzed in a single experiment due to the additional time this approach will require.

Positive and negative controls.—Every experiment requires a positive control and a negative control. If a genetically modified mouse is being used, it should be compared against a corresponding wild type mouse of the same gender and age. Additionally, littermate controls are ideal and should be used whenever possible. When activating a particular inflammasome *in vitro*, the positive control should be wild type cells stimulated with a known activator of that inflammasome (e.g., LPS + ATP for the NLRP3 inflammasome). The optimal negative controls are treatment with media only and treatment with a known activator of the inflammasome of interest in cells that are genetically deficient for the inflammasome sensor (e.g., LPS + ATP treatment in *Nlrp3*^{-/-} cells for the NLRP3 inflammasome). Best results are obtained by paying attention to cellular morphology and the readouts from experimental controls. BMDM morphology during differentiation (Steps 19–30) can indicate whether the cells are healthy and the experimental treatments can proceed. Cell death or caspase-1 activation in the absence of stimulation indicate that the cells are likely stressed and are not fit for the experiment. Being attentive to these details avoids the potential false positives and helps to ensure the data are accurate and reproducible.

Materials

Biological materials

- Adult wild type mice (6 to 8 weeks old) and/or a transgenic mouse line. In our protocol, we generally use C57BL/6J and transgenic mice that have been

extensively backcrossed (10 times) to the C57BL/6J background unless otherwise necessitated. (The Jackson Laboratory)

- Optional; Sharpin^{cpdm} mice (10. weeks old) for studying skin disease in Step 1 Option K (The Jackson Laboratory, stock no. 007599).
- Optional: *Pstpip2*^{mo} mice (60–120 days old) for studying bone disease in Step 1 Option L. (The Jackson Laboratory, stock no. 002864)
- Optional: TPS mice that overexpress human Tau (1N4R) with the P301S mutation (11 months old) for studying Tau-related neurodegenerative disease in Step 1 Option Q (The Jackson Laboratory, stock no. 008169)
- Optional: *App* knock-in mice (*App*^{NL-F} and *App*^{NL-G-F}) (2–12 months old)¹⁰² for studying Tau-related neurodegenerative disease in Step 1 Option Q (Available upon request from Dr. Takaomi Saido, RIKEN Center for Brain Science)
- Optional: BALB/c or transgenic C57BL/6J mice for studying the NLRP1b inflammasome, which is not functional in wild type C57BL/6J mice (The Jackson Laboratory, stock no. 000651 [BALB/c] or stock no. 006840 [C57BL/6J transgenic])

(Caution—Experiments involving mice must conform to institutional and governmental guidance. The procedures in this protocol were approved by the animal care and use committee at St. Jude Children’s Research Hospital and performed in accordance with the *Guide for the Care and Use of Laboratory Animals* from the National Institutes of Health and all other relevant ethical guidelines.)

Biological materials for inflammasome activation

(Caution—All bacterial and fungal species and viral strains included in this protocol are potentially pathogenic and should be handled with the appropriate precautions in a BSL2 facility with approval from the relevant institutional and governmental authorities.)

- L929 cells (ATCC, cat. no. CCL-1; RRID: https://scicrunch.org/resolver/CVCL_0462) for making BMDM culture media (see Reagent Setup)
- *Citrobacter rodentium* DBS100 (ATCC, cat. no. 51459) for Step 1 Option A and for Step 31 Option H (see Reagent Setup)
- *Pseudomonas aeruginosa* PA01 (ATCC, cat. no. 47085) for Step 1 Option B and for Step 31 Option H or Option I (see Reagent Setup)
- *Salmonella enterica* serovar Typhimurium SL1344 (ATCC, cat. no. 700720) for Step 1 Option C and for Step 31 Option I (see Reagent Setup)
- *Burkholderia thailandensis* E264 (ATCC, cat. no. 700388) for Step 1 Option D and for Step 31 Option H or Option I (see Reagent Setup)
- *Francisella novicida* U112 (BEI Resources, cat. no. NR-13) for Step 1 Option E and for Step 31 Option J (see Reagent Setup)

- Influenza A virus (A/Puerto Rico/8/34, H1N1 [PR8]) (constructed per Hoffmann et al.¹⁰³) for Step 1 Option F and for Step 31 Option K (see Reagent Setup)
- Embryonated chicken eggs (Charles River, cat. no. 10100326) for multiplying the influenza virus for Step 1 Option F and for Step 31 Option K (see Reagent Setup)
- MDCK cells (ATCC, cat. no. CCL-34; RRID: https://scicrunch.org/resolver/CVCL_0422) for determining the influenza virus titer for Step 1 Option F and Step 31 Option K (see Reagent Setup)
- *Aspergillus fumigatus* FGSC A1163, FGSC A1160, and FGS A1100 (Af293)¹⁰⁴ (Available upon request from Pr. Jean-Paul Latge, Institut Pasteur) for Step 1 Option G and for Step 31 Option M (see Reagent Setup)
- *Candida albicans* SC5314 (ATCC, cat. no. MYA-2876) for Step 1 Option H and for Step 31 Option N (see Reagent Setup)
- *Clostridium difficile* r20291 AB– and AB+ strains¹⁰⁵ (Available upon request from Dr. N. Minton, University of Nottingham) for Step 31 Option E (see Reagent Setup)
- *Escherichia coli* NCTC 9001 (ATCC, cat. no. 11775) for Step 31 Option H (see Reagent Setup)
- Murine cytomegalovirus MSGV Smith strain (ATCC, cat. no. VR-1399) for Step 31 Option L (see Reagent Setup)

Reagents

Bacterial triggers for inflammasome activation (Step 1 Options A-E and Step 31 Options H-J)

- LB broth (MP Biomedicals, cat. no. 3002–031)
- LB agar (Fisher Scientific, cat. no. BP9724–500)
- Tryptic soy broth (Fisher Scientific, cat. no. R07224)
- L-cysteine (Millipore Sigma, cat. no. C7352)
- Brain heart infusion agar (BD Biosciences, cat. no. L007442)
- Tryptone-yeast extract medium (Fisher Scientific, cat. no. DF0440–17)
- Gentamicin (Gibco, cat. no. 15750060)

Viral triggers for inflammasome activation (Step 1 Option F and Step 31 Options K-L)

- High glucose DMEM (Sigma, cat. no. D6171)
  - 2,2,2-tribromethanol (Sigma, cat. no. T48402)
- (Caution—2,2,2-tribromethanol is dangerous if inhaled. Wear appropriate respiratory protection when handling. Use of this reagent as an anesthetic must be approved by the appropriate institutional authorities.)

- 2-methyl-2-butanol (Sigma, cat. no. 240486)
(Caution—2-methyl-2-butanol is flammable. Keep away from open flames. It is also dangerous if inhaled. Wear appropriate respiratory protection when handling.)

Fungal triggers for inflammasome activation (Step 1 Options G-H and Step 31 Options M-N)

- Maltose (MP Biomedicals, cat. no. SKU 0219023780)
- Sabouraud broth (Sigma, cat. no. S3306)
- RPMI-1640 (Corning, cat. no. 196196006)
- Tween-20 (Sigma, cat. no. P1379)
- Cyclophosphamide monohydrate (Sigma, cat. no. C0768)
- Cortisone 21-acetate (Sigma, cat. no. C3130)

Inflammatory disease triggers for inflammasome activation (Step 1 Options I-Q)

- Type II collagen (Chondrex; cat. no. 20011)
- Acetic acid (Sigma, cat. no. 695092)
(Caution—Acetic acid is caustic. Use caution when handling. Wear gloves, splash goggles, and a synthetic apron or lab coat, and handle in a fume hood.)
- Complete Freund Adjuvant (CFA; BD Biosciences, cat. no. 231131)
- Heat-killed *Mycobacterium tuberculosis* H37 Ra (BD Biosciences, cat. no. 231141)
- High-fat rodent chow (Research Diets Incorporated, cat. no. D12451)
- High-sugar rodent chow (Research Diets Incorporated, custom order, 65% of calories from sucrose)
- Western-type diet rodent chow (Research Diets Incorporated, cat. no. D12079B)

Cancer triggers for inflammasome activation (Step 1 Option R)

- Azoxymethane (Sigma, cat. no. A5486)
(Caution—Azoxymethane is carcinogenic. Wear gloves and avoid contact with eyes, nose, and mouth.)
- Dextran sodium sulfate (Thermo Fisher Scientific, cat. no. 9011-18-1)

Bone marrow progenitor cell isolation and BMDM differentiation (Steps 2–30)

- IMDM (Thermo Fisher Scientific, cat. no. 12440-053)
- Non-essential amino acids (Gibco, cat. no. 11140050)
- Fetal bovine serum (FBS) (Biowest, cat. no. S1620)
- Sodium pyruvate (Gibco, cat. no. 11360-070)

- Penicillin and streptomycin (Sigma, cat. no. P4333)
- DMEM (Thermo Fisher Scientific, cat. no. 11995-073)
- Ethanol (Pharmco, cat. no. 111000200)
(Caution—Ethanol is flammable. Keep away from open flames.)
- DPBS (Gibco, cat. no. 14190-144)

Ligand-based triggers for *in vitro* inflammasome activation (Step 31)

- Ultrapure LPS from *E. coli* 0111:B4 (Invivogen, cat. no. tlrl-3pelps)
- ATP (Invivogen, cat. no. tlrl-atpl)
- Nigericin (Sigma, cat. no. N7143)
- *Bacillus anthracis* protective antigen (List Biological Laboratories, Inc., cat. no. 171E)
- *Bacillus anthracis* lethal factor (List Biological Laboratories, Inc., cat. no. 172B)
- TcdA toxin (List Biological Laboratories, Inc., cat. no. 152C)
- TcdB toxin (List Biological Laboratories, Inc., cat. no. 155A)
- Opti-MEM (Thermo Fisher Scientific, cat. no. 31985-070)
- DOTAP (Roche, 11202375001)
- Recombinant PrgI from *S. typhimurium* (Mybiosource, cat. no. MBS1177087)
- Recombinant PrgJ *S. typhimurium* (Mybiosource, cat. no. MBS1061410)
- Ultrapure flagellin from *S. typhimurium* (Invivogen tlrl-epstfla-5)
- Xfect (Clontech Laboratories, cat. no. 631318)
- Poly(dA:dT) (Invivogen, cat. no. tlrl-patn)

Real-time cell death analysis (Step 32 Option A)

- SYTOX Green (Thermo Fisher Scientific, cat. no. S7020)
- Nuclear-ID (Enzo Life Sciences, cat. no. ENZ-52406)

Fixing cells for microscopy (Step 32 Option B)

- 4% (wt/vol) paraformaldehyde (ChemCruz, cat. no. sc-281692)
(Caution—Paraformaldehyde is carcinogenic. Wear gloves, splash goggles, and a synthetic apron or lab coat, and handle in a fume hood.)
- Normal goat serum (Sigma, cat. no. NS02L)
- Triton X-100 (Sigma, cat. no. X100)

Supernatant and protein lysate collection (Step 32 Options C-D)

- 2-Mercaptoethanol (BME; Sigma, cat. no. M6250)

(Caution—BME is carcinogenic. Wear gloves, splash goggles, and a synthetic apron or lab coat, and handle in a fume hood.)

- Bromophenol blue (Sigma, cat. no. BO126)
- NP-40 Alternative (Sigma, cat. no. 492016)
- Glycerol (Sigma, cat. no. G7893)
- Protease inhibitor cocktail (Sigma, cat. no. S8820)

ASC speck analysis (Step 33 Option A)

- Anti-ASC primary antibody (AdipoGen, cat. no. AG-25B-006-C100; RRID: https://scicrunch.org/resolver/AB_2490440)
- Alexa Fluor 568-conjugated anti-rabbit IgG (Life Technologies, cat. no. A11036; RRID: https://scicrunch.org/resolver/AB_143011)
- Alexa Fluor 488-conjugated anti-rabbit IgG (Life Technologies, cat. no. A11034; RRID: https://scicrunch.org/resolver/AB_2576217)
- VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, cat. no. H1200)

LDH assay (Step 33 Option B)

- CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega, cat. no. G1780)

Caspase-1 and IL-18 sandwich ELISAs (Step 33 Option C)

- Caspase-1 ELISA kit (AdipoGen, cat. no. AG-45B-0002-KI01)
- IL-18 ELISA kit (Invitrogen, BMS618–3)

IL-1 β multiplex ELISA (Step 33 Option D)

- IL-1 β multiplex ELISA kit, MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore, cat. no. MCYTOMAG-70K)

Caspase-1 and gasdermin D western blot (Step 33 Option E)

- Anti-caspase-1 antibody (AdipoGen, cat. no. AG-20B-0042-C100; RRID: https://scicrunch.org/resolver/AB_2755041)

(Critical—While anti-caspase-1 antibodies from other sources do work, we have found that this one is best.)

- Anti-gasdermin D antibody (Abcam, cat. no. ab209845; RRID: https://scicrunch.org/resolver/AB_2783550)

(Critical—While anti-gasdermin D antibodies from other sources do work, we have found that this one is best.)

- Anti- β -actin antibody (Cell Signaling Technology, cat. no. 8457; RRID: https://scicrunch.org/resolver/AB_10950489)

(Critical—While anti- β -actin antibodies from other sources do work, we have found that this one is best.)

- Anti-mouse HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, cat. no. 315-035-047; RRID: https://scicrunch.org/resolver/AB_2340068)
- Anti-rabbit HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, cat. no. 111-035-047; RRID: https://scicrunch.org/resolver/AB_2337940)
- Luminata Forte Western HRP Substrate (Millipore, cat. no. WBLUF0500)
- Methanol (Sigma, cat. no. 322415)

(Caution—Methanol is flammable. Keep away from open flames.)

- Tris base (Sigma, cat. no. TRIS-RO)
- Glycine (Sigma, cat. no. G8898)
- Sodium dodecyl sulfate (SDS) (Sigma, cat. no. L3771)

(Caution—SDS is caustic. Use caution when handling. Wear gloves, splash goggles, and a synthetic apron or lab coat, and handle in a fume hood.)

- Sodium chloride (NaCl) (Sigma, cat. no. S9888)
- Sodium deoxycholate (Sigma, cat. no. 30970)
- Sodium hydroxide (NaOH) (Sigma, cat. no. 72068)

(Caution—Sodium hydroxide is a strong base. Use caution when handling. Wear gloves, splash goggles, and a synthetic apron or lab coat, and handle in a fume hood.)

- Hydrochloric acid (HCl) (Sigma, cat. no. H9892)

(Caution—Hydrochloric acid is caustic. Use caution when handling. Wear gloves, splash goggles, and a synthetic apron or lab coat, and handle in a fume hood.)

- Non-fat dried milk¹⁰⁶

Equipment

General equipment

- Incubator (Thermo Fisher Scientific, cat. no. 51026282)
- Shaking incubator (New Brunswick Scientific, cat. no. M1352-0004)
- Tabletop centrifuge for 50 mL and 15 mL conical tubes (Thermo Fisher Scientific, cat. no. 75004524)
- Microcentrifuge (Thermo Fisher Scientific, cat. no. 75002401)
- Vortex mixer (Fisher Scientific, cat. no. 02-215-418)

- Plate shaker (Fisher Scientific, cat. no. 88-861-023)
- Plate rocker (Stellar Scientific, cat. no. LI-PRB-S2025-XLP)
- Filter unit, 0.45 µm pore size (Millipore, cat. no. SCHVU05RE)
- Filter unit, 0.22 µm pore size (Millipore, cat. no. SCGVU02RE)
- Cell strainer, 40 µm pore size (Fisher Scientific, cat. no. 22363547)
- Conical screw cap tubes (50 mL; Fisher Scientific, cat. no. 50-809-218)
- Conical screw cap tubes (15 mL; Fisher Scientific, cat. no. NC9531248)
- 1.5 mL microfuge tubes (Fisher Scientific, cat. no. 05-408-129)
- Candle wax¹⁰⁶
- Paper towels¹⁰⁶
- Aluminum foil¹⁰⁶
- GraphPad Prism (<https://www.graphpad.com>)

In vivo Inflammasome activation (Step 1)

- Isoflurane pump (VetEquip, cat. no. 901808)
- 30-gauge needle (BD Biosciences, cat. no. 305106)
- 20-gauge needle (BD Biosciences, cat. no. 305175)
- Oral gavage needle, 22-gauge (Fisher Scientific, cat. no. 01-290-2A)
- Oral gavage needle, 20-gauge (Fisher Scientific, cat. no. 01-290-3A)
- Oral gavage needle, 18-gauge (Fisher Scientific, cat. no. 14-818-107B)
- Insulin syringe (BD Biosciences, cat. no. 309659)
- PE-10 tubing (Braintree Scientific Inc., cat. no. PE10)
- Heating block (Fisher Scientific, cat. no. 23-043-160)

Bone marrow progenitor cell isolation and BMDM differentiation (Steps 2–30)

- Cell strainer, 70 µm pore size (Corning, cat. no. 431751)
- Tissue culture plates (12 well; Corning, cat. no. 07-200-82)
- Tissue culture flasks (182-cm²; Genesee Scientific, cat. no. 25-211)
- 150 mm × 25 mm tissue culture dishes (Corning, cat. no. 430597)
- Hemocytometer (Fisher Scientific, cat. no. 02-671-518)
- Dissecting scissors (Fisher Scientific, cat. no. 08-940)
- Forceps (Fisher Scientific, cat. no. 22-327379)
- Petri dish (Fisher Scientific, cat. no. 07-202-011)
- 25-gauge needle (BD Biosciences, cat. no. 305122)

- 18-gauge needle (BD Biosciences, cat. no. 305195)
- 10 mL sterile syringe (BD Biosciences, cat. no. 309604)
- Cell scrapers (CELLTREAT Scientific Products, cat. no. 229315)

Real-time cell death analysis (Step 32 Option A)

- IncuCyte Zoom incubator imaging system (Essen Biosciences, cat. no. 4647). Alternatively, any microscope with live-cell fluorescence imaging functionality can be used.
- IncuCyte software (<https://www.essenbioscience.com/en/products/software/incucyte-base-software/>)

Fixing cells for microscopy (Step 32 Option B)

- 10 mm glass coverslips (MatTek Corporation, cat. no. P12G-1.5–10-F)

ASC speck analysis (Step 33 Option A)

- Kimwipes (Kimtech, cat. no. 34133)
- Fluorescence microscope (Leica Microsystems)

LDH assay and caspase-1 and IL-18 sandwich ELISAs (Step 33 Options B-C)

- Spectrophotometer (Bio-Rad, cat. no. 170–2525)
- Flat clear bottom 96-well plate (Corning, cat. no. 9017)

IL-1 β multiplex ELISA (Step 33 Option D)

- Luminex 200 (Luminex Corporation, cat. no. LX20-XPON-IVD)
- Magnetic separation block (EMD Millipore, cat. no. 40–285)

Caspase-1 and gasdermin D western blot (Step 33 Option E)

- Electrophoresis apparatus (Bio-Rad, cat. no. 1658004)
- Trans-Blot semi-dry transfer apparatus (Bio-Rad, cat. no. 170–3940)
- Power source (Bio-Rad, cat. no. 164–5052)
- 10% (wt/vol) polyacrylamide gel with 15 wells (Bio-Rad, cat. no. 4561036)
- 12% (wt/vol) polyacrylamide gel with 10 wells (Bio-Rad, cat. no. 4561043)
- PVDF membrane (Millipore, cat. no. IPVH00010)
- Filter paper (Bio-Rad, cat. no. 1703965)
- Gel releaser (Bio-Rad, cat. no. 1653320)

Reagent setup

70% (vol/vol) ethanol—Combine 700 mL ethanol with enough water to reach 1 L in total volume and mix. This solution can be stored at room temperature for up to 1 week.

Bacterial cultures (Step 1 Options A-D and Step 31 Options H-I)—CRITICAL:

The procedure below can be used to prepare bacterial cultures for *Escherichia coli*, *Citrobacter rodentium*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, and *Burkholderia thailandensis*. To prepare bacterial cultures for *Francisella novicida*, see the following Reagents Setup item.

1. Using a frozen stock of bacteria, streak an LB agar plate and grow overnight at 37 °C.
2. **CRITICAL:** A fresh plate should be streaked from the frozen stock for each experiment.
3. Pick a single colony to inoculate 5 mL liquid LB broth in a 15 mL conical tube and grow overnight at 37 °C and at 220 rpm in a shaking incubator.
4. Subculture 500 µL into 5 mL fresh LB broth in a 15 mL conical tube and incubate at 37 °C for another 3–4 hours.
5. Measure the OD₆₀₀. In the table below, we provide the OD₆₀₀ readings with their corresponding CFUs as determined in our lab.

OD ₆₀₀	CFU/mL	Volume (µL) required for 1 MOI	Volume (µL) required for 5 MOI	Volume (µL) required for 10 MOI	Volume (µL) required for 20 MOI
0.1	0.05×10^9	20	100	200	400
1	0.5×10^9	2	10	20	40

- a. It is best practice to determine a liquid culture growth curve within your own lab to ensure accuracy. To generate this curve, begin by inoculating a flask containing 50 mL LB broth with a single colony picked from a LB agar plate grown overnight.
- b. Incubate the flask at 37 °C and at 220 rpm in a shaking incubator. Every 30 minutes determine the OD₆₀₀ and collect a 1 mL aliquot to serially dilute and inoculate onto a series of fresh LB agar plates. Incubate these plates at 37 °C for 18 hours.
- c. Count the number of colonies on the plates to determine the number of CFU/mL that corresponds to each OD₆₀₀.
6. Add the appropriate volume of bacteria necessary to BMDM culture media without antibiotics to obtain the MOI desired for *in vitro* stimulations or to PBS to obtain the CFU desired for *in vivo* infections.

Avertin stock solution (Step 1 Options B and F)—Combine 250 mg of 2,2,2-tribromoethanol with 0.5 mL of 2-methyl-2-butanol and vortex until dissolved. Then add 20 mL of room temperature (20–23°C) ultrapure water while vortexing slowly. Once all the water is added, vortex an additional 1–2 minutes, or until completely solubilized. Sterile filter through a 0.22 µm filter. Store at 4 °C and use within 1 week.

Bacterial cultures for *Francisella novicida* (Step 1 Option E and Step 31 Option J)

1. Using a frozen stock of bacteria, streak a TSBC agar plate and grow overnight at 37 °C.

CRITICAL: A fresh plate should be streaked from the frozen stock for each experiment.

2. Pick a single colony to inoculate 5 mL liquid TSBC broth in a 15 mL conical tube and grow overnight at 37 °C.
3. Subculture 500 µL into 5 mL fresh TSBC broth in a 15 mL conical tube and incubate at 37 °C for another 3–4 hours.
4. Measure the OD₆₀₀. In the table below, we provide the OD₆₀₀ readings with their corresponding CFUs as determined in our lab.

OD ₆₀₀	CFU/mL	Volume (µL) required for 1 MOI	Volume (µL) required for 50 MOI	Volume (µL) required for 100 MOI
1	1×10^9	1	50	100
1.5	1.5×10^9	0.67	33.33	66.67

- a. It is best practice to determine a liquid culture growth curve within your own lab to ensure accuracy. To generate this curve, begin by inoculating a flask containing 50 mL TSBC broth with a single colony picked from a TSBC agar plate grown overnight.
  - b. Incubate the flask at 37 °C. Every 30 minutes determine the OD₆₀₀ and collect a 1 mL aliquot to serially dilute and inoculate onto a series for fresh TSBC agar plates. Incubate these plates at 37 °C for 18 hours.
  - c. Count the number of colonies on the plates to determine the number of CFU/mL that corresponds to each OD₆₀₀.
5. Add the appropriate volume of bacteria necessary to BMDM culture media without antibiotics to obtain the MOI desired for *in vitro* stimulations or to PBS to obtain the CFU desired for *in vivo* infections.

Tryptic soy broth supplemented with L-cysteine (TSBC) (Step 1 Option E and Step 31 Option J)—Dissolve 0.1 g L-cysteine in 100 mL tryptic soy broth. Filter sterilize (do not autoclave) using a 0.45 µm filter. This solution can be stored at 4 °C for up to 1 week. Note that cysteine can crystallize in the solution, resulting in polka dot-like spots in the plates. These are fine to use and will not affect growth.

1. Influenza A virus (Step 1 Option F and Step 31 Option K) Dilute virus to a concentration of 1×10^3 PFU/mL in PBS.

2. Using a 20-gauge needle, make a hole in the shell of a 9- to 11-day old embryonated chicken egg above the air sack. **CRITICAL STEP** Take care to only puncture the shell and not the shell membrane.
3. Spray the egg with 70% (vol/vol) ethanol and incubate for 1 minute.
4. Using a 25-gauge needle, inject 100 μ L of virus through the hole at a 45° angle. Seal the hole in the shell with melted candle wax. Incubate the egg at 37 °C for 2 days.
5. Then transfer the egg to 4 °C for at least 12 hours.
6. Spray the egg with 70% (vol/vol) ethanol, then crack open the shell above the air sack using tweezers.
7. Peel back the air sack membrane to expose the allantoic fluid. Use a serological pipette to collect the allantoic fluid into a 15 mL conical tube.
8. Centrifuge the tube at $3000 \times g$ for 10 minutes at 4 °C and collect the supernatant, which will contain the virus. Using this protocol we expect to collect approximately 10 mL allantoic fluid with a viral titer of 1×10^9 PFU/mL. This supernatant can be aliquoted, frozen on dry ice, and stored at –80 °C. The concentrated virus stock can be stored at –80 °C for up to 1 year. Fresh virus should be thawed and used each time.
9. Measure the viral titer using a plaque assay in MDCK cells and dilute to an MOI of 20 in high glucose DMEM without pyruvate and FBS for *in vitro* stimulations or dilute to 1 PFU/ μ L in PBS for *in vivo* infections.

***Aspergillus fumigatus* culture (Step 1 Option G and Step 31 Option M)**

1. Grow *A. fumigatus* on 2% (wt/vol) maltose, 2% (wt/vol) agar slants for 1 week at 37 °C.
CRITICAL: A fresh slant should be prepared for each experiment.
2. Harvest conidia in 12 mL PBS containing 0.1% (vol/vol) Tween-20. Filter the conidia twice through a 0.40 μ m sterile cell strainer.
3. Count the number of resting conidia per mL using a hemocytometer and dilute to the desired MOI in BMDM stimulation media for *in vitro* stimulations or to the desired number of conidia in PBS with 0.1% (vol/vol) Tween-20 with a final volume of 30 μ L per mouse for *in vivo* infections. Using this protocol we expect to collect $1\text{--}6 \times 10^8$ resting conidia per mL.

***Candida albicans* culture (Step 1 Option H and Step 31 Option N)**

1. Grow *C. albicans* overnight in 10 mL Sabouraud broth at 28 °C in a 50 mL conical tube in a shaking incubator at 200 rpm. **CRITICAL STEP:** Fresh fungus should be used each time.
2. Centrifuge the culture at $1950 \times g$ for 5 minutes at room temperature. Remove the supernatant and resuspend the pellet in 10 mL DPBS.

3. Centrifuge the suspension at $1950 \times g$ for 5 minutes at room temperature. Remove the supernatant and resuspend the pellet in 10 mL DPBS.
4. Centrifuge the suspension at $1950 \times g$ for 5 minutes at room temperature. Remove the supernatant and resuspend the pellet in 5 mL of DPBS.
5. Remove 100 μ L and dilute in 900 μ L DPBS, then count the number of fungi using a hemocytometer.
6. Dilute the stock to 2×10^5 blastoconidia in 100 μ L PBS for *in vivo* infections. For *in vitro* stimulations, use the live *Candida* blastoconidia at 1–5 MOI.

LPS preparation for LPS shock (Step 1 Options I-J)—Resuspend the lyophilized LPS to a concentration of 2.5 mg/mL using endotoxin-free PBS. This solution can be stored at -20°C for up to 2 years. Weigh each mouse before LPS administration. To inject 25 mg/kg, each mouse will be intraperitoneally injected with 10 μ L per gram body weight. To inject 10 mg/kg, each mouse will be injected with 4 μ L per gram body weight.

Collagen emulsion (Step 1 Option M)—Follow the protocol previously established by Brand et al.¹⁰⁷ to create the collagen emulsion. Use type II collagen at 4 mg/mL (dissolved overnight at 4°C in 10 mM acetic acid) and emulsify in an equal volume of CFA with 4 mg/mL heat-killed *Mycobacterium tuberculosis*. The collagen emulsion should be prepared fresh each time.

Azoxymethane (AOM) solution (Step 1 Option R)—Prepare a 1 μ g/ μ L solution of AOM by diluting 5 μ L AOM in 5 mL PBS. Filter the solution using a 0.45 μ m filter. Aliquots of the solution can be stored at -20°C for up to 1 year. Each mouse will be injected with 10 μ L per gram body weight to achieve the 10 mg/kg dose.

Dextran sodium sulfate (DSS) solutions (Step 1 Option R)—Prepare a 3% (wt/vol) DSS solution by dissolving 30 g DSS into 1 L of water. Filter the solution using a 0.45 μ m filter. Prepare a 2% (wt/vol) DSS solution by dissolving 20 g DSS into 1 L of water. Filter the solutions using a 0.45 μ m filter. DSS solutions should be prepared fresh for each use.

L929-conditioned media (Steps 10–31)—Plate 1×10^6 L929 cells in 50 mL IMDM cell culture media containing 5 mL heat-inactivated FBS (heat-inactivation is achieved by incubating FBS at 56°C for 30 minutes), 0.5 mL sodium pyruvate, 0.5 mL non-essential amino acids, and 0.5 mL penicillin and streptomycin in a 182-cm² tissue culture flask. Grow the cells in a humidified incubator with 5% CO₂ at 37°C for 7 days. Collect the supernatant and filter using a 0.45 μ m filter. Aliquots of L929-conditioned media can be stored frozen at -80°C for up to 1 year. L929-conditioned media is expected to contain approximately 20 ng/mL M-CSF. **CRITICAL** As an alternative to reduce potential variation, 500 UI/mL commercial M-CSF can be added directly to the complete cell culture media. However, using commercial M-CSF is more expensive.

BMDM culture media (Steps 10–31)—Combine 290 mL IMDM cell culture media with 150 mL L929-conditioned media, 50 mL heat-inactivated FBS (heat-inactivation is achieved

by incubating FBS at 56 °C for 30 minutes), 5 mL non-essential amino acids, and 5 mL penicillin and streptomycin. BMDM culture media can be stored at 4 °C for up to 1 week.

BMDM stimulation media (Steps 30–31)—Combine 445 mL DMEM with 50 mL heat-inactivated FBS (heat-inactivation is achieved by incubating FBS at 56 °C for 30 minutes) and 5 mL penicillin and streptomycin. BMDM stimulation media can be stored at 4 °C for up to 1 week.

ATP stock solution (Step 31 Option A)—Dissolve the 1 gram lyophilized ATP to a concentration of 0.5 M in 3.63 mL of the manufacturer supplied endotoxin-free water. This solution can be aliquoted and stored at –20 °C for up to 2 years.

Nigericin stock solution (Step 31 Option B)—Resuspend the 5 mg lyophilized nigericin to a concentration of 5 mM in 1.34 mL of ethanol. This solution can be aliquoted and stored at –20 °C for up to 2 years.

Xfect and LPS mix (Step 31 Option C)—Dissolve the lyophilized LPS to a concentration of 5 mg/mL using the manufacturer supplied endotoxin-free water. This solution can be stored at –20 °C for up to 2 years. For each well of 1×10^6 BMDMs, combine 0.2 μ L (1 μ g/well) LPS and 99.5 μ L buffer and vortex. Add 0.3 μ L Xfect and vortex immediately. Incubate for 15 minutes at room temperature before adding 100 μ L per well to cells. This mixture should be prepared fresh for each use.

PrgI or PrgJ and DOTAP mix (Step 31 Option D)—Thaw the PrgI or PrgJ stock solution from the manufacturer (1 mg/mL) on ice. For each well of 1×10^6 BMDMs, combine 19 μ L PBS, 1 μ L (1 μ g/well) PrgI or PrgJ, and 20 μ L DOTAP and vortex. Incubate for 60 minutes at 37 °C before adding 40 μ L per well to cells. This mixture should be prepared fresh for each use. Lipofectamine or Xfect can be used in place of DOTAP if desired.

Flagellin and DOTAP mix (Step 31 Option D)—Dissolve the lyophilized flagellin to a concentration of 0.5 mg/mL using the manufacturer supplied endotoxin-free water. This solution can be stored at –20 °C for up to 2 years. For each well of 1×10^6 BMDMs, combine 19 μ L PBS, 1 μ L (0.5 μ g/well) flagellin, and 20 μ L DOTAP and vortex. Incubate for 60 minutes at 37 °C before adding 40 μ L per well to cells. This mixture should be prepared fresh for each use. Lipofectamine or Xfect can be used in place of DOTAP if desired.

***Clostridium difficile* toxin (Step 31 Option E)**—**CRITICAL** Manufacturer supplied toxin can be used (List Biological Laboratories, Inc.), This will provide a more pure and homogeneous reagent than toxin production directly from the bacteria. In order to do so, resuspend the lyophilized TcdA or TcdB in Opti-MEM at a concentration of 100 μ g/mL. Aliquot this stock into single-use aliquots to store at –80 °C for up to 2 years, as the toxin does not tolerate more than one freeze-thaw cycle.

CRITICAL For direct production of the toxin from the bacteria, in this protocol, we use the *C. difficile* AB+ strain; we recommend processing the AB– strain in parallel to serve as a negative control.

1. Using a frozen stock of bacteria, streak a brain heart infusion agar plate and grow overnight at 37 °C under anaerobic conditions.
2. Pick a single colony to inoculate 5 mL liquid tryptone-yeast extract medium in a 15 mL conical tube and grow overnight at 37 °C under anaerobic conditions.
3. Spin down the bacteria at $14,500 \times g$ for 10 minutes at room temperature.
4. Collect the toxin-containing supernatant and pass it through a 0.22 µm filter.

The filtered supernatant can be stored at 4 °C for up to 1 week.

Xfect and poly(dA:dT) mix (Step 31 Option G)—Dissolve the lyophilized poly(dA:dT) to a concentration of 1 mg/mL using the manufacturer supplied endotoxin-free water. This solution can be stored at –20 °C for up to 2 months. For each well of 1×10^6 BMDMs, combine 2 µL (2 µg/well) poly(dA:dT) and 47.7 µL Xfect buffer and vortex. Add 0.25 µL Xfect and vortex immediately. Incubate for 15 minutes before adding 50 µL per well to cells. This mixture should be prepared fresh for each use.

SYTOX Green solution (Step 31 all options)

1. Mix 10 µL of the 5 mM SYTOX Green reagent from the manufacturer with 990 µL BMDM stimulation media. This will create a 50 µM stock solution. Prepare this stock solution fresh for each use. **CRITICAL STEP** Protect this solution from light at all times. **CRITICAL STEP** Propidium iodide at a final concentration of 500 nM, 7-AAD at a final concentration of 1 µg/mL or DRAQ7 at a final concentration of 3 µM can be used in place of SYTOX Green if desired.
2. When adding this stock solution to the wells of BMDMs during inflammasome stimulation, add 0.2 µL per well (each well contains 500 µL total media) to achieve a 20 nM concentration. **CRITICAL STEP** The 0.2 µL per well should be added to the BMDM media containing the inflammasome stimulant before the stimulant is added to the wells rather than being pipetted directly into individual wells to minimize concentration variations between wells.

10% (vol/vol) normal goat serum solution (Step 32 Option B and Step 33

Option A)—Reconstitute the lyophilized goat serum in 1 mL water and mix every 20 minutes. Incubate at 4 °C for at least 2 hours. Then dilute 1 mL of the normal goat serum in 9 mL PBS. Store diluted solution at 4 °C for up to 1 week. The remaining concentrated serum can be stored at –20 °C for 6–8 weeks. **CRITICAL** If cells are being counterstained with any goat antibodies, FBS or BSA should be used instead of normal goat serum.

Permeabilization solution (Step 32 Option B and Step 33 Option A)—Dilute 5 µL Triton X-100 in 50 mL PBS. This solution can be stored at 4 °C for up to 1 month.

4× SDS buffer (Step 32 Options C-D)

1. Prepare a 1 M Tris buffer by dissolving 12.11 g Tris in 80 mL water. Adjust the pH to 6.8 using HCl while stirring, then adjust the volume to 100 mL total with water. The Tris buffer stock solution can be stored at 4 °C for up to 1 month.
2. Combine 60 mL 1 M Tris (pH 6.8) with 24 mg SDS, 100 mg bromophenol blue, 120 mL glycerol, 8.4 mL BME, and 90 mL water. Stir and warm until all salts go into solution.
3. Adjust the final volume to 300 mL with water. This solution can be aliquoted and stored at −20 °C for up to 1 year.

DTT solution (Step 32 Option C)—To obtain a 1 M (100×) stock of DTT, dissolve 0.154 g DTT powder in 1 mL PBS. This solution can be aliquoted and stored at −20 °C for up to 1 year.

Protease inhibitor cocktail solution (Step 32 Option C)—To obtain a 100× working stock, dissolve one protease inhibitor tablet in 1 mL water. This stock solution can be stored at 4 °C for up to 2 weeks.

Caspase lysis buffer (Step 32 Option C)—To make 50 mL of caspase lysis buffer, add 3.57 mL of the 70% (vol/vol) NP-40 solution (5% (vol/vol) final concentration), 500 µL of the 100× DTT working stock solution (10 mM final concentration), and 500 µL protease inhibitor solution (1× final concentration) to 41.86 mL water. This solution can be stored at 4 °C for up to 1 month.

RIPA buffer (Step 32 Option D)

1. Prepare a 1.5 M Tris buffer by dissolving 18.2 g Tris in 80 mL water. Adjust the pH to 8.8 using NaOH while stirring, then adjust the volume to 100 mL total with water. The Tris buffer stock solution can be stored at 4 °C for up to 1 month.
2. Prepare a 10% (wt/vol) SDS solution by dissolving 10 g SDS in 80 mL water and stir until dissolved. Adjust the volume to 100 mL total with water. The SDS stock solution can be stored at room temperature for up to 6 months.
3. To prepare a 2× RIPA buffer stock, combine 1 mL NP-40, 0.5 g sodium deoxycholate, 1 mL 10% (wt/vol) SDS, 0.876 g NaCl, and 3.3 mL 1.5 M Tris (pH 8.8) in 46 mL water. Stir until all salts go into solution. Adjust the final volume to 50 mL with water. This solution can be stored at 4 °C for up to 1 month.
4. Before using, dilute the 2× RIPA buffer stock in an equal volume of water.

Anti-ASC antibody solution (Step 33 Option A)—Dilute the anti-ASC antibody 1:250 (vol:vol) for microscopy to a concentration of 4 µg/mL. To stain each well of 1×10^6 BMDMs, add 1 µL of antibody to 249 µL of the 10% (vol/vol) normal goat serum solution. This antibody dilution should be prepared fresh each time.

Microscopy secondary antibody solution (Step 33 Option A)—Dilute the secondary antibody 1:250 (vol:vol) for microscopy to a concentration of 8 µg/mL. To stain each well of 1×10^6 BMDMs, add 1 µL of antibody to 249 µL of the 10% (vol/vol) normal goat serum solution. The diluted antibody should be prepared fresh each time. Protect this solution from light at all times.

CytoTox96 reagent (Step 33 Option B)—Thaw the assay buffer from the CytoTox96 assay kit. Remove 12 mL of the assay buffer and warm to room temperature. Return the remaining assay buffer to storage at -20°C . Once the 12 mL of assay buffer has reached room temperature, add this buffer to the bottle of Substrate Mix from the kit; gently shake and invert the bottle to dissolve the Substrate Mix. This creates the CytoTox96 reagent. Protect this solution from light at all times. Unused CytoTox96 reagent (the combination of assay buffer and Substrate Mix) can be stored at -20°C for 6–8 weeks.

Maximum LDH release control (Step 33 Option B)—Dilute 50 µL of the $10\times$ lysis buffer from the CytoTox96 kit in 450 µL BMDM culture media. Add to a well of 1×10^6 BMDMs and incubate at 37°C for 45 minutes. Then collect the supernatant to be used as the maximum LDH release control. A fresh control should be made for each experiment.

Caspase-1 ELISA kit solution (Step 33 Option C)

1. To make 100 mL of wash buffer, add 10 mL of the manufacturer supplied $10\times$ wash buffer to 90 mL deionized water. The wash buffer stock solution can be stored at 4°C for up to 2 months.
2. To make 100 mL of ELISA buffer, add 10 mL of the manufacturer supplied $10\times$ ELISA buffer to 90 mL deionized water. The ELISA buffer stock solution can be stored at 4°C for up to 2 months.
3. To make 2 mL of diluted detection antibody solution, add 4 µL of the manufacturer supplied detection antibody to 2 mL of the ELISA buffer. The diluted detection antibody solution cannot be stored.
4. Reconstitute the manufacturer supplied STREP-HRP in 100 µL of ELISA buffer. This solution should be aliquoted (we suggest 50 µL per aliquot) and stored at -20°C for up to 6 months; this solution should not undergo freeze/thaw cycles.
5. To make 10 mL of the STREP-HRP working solution, add 50 µL of the reconstituted STREP-HRP to 10 mL of the ELISA buffer. The STREP-HRP working solution cannot be stored.
6. Reconstitute the manufacturer supplied mouse caspase-1 standard in 100 µL of the ELISA buffer and mix well (the stock concentration is 10 µg/mL). Incubate the standard at room temperature for 15 minutes before use. Reconstituted standard can then be aliquoted and stored at -20°C for up to 6 months.
7. Prepare the following caspase-1 standard curve solutions:

Final concentration	Amount to Add	Diluent
100 ng/mL	10 µL of 10 µg/mL caspase-1	990 µL ELISA buffer
10 ng/mL	100 µL of 100 ng/mL caspase-1	900 µL ELISA buffer
5 ng/mL	300 µL of 10 ng/mL caspase-1	300 µL ELISA buffer
2.5 ng/mL	300 µL of 5 ng/mL caspase-1	300 µL ELISA buffer
1.25 ng/mL	300 µL of 2.5 ng/mL caspase-1	300 µL ELISA buffer
0.625 ng/mL	300 µL of 1.25 ng/mL caspase-1	300 µL ELISA buffer
0.312 ng/mL	300 µL of 0.625 ng/mL caspase-1	300 µL ELISA buffer
0.156 ng/mL	300 µL of 0.312 ng/mL caspase-1	300 µL ELISA buffer
0 ng/mL	0 µL of caspase-1	300 µL ELISA buffer

IL-18 ELISA kit solution (Step 33 Option C)

1. To make 500 mL of wash buffer, add 25 mL of the manufacturer supplied 20× wash buffer to 475 mL deionized water. Wash buffer can be stored at room temperature for 30 days.
2. To make 100 mL of assay buffer, add 5 mL of the manufacturer supplied 20× assay buffer to 95 mL deionized water. Assay buffer can be stored at 4 °C for 30 days.
3. To make 3 mL of diluted Biotin-Conjugate solution, add 30 µL of the manufacturer supplied Biotin-Conjugate to 2.97 mL of the assay buffer. This solution should be used right away, within 30 minutes of dilution, and cannot be stored.
4. To make 6 mL of the diluted Streptavidin-HRP solution, add 30 µL of the manufacturer supplied Streptavidin-HRP to 5.97 mL of the assay buffer. This solution should be used right away, within 30 minutes of dilution, and cannot be stored.
5. Reconstitute the manufacturer supplied mouse IL-18 standard in the volume indicated on the label of the tube and mix well (the stock concentration is 4 µg/mL). Incubate the standard at room temperature for 15 minutes before use. Reconstituted standard should be used immediately and cannot be stored.
6. Prepare the following IL-18 standard curve solutions:

Final concentration	Amount to Add	Diluent
2000 pg/mL	225 µL of 4 µg/mL stock IL-18	225 µL sample diluent
1000 pg/mL	225 µL of 2000 pg/mL IL-18	225 µL sample diluent
500 pg/mL	225 µL of 1000 pg/mL IL-18	225 µL sample diluent
250 pg/mL	225 µL of 500 pg/mL IL-18	225 µL sample diluent
125 pg/mL	225 µL of 250 pg/mL IL-18	225 µL sample diluent

Final concentration	Amount to Add	Diluent
62.5 pg/mL	225 μ L of 125 pg/mL IL-18	225 μ L sample diluent
31.3 pg/mL	225 μ L of 62.5 pg/mL IL-18	225 μ L sample diluent
0 pg/mL	0 μ L of IL-18	225 μ L sample diluent

IL-1 β ELISA kit solution (Step 33 Option D)—We use a multiplex ELISA kit to measure IL-1 β levels; therefore, this ELISA kit also analyzes IL-13, which has a different standard concentration than the other cytokines. This analysis can also be done with a standard IL-1 β sandwich ELISA, such as the IL-1 β mouse ELISA kit from Invitrogen [cat. no. BMS6002]. Follow the procedure below to prepare the IL-1 β multiplex ELISA kit solutions:

1. To make 100 mL of wash buffer, add 10 mL of the manufacturer supplied 10 $\times$ wash buffer to 90 mL deionized water. The wash buffer stock solution can be stored at room temperature for up to 2 months.
2. Reconstitute the manufacturer supplied mouse cytokine/chemokine standard in 250 μ L of water and mix well. Vortex for 15 seconds. Incubate the standard at room temperature for 10 minutes before use. Unused reconstituted standard can be stored at -20°C for up to 1 month.
3. Prepare the following mouse cytokine/chemokines standard curve solutions:

Final concentration (IL-13; All other analytes)	Amount to Add	Diluent
40,000 pg/mL; 10,000 pg/mL	Lyophilized standard	250 μ L water
8000 pg/mL; 2000 pg/mL	50 μ L of solution above	200 μ L assay buffer
1600 pg/mL; 400 pg/mL	50 μ L of solution above	200 μ L assay buffer
320 pg/mL; 80 pg/mL	50 μ L of solution above	200 μ L assay buffer
64 pg/mL; 16 pg/mL	50 μ L of solution above	200 μ L assay buffer
12.8 pg/mL; 3.2 pg/mL	50 μ L of solution above	200 μ L assay buffer
0 pg/mL; 0 pg/mL	0 μ L of solution above	250 μ L assay buffer

Western blot running buffer solution (Step 33 Option E)—Prepare a 5 $\times$ running buffer stock by combining 15 g Tris base, 72 g glycine, and 5 g SDS in the volume of water required to bring the solution to 1 L total volume. This solution can be stored at room temperature for up to 6 months. To prepare 1 $\times$ running buffer for use in western blotting, combine 800 mL deionized water and 200 mL of the 5 $\times$ running buffer stock. The 1 $\times$ solution should be made fresh each time.

Western blot transfer buffer solution (Step 33 Option E)—Prepare a 10 $\times$ transfer buffer stock by combining 12 g Tris base and 72 g glycine in the volume of water required to

bring the solution to 1 L total volume. This solution can be stored at room temperature for up to 3 months. To prepare 1× transfer buffer for use in western blotting, combine 700 mL deionized water and 100 mL of the 10× transfer buffer stock and 200 mL methanol. The 1× solution should be made fresh each time.

TBST solution (Step 33 Option E)—Dissolve 6.05 g Tris base and 8.76 g sodium chloride in 850 mL water. Then adjust the pH to 7.4 using HCl. Add enough water to bring the total volume up to 1 L. Finally, add 0.5 mL Tween-20 (0.05% (vol/vol) final concentration). This solution can be stored at room temperature for up to 1 month.

5% (wt/vol) skim milk blocking solution (Step 33 Option E)—To make 100 mL of a 5% (wt/vol) skim milk blocking solution, dissolve 5 g nonfat dried milk powder in 100 mL TBST. This solution can be stored at 4 °C for up to 1 week.

Western blot caspase-1 primary antibody (Step 33 Option E)—To make 10 mL of diluted caspase-1 primary antibody, dilute the Adipogen anti-caspase-1 antibody 1:1000 (vol:vol) to a concentration of 1 µg/mL by adding 10 µL of the antibody to 10 mL of 5% (wt/vol) skim milk blocking solution. The antibody dilution should be prepared fresh each time.

Western blot gasdermin D primary antibody (Step 33 Option E)—To make 10 mL of diluted gasdermin D primary antibody, dilute the Abcam anti-gasdermin D antibody 1:500 (vol:vol) to a concentration of 1.2 µg/mL by adding 20 µL of the antibody to 10 mL of 5% (wt/vol) skim milk blocking solution. The antibody dilution should be prepared fresh each time.

Western blot HRP-conjugated secondary antibody (Step 33 Option E)—To make 10 mL of diluted secondary antibody, dilute the Jackson Immuno Research Laboratories HRP-conjugated secondary antibody 1:5000 (vol:vol) to a concentration of 0.16 µg/mL by adding 2 µL of the antibody to 10 mL of 5% (wt/vol) skim milk blocking solution. The antibody dilution should be prepared fresh each time.

Western blot stripping solution (Step 33 Option E)—Dissolve 15 g glycine, 1 g SDS, and 10 mL Tween-20 in 800 mL water. Then adjust the pH to 2.2 using HCl. Add enough water to bring the total volume up to 1 L. This solution can be stored at room temperature for up to 1 month.

Western blot β-actin loading control primary antibody (Step 33 Option E)—To make 10 mL of diluted β-actin primary antibody, dilute the Cell Signaling Technology anti-β-actin antibody 1:1000 (vol:vol) to a concentration of 0.43 µg/mL by adding 10 µL of the antibody to 10 mL of 5% (wt/vol) skim milk blocking solution. The antibody dilution should be prepared fresh each time.

Procedure

(Optional): *in vivo* inflammasome activation—CRITICAL: Step 1 describes the procedure for *in vivo* inflammasome activation assays in mice. When performing *in vitro* inflammasome activation assays, skip this step and start the procedure at Step 2.

1. Stimulate inflammasome activation in mice *in vivo* (see Table 1). For investigation of inflammasome activation in response to bacterial infection, follow options A–E. For investigation of inflammasome activation in response to viral infection, follow option F. For investigation of inflammasome activation in response to fungal infection, follow options G–H. For investigation of inflammasome activation in response to inflammatory diseases, follow options I–Q. For investigation of inflammasome activation in response to cancer, follow option R.

CAUTION: Ensure that all *in vivo* procedures with pathogens are carried out with the proper precautions and personal protective equipment (including a disposable gown, shoe covers, gloves, and a face mask) in a BSL2 facility. Infections should be performed in a biosafety cabinet.

A. *Citrobacter rodentium*

(Timing 10 days)

- i. Eight hours prior to the planned infection, remove food and water from the mouse cage.
- ii. Load a syringe with 500 μ L PBS containing 1×10^{10} CFU *C. rodentium* (see Reagent Setup).
- iii. Attach the oral gavage needle to the syringe, depending on the weight of the mouse:

Weight (g)	Needle
15–20	22-gauge, 1.25 mm ball diameter
20–25	20-gauge, 2.25 mm ball diameter
25	18-gauge, 2.25 mm ball diameter

- iv. Scruff the mouse securely.
- v. Palpate the mouse's last rib (the stomach will be just past this) and lay the gavage needle along the exterior of the mouse's body with the tip at the stomach and the syringe end at the mouth to determine how far the needle will need to be inserted from the mouth to reach the stomach.
- vi. Insert the ball tip of the needle into the mouth and guide the ball tip along the roof of the mouth and down the esophagus.

CRITICAL STEP The needle should progress smoothly; if it does not, gently remove and start again.

- vii. Once the needle has reached the stomach, inject the solution containing the bacteria slowly.
- viii. Remove the needle by pulling straight out slowly and gently and monitor the mouse to ensure there is no injury or respiratory distress.
- ix. One hour post-infection, return food and water
- x. Allow the infection to progress for 10 days.
- xi. Sacrifice the mouse by asphyxiation by placing it into a CO₂ chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cardiac puncture.
- xii. Collect serum by cardiac puncture and colon tissue by dissection and homogenization to analyze cytokine release via ELISA in Step 33 Options C-D. Fecal and colon lysates can also be collected for CFU quantification by serial dilution of lysates LB agar plates followed by incubation at 37 °C for 24 h and colony counting to monitor the contribution of inflammasome activation on pathogen clearance, and colon lysates can be used for western blotting¹⁰⁸ in Step 33 Option E. Proceed directly to Step 33 for downstream inflammasome activation assays.

(PAUSE POINT) While using freshly collected serum and lysates is preferable, these can be stored at –80 °C for up to 1 year. Avoid multiple freeze-thaw cycles.

B. *Pseudomonas aeruginosa*

(Timing 8–50 h)

- i. Load a syringe with 200 µL containing 250 mg/kg Avertin and attach a 25-gauge needle.
- ii. Scruff the mouse securely.
- iii. Insert the needle bevel-side up at a 20° angle relative to the mouse's body into the peritoneal cavity (only insert the needle about 4 mm beyond the skin) and inject the Avertin to anesthetize the mouse.
- iv. Attach 2.5 cm of PE-10 tubing to the needle of an insulin syringe. Preload the syringe with at least 50 cc of air. Then load the tubing with 50 µL PBS containing 5×10^5 CFU *P. aeruginosa* (see Reagent Setup).

- v. Once the mouse is deeply anesthetized, place it by its incisors on the intubating platform.
- vi. Use blunt-end forceps to move the mouse's tongue up and to the left to allow visualization beyond the tongue and into the larynx. Use of highly focused bright light can improve visualization.
- vii. Place the PE-10 tubing on the insulin syringe about 1 cm into the trachea.
- viii. Expel the PBS solution and the air from the syringe and wait about 5 seconds before removing the tubing from the trachea.
- ix. Keep the mouse angled on the intubating platform for about 45 seconds before returning it to the cage to recover from anesthesia with supervision.
- x. Allow the infection to progress for 6–48 hours.
- xi. Sacrifice the mouse by asphyxiation by placing it into a CO₂ chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cardiac puncture.
- xii. Collect serum by cardiac puncture and lung tissue by dissection and homogenization to analyze cytokine release via ELISA in Step 33 Options C-D. Lung, spleen and liver lysates can also be collected for CFU quantification by serial dilution of lysates LB agar plates followed by incubation at 37 °C for 24 h and colony counting to monitor the contribution of inflammasome activation on pathogen clearance, and they can be used for western blotting¹⁰⁹ in Step 33 Option E. Proceed directly to Step 33 for downstream inflammasome activation assays.

(PAUSE POINT) While using freshly collected serum and lysates is preferable, these can be stored at –80 °C for up to 1 year. Avoid multiple freeze-thaw cycles.

C. *Salmonella enterica* serovar Typhimurium

(Timing 7 days)

- i. Eight hours prior to the planned infection, remove food and water from the mouse cage.
- ii. Load a syringe with 500 µL PBS containing 1×10^5 CFU *S. Typhimurium* (see Reagent Setup).
- iii. Deliver the bacteria via oral gavage as described in Step 1 Option A.iii–ix.

- iv. Allow the infection to progress for 7 days.
- v. Sacrifice the mouse by asphyxiation by placing it into a CO₂ chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cardiac puncture.
- vi. Collect serum by cardiac puncture and spleen tissue by dissection and homogenization to analyze cytokine release via ELISA in Step 33 Options C-D. Spleen and liver lysates can also be collected for CFU quantification by serial dilution of lysates LB agar plates followed by incubation at 37 °C for 24 h and colony counting to monitor the contribution of inflammasome activation on pathogen clearance, and they can be used for western blotting³⁵ in Step 33 Option E. Proceed directly to Step 33 for downstream inflammasome activation assays.

(PAUSE POINT) While using freshly collected serum and lysates is preferable, these can be stored at –80 °C for up to 1 year. Avoid multiple freeze-thaw cycles.

D. *Burkholderia thailandensis*

(Timing 2 days)

- i. Anesthetize a mouse by isoflurane inhalation in an isoflurane chamber containing 3.0% isoflurane for 2–5 minutes with observation.
- ii. Once the mouse is deeply anesthetized and has stopped moving, scruff the mouse and immediately proceed to inoculation.
- iii. Using a micropipette, gradually release 50 µL PBS containing 5×10^5 CFU *B. thailandensis* (see Reagent Setup) into the nostrils. Be sure to go slowly enough that the mouse can inhale the inoculum.
- iv. Place the mouse back in its cage to recover from anesthesia with supervision.
- v. Allow the infection to progress for 2 days.
- vi. Sacrifice the mouse by asphyxiation by placing it into a CO₂ chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cardiac puncture.
- vii. Collect serum by cardiac puncture and lung, liver and spleen tissue by dissection and homogenization to analyze cytokine release via ELISA in Step 33 Options C-D. Lung, spleen and

liver lysates can also be collected for CFU quantification by serial dilution of lysates LB agar plates followed by incubation at 37 °C for 24 h and colony counting to monitor the contribution of inflammasome activation on pathogen clearance, and they can be used for western blotting^{35,50} in Step 33 Option E. Proceed directly to Step 33 for downstream inflammasome activation assays.

(PAUSE POINT) While using freshly collected serum and lysates is preferable, these can be stored at –80 °C for up to 1 year. Avoid multiple freeze-thaw cycles.

E. Francisella novicida

(Timing 2–7 days)

- i.** Load a syringe with 100 µL PBS containing 1×10^5 CFU *F. novicida* (see Reagent Setup) and attach a 25-gauge needle.
- ii.** Restrain the mouse while grasping the skin along its back and pulling it gently up and away from the body to collect the loose skin over the neck between your thumb and finger to create a tent.
- iii.** With the needle parallel to the mouse's spine and pointing toward the tail, insert the needle subcutaneously at the base of the neck, into the tent between your thumb and finger.
- iv.** Pull back on the syringe plunger to check for negative pressure. If blood is aspirated, the needle placement is not correct. If there is negative pressure, inject the bacteria.
- v.** Allow the infection to progress for 2–7 days.
- vi.** Sacrifice the mouse by asphyxiation by placing it into a CO₂ chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cardiac puncture.
- vii.** Collect serum by cardiac puncture to analyze cytokine release via ELISA in Step 33 Options C-D. Spleen and liver lysates can also be collected by dissection and homogenization for CFU quantification by serial dilution of lysates on TSBC agar plate followed by incubation at 37 °C for 24 h and colony counting to monitor the contribution of inflammasome activation on pathogen clearance, and they can be used for western blotting⁴⁶ in Step 33 Option E. Proceed directly to Step 33 for downstream inflammasome activation assays.

(PAUSE POINT) While using freshly collected serum and lysates is preferable, these can be stored at -80°C for up to 1 year. Avoid multiple freeze-thaw cycles.

F. Influenza A virus (IAV)

(Timing 4–8 days)

- i. Load a syringe with 200 μL containing 250 mg/kg Avertin and attach a 25-gauge needle.
- ii. Scruff the mouse securely.
- iii. Insert the needle bevel-side up at a 20° angle relative to the mouse's body into the peritoneal cavity (only insert the needle about 4 mm beyond the skin) and inject the Avertin to anesthetize the mouse.
- iv. Once the mouse is deeply anesthetized, scruff the mouse.
- v. Using a micropipette, gradually release 50 μL PBS containing 50 PFU Influenza A virus (see Reagent Setup) into the nostrils. Be sure to go slowly enough that the mouse can inhale the inoculum.
- vi. Place the mouse back in its cage to recover from anesthesia with supervision
- vii. Allow the infection to progress for 4–8 days.
- viii. Sacrifice the mouse by asphyxiation by placing it into a CO_2 chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cardiac puncture.
- ix. Collect serum by cardiac puncture to analyze cytokine release via ELISA in Step 33 Options C-D. Lung lysates can also be collected by dissection and homogenization for viral titer quantification by plaque assay in MDCK cells to monitor the contribution of inflammasome activation on pathogen clearance, and they can be used for western blotting⁵³ in Step 33 Option E. Proceed directly to Step 33 for downstream inflammasome activation assays.

(PAUSE POINT) While using freshly collected serum and lysates is preferable, these can be stored at -80°C for up to 1 year. Avoid multiple freeze-thaw cycles.

G. Aspergillus fumigatus

(Timing 2–5 days)

- i. (Optional) Mice should be immunocompromised to model the pathogenesis of *A. fumigatus* in patients with

immunocompromising conditions, such as cancer, or undergoing immunosuppressive treatments.: In order to do so, inject the mouse intraperitoneally with 150 mg/kg cyclophosphamide monohydrate in PBS and subcutaneously with 112 mg/kg cortisone 21-acetate in 0.1% (vol/vol) Tween-20 in PBS 2 days before infection and on the day of infection. This step can be omitted to model the pathogenesis of *A. fumigatus* in immunocompetent individuals.

- ii. Anesthetize a mouse by isoflurane inhalation in an isoflurane chamber containing 3.0% isoflurane for 2–5 minutes with observation.
- iii. Once the mouse is deeply anesthetized and has stopped moving, scruff the mouse and immediately proceed to inoculation.
- iv. Using a micropipette, gradually release 5.5×10^5 *A. fumigatus* conidia (see Reagent Setup) in 30 μ L PBS with 0.1% (vol/vol) Tween-20 (immunocompromised mouse) or 1×10^7 *A. fumigatus* conidia in 50 μ L PBS with 0.1% (vol/vol) Tween-20 (immunocompetent mouse) into the nostrils. Be sure to go slowly enough that the mouse can inhale the inoculum.
- v. Place the mouse back in its cage to recover from anesthesia with supervision.
- vi. Allow the infection to progress for 2 days.
- vii. Sacrifice the mouse by asphyxiation by placing it into a CO₂ chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cardiac puncture.
- viii. Collect serum by cardiac puncture to analyze cytokine release via ELISA in Step 33 Options C-D. Lung lysates can also be collected by dissection and homogenization for fungal burden quantification by RTPCR (as described by Werner et al.¹¹⁰) to monitor the contribution of inflammasome activation on pathogen clearance, and they can be used for western blotting^{56,57} in Step 33 Option E. Proceed directly to Step 33 for downstream inflammasome activation assays.

(PAUSE POINT) While using freshly collected serum and lysates is preferable, these can be stored at –80 °C for up to 1 year. Avoid multiple freeze-thaw cycles.

H. *Candida albicans*

(Timing 3–7 days)

- i. Load a syringe with 100 μ L containing 2×10^5 *C. albicans* blastoconidia (see Reagent Setup) and attach a 27-gauge needle.
- ii. Place the mouse in a restrainer and ensure that the mouse is immobilized but not being injured; the mouse should be able to breathe without difficulty while in the restrainer.
- iii. Grasp the mouse's tail and ensure it is straight.
- iv. Insert the needle pointing toward the head bevel-side up at a 20° angle relative to the mouse's tail into the tail vein and inject the fungi.
- v. When the needle is removed, press absorbent gauze firmly against the injection site to stop bleeding.
- vi. Remove the mouse from the restrainer and place it back in its cage to recover with supervision.
- vii. Allow the infection to progress for 3–7 days.
- viii. Sacrifice the mouse by asphyxiation by placing it into a CO₂ chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cardiac puncture.
- ix. Collect serum by cardiac puncture to analyze cytokine release via ELISA in Step 33 Options C–D. Kidney lysates can also be collected by dissection and homogenization for fungal burden quantification by serial dilution of lysates on Sabouraud agar plates followed by incubation at 37 °C for 24 h and colony counting to monitor the contribution of inflammasome activation on pathogen clearance, and they can be used for western blotting⁵⁸ in Step 33 Option E. Proceed directly to Step 33 for downstream inflammasome activation assays.

(PAUSE POINT) While using freshly collected serum and lysates is preferable, these can be stored at –80 °C for up to 1 year. Avoid multiple freeze-thaw cycles.

I. Sepsis—LPS shock

(Timing 6.5–72.5 h)

- i. Load a syringe with 200 μ L PBS containing 25 mg/kg LPS (see Reagent Setup) and attach a 25-gauge needle.
- ii. Scruff the mouse securely.
- iii. Insert the needle bevel-side up at a 20° angle relative to the mouse's body into the peritoneal cavity (only insert the needle about 4 mm beyond the skin) and inject the LPS.

- iv. Allow sepsis to develop for 6–72 hours. **CRITICAL STEP**
Within 8–12 hours of LPS injection, mice will appear very sick and almost stop moving; however, they can recover and regain normal activity over time depending on their sensitivity to LPS. Timepoints as early as 2 and 4 hours can also be analyzed, as activation occurs quickly in this model.
- v. Sacrifice the mouse by asphyxiation by placing it into a CO₂ chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cardiac puncture.
- vi. Collect serum by cardiac puncture to analyze cytokine release via ELISA in Step 33 Options C–D. Livers can also be collected for sectioning and hematoxylin and eosin staining to monitor pathology (standard H&E staining can be used; detailed methods are not described here). Spleen and liver lysates can be collected by dissection and homogenization to be used for western blotting⁵⁹ in Step 33 Option E. Proceed directly to Step 33 for downstream inflammasome activation assays.

(PAUSE POINT) While using freshly collected serum and lysates is preferable, these can be stored at –80 °C for up to 1 year. Avoid multiple freeze-thaw cycles.

J. Sepsis—Low dose LPS shock (TAK1 deficiency)

(Timing 8.5–52.5 h)

- i. Load a syringe with 50 µL DMSO containing 50 mg/kg TAK1 inhibitor (TAK1-i) 5Z-7-Oxozeaenol and attach a 25-gauge needle. TAK1-i is a potent and selective inhibitor of the mitogen-activated protein kinase kinase kinase 7 (MAP3K7), also called transforming growth factor β -activated kinase 1 (TAK1), and it sensitizes cells to undergo inflammatory cell death (PANoptosis) in response to LPS treatment.
- ii. Scruff the mouse securely.
- iii. Insert the needle bevel-side up at a 20° angle relative to the mouse's body into the peritoneal cavity (only insert the needle about 4 mm beyond the skin) and inject the TAK1-i.
- iv. After two hours of TAK1 inhibitor treatment, load a syringe with PBS containing 0.5 µg/kg LPS (see Reagent Setup) and attach a 25-gauge needle.
- v. Scruff the mouse securely.

- vi. Insert the needle bevel-side up at a 20° angle relative to the mouse's body into the peritoneal cavity (only insert the needle about 4 mm beyond the skin) and inject the LPS.
- vii. Allow sepsis to develop for 6–50 hours.
- viii. Sacrifice the mouse by asphyxiation by placing it into a CO₂ chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cardiac puncture.
- ix. Collect serum by cardiac puncture to analyze cytokine release via ELISA in Step 33 Options C-D. Livers can also be collected for sectioning and hematoxylin and eosin staining to monitor pathology (standard H&E staining can be used; detailed methods are not described here). Spleen and liver lysates can be collected by dissection and homogenization to be used for western blotting⁵⁹ in Step 33 Option E. Proceed directly to Step 33 for downstream inflammasome activation assays.

(PAUSE POINT) While using freshly collected serum and lysates is preferable, these can be stored at –80 °C for up to 1 year. Avoid multiple freeze-thaw cycles.

K. Skin disease—chronic proliferative dermatitis model

(Timing 10 weeks)

- i. Age *Sharpin*^{cpdm} mice (which will spontaneously develop an inflammatory disease characterized by severe dermatitis and inflammation by 10 weeks of age) for 10 weeks or until moribund.
- ii. Sacrifice the mice by asphyxiation by placing it into a CO₂ chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cardiac puncture.
- iii. Collect serum by cardiac puncture to analyze cytokine release via ELISA in Step 33 Options C-D. Spleens and skin can also be collected for sectioning and hematoxylin and eosin staining to monitor pathology (standard H&E staining can be used; detailed methods are not described here).. Spleen size and weight can also be recorded. Spleen and skin lysates can also be collected by dissection and homogenization to be used for western blotting⁶⁰ in Step 33 Option E. Proceed directly to Step 33 for downstream inflammasome activation assays.

(PAUSE POINT) While using freshly collected serum and lysates is preferable, these can be stored at -80°C for up to 1 year. Avoid multiple freeze-thaw cycles.

L. Bone disease—chronic recurrent multifocal osteomyelitis model

(Timing 60–120 days)

- i. Age *Pstpip2^{mo}* mice (which will spontaneously develop a chronic bone disease featuring bone deformity and inflammation by day 60–120) for 120 days or until moribund.
- ii. Sacrifice the mice by asphyxiation by placing it into a CO_2 chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cardiac puncture.
- iii. Collect serum by cardiac puncture and footpad tissue by dissection and homogenization to analyze cytokine release via ELISA in Step 33 Options C–D. Footpads tissue homogenates can also be used for western blotting in Step 33 Option E, and footpad tissue can also be collected for sectioning and hematoxylin and eosin staining to monitor pathology⁶¹ (standard H&E staining can be used; detailed methods are not described here). Proceed directly to Step 33 for downstream inflammasome activation assays.

(PAUSE POINT) While using freshly collected serum and lysates is preferable, these can be stored at -80°C for up to 1 year. Avoid multiple freeze-thaw cycles.

M. Bone disease—collagen-induced arthritis model

(Timing 7–42 days)

- i. Load a syringe with 25 μL of a 200 mg type II collagen/100 mL emulsion (see Reagent Setup) and attach a 30-gauge needle.
- ii. Anesthetize the mouse with isoflurane inhalation in an isoflurane chamber containing 3% isoflurane for 2–5 minutes with observation. Once the mouse is deeply anesthetized and has stopped moving, immediately proceed to inoculation.
- iii. Wipe the skin on the tail with 70% (vol/vol) ethanol.
- iv. Select an injection site about 1.5 cm distal from the base of the tail and pull the skin taut between the thumb and index finger.
- v. Insert the needle bevel-side up at a 10° angle relative to the mouse's body (only insert the needle about 1 mm, just under the superficial layer of the skin) and inject the emulsion.

- vi. Place the mouse back in its cage to recover from anesthesia with supervision.
- vii. Allow the development of arthritis for up to 42 days.
- viii. Sacrifice the mouse by asphyxiation by placing it into a CO₂ chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cardiac puncture.
- ix. Collect serum by cardiac puncture to analyze cytokine release via ELISA Step 33 Options C-D. Pathology can also be monitored using computed microtomography scanning of the hind limbs and by using clinical scoring of arthritis (standard arthritis pathology methods can be used and are not described in detail here). Footpad and knee and ankle joint lysates can also be collected by dissection and homogenization to be used in western blotting⁶³ in Step 33 Option E. Proceed directly to Step 33 for downstream inflammasome activation assays.

(PAUSE POINT) While using freshly collected serum and lysates is preferable, these can be stored at –80 °C for up to 1 year. Avoid multiple freeze-thaw cycles.

N. High-fat diet (obesity model)

(Timing 16 weeks)

- i. Once mice have reached 8 weeks of age, change chow to a high-fat diet, in which 45% of calories come from fat.
- ii. Allow mice to eat high-fat diet chow ad libitum for 16 weeks.
- iii. Sacrifice the mouse by asphyxiation by placing it into a CO₂ chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cardiac puncture.
- iv. Collect serum by cardiac puncture to analyze cytokine release via ELISA in Step 33 Options C-D. Collect epididymal white adipose tissue by dissection and homogenization for western blotting in Step 33 Option E. Serum concentrations of insulin, leptin and resistin and the insulin tolerance and glucose tolerance tests can be used to monitor pathology⁶⁴ (standard pathological evaluation of these markers in serum can be used and are not described in detail here). Proceed directly to Step 33 for downstream inflammasome activation assays.

(PAUSE POINT) While using freshly collected serum and lysates is preferable, these can be stored at –80 °C for up to 1 year. Avoid multiple freeze-thaw cycles.

O. High-sugar diet (diabetes/glucose intolerance model)

(Timing 16 weeks)

- i.** Once mice have reached 8 weeks of age, change chow to a high-sugar diet, in which 50% of calories come from sucrose.
- ii.** Allow mice to eat high-sugar diet chow ad libitum for 16 weeks.
- iii.** Sacrifice the mouse by asphyxiation by placing it into a CO₂ chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cardiac puncture.
- iv.** Collect serum by cardiac puncture to analyze cytokine release via ELISA in Step 33 Options C-D. Collect epididymal white adipose tissue by dissection and homogenization for western blotting in Step 33 Option E. Serum concentrations of insulin, leptin and resistin and the insulin tolerance and glucose tolerance tests can be used to monitor pathology⁶⁴ (standard pathological evaluation of these markers in serum can be used and are not described in detail here). Proceed directly to Step 33 for downstream inflammasome activation assays.

(PAUSE POINT) While using freshly collected serum and lysates is preferable, these can be stored at –80 °C for up to 1 year. Avoid multiple freeze-thaw cycles.

P. Atherosclerosis

(Timing 10 weeks)

- i.** Once mice have reached 8 weeks of age, change chow to a Western-type diet containing 0.1% (wt/wt) cholesterol to induce atherosclerosis.
- ii.** Allow mice to eat Western-type diet chow ad libitum for 10 weeks.
- iii.** Sacrifice the mouse by asphyxiation by placing it into a CO₂ chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cardiac puncture.
- iv.** Collect serum by cardiac puncture to analyze cytokine release via ELISA in Step 33 Options C-D. Atherosclerotic plaque lesion size can be used to monitor pathology¹¹¹ (standard atherosclerosis pathology methods can be used and are not described in detail here). Proceed directly to Step 33 for downstream inflammasome activation assays.

(PAUSE POINT) While using freshly collected serum and lysates is preferable, these can be stored at -80°C for up to 1 year. Avoid multiple freeze-thaw cycles.

Q. Tau-related neurodegenerative disease (Alzheimer's model)

(Timing 2–12 months)

- i.** Age TPS mice that overexpress human Tau (1N4R) with the P301S mutation (which will spontaneously develop Tauopathies, including the manifestations of Alzheimer's disease, starting around 11 months of age) or *App* knock-in mice (*App*^{NL-F} and *App*^{NL-G-F} which develop pathological A β 42 at 2–12 months of age) for 12 months or until moribund.
- ii.** Sacrifice the mouse by asphyxiation by placing it into a CO₂ chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cervical dislocation.
- iii.** Isolate microglial cells (follow the protocol previously established by Cardona et al.¹¹²) or astrocytes (follow the protocol previously established by He et al.¹¹³) to assess inflammasome activation. Microglial cells and astrocytes can be used in the same *in vitro* assays as BMDMs outlined in the Steps 31–33 of this protocol.

R. Colorectal cancer

(Timing 80 days)

- i.** Load a syringe with 200 μL PBS containing 10 mg/kg AOM (see Reagent Setup) and attach a 25-gauge needle.
- ii.** Scruff the mouse securely.
- iii.** Insert the needle bevel-side up at a 20° angle relative to the mouse's body into the peritoneal cavity (only insert the needle about 4 mm beyond the skin) and inject the AOM.
- iv.** Five days after AOM injection, remove the drinking water in the mouse cage and replace with a 3% (wt/vol) DSS solution in water. Allow the mice to drink ad libitum for 6 days.
- v.** Remove the 3% (wt/vol) DSS solution on day 11 and return mice to normal drinking water ad libitum for 14 days.
- vi.** Remove the drinking water on day 25 and replace with a 2% (wt/vol) DSS solution in water. Allow the mice to drink ad libitum for 6 days.
- vii.** Remove the 2% (wt/vol) DSS solution on day 31 and return mice to normal drinking water ad libitum for 14 days.

- viii. Remove the drinking water on day 45 and replace with a 2% (wt/vol) DSS solution in water. Allow the mice to drink ad libitum for 6 days.
- ix. Remove the 2% (wt/vol) DSS solution on day 51 and return mice to normal drinking water ad libitum for 29 days.
- x. Sacrifice the mouse by asphyxiation by placing it into a CO₂ chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cardiac puncture.
- xi. Examine whole colons to determine tumor burden and colon length (standard colorectal cancer pathology methods can be used and are not described in detail here). Collect serum by cardiac puncture and colon tissue homogenate by dissection and homogenization to analyze cytokine release via ELISA in Step 33 Options C-D. Colon lysates can also be used for western blotting⁶⁸ in Step 33 Option E. Proceed directly to Step 33 for downstream inflammasome activation assays.

(PAUSE POINT) While using freshly collected serum and lysates is preferable, these can be stored at –80 °C for up to 1 year. Avoid multiple freeze-thaw cycles.

(Optional) Bone marrow progenitor cell isolation—(Timing 4 hours to process 2 mice for bone marrow isolation; add an additional 30 minutes per mouse to process more)

Critical – When performing *in vitro* inflammasome activation, start the procedure here. If *in vivo* inflammasome procedures have been used (as described in the different Options of Step 1), proceed directly to Step 33 instead.

(Critical—several other protocols have been published to isolate BMDMs [such as that by Weischenfeldt and Porse¹²⁰] and BMDCs [such as that by Roney¹²¹]; these can also be used. BMDCs should be especially considered when following Step 31 Option M.)

(Critical—sterile technique is required for the isolation of BMDMs; all tools should be rinsed with ethanol, and procedures should be performed in a laminar flow hood.)

(Critical—If using *Bacillus anthracis* protective antigen and lethal factor to stimulate the NLRP1b inflammasome in Step 31 Option F, BALB/c or transgenic C57BL/6J mice must be used.)

2. Sacrifice a 6–8 week old mouse by asphyxiation by placing it into a CO₂ chamber with a flow rate to displace 10% to 30% of the cage volume per minute for 2–3 minutes followed by cervical dislocation.
3. Pin the mouse to expose the abdomen. Sterilize the hind legs and abdomen by spraying with 70% (vol/vol) ethanol.

4. Using scissors, cut the midline of the abdomen and continue cutting to expose the femurs.
5. Unpin the right leg and peel the skin off toward the midline. Cut the adductor muscles toward the midline and sever the leg between the hip joint and the spine. Once the leg is detached from the body, remove excess tissue (i.e., muscle and fat) from the bone, cut the paw off distal to the ankle, and peel off the remaining skin.
6. Repeat step 5 for the left leg.
7. Spray both legs with 70% (vol/vol) ethanol.
8. Working on an 70% (vol/vol) ethanol-soaked towel, hold the distal end of the tibia with forceps so that knee joint is pointing away from you and the leg is vertical. Using the tip of slightly opened scissors, strip the calf tissue by pushing it down toward the knee. Grip the tibia and femur with two sets of forceps and gently push against the direction of the knee joint until the tibia breaks right at the knee. Remove any hanging tissue and place the tibia on the towel. Recover the femur in the same way, snapping the knee off.
9. To remove remaining flesh from the bones, hold the fleshy end of each piece in dry paper towel and pull the bone with forceps, leaving the flesh in the towel. Then squeeze the remaining fleshy part in the towel and rub it with your fingers (holding it in the towel) to clean the bone off.

Troubleshooting

10. Spray the bones with 70% (vol/vol) ethanol and place them in a sterile petri dish with 10 mL BMDM culture media. Rinse the bones by moving the dish back and forth.
11. Collect 10 mL fresh BMDM culture media in a 10 mL syringe and attach a 25-gauge needle.
12. Remove the tibia from the petri dish and cut off at the ankle joint at an angle.
13. (Critical step—The cut needs to be made at an angle; a straight cut can splinter the bone.)
14. Hold the tibia over a fresh 50 mL tube, with the narrow end of the tibia pointing down. Using the needle, squirt media on the tibia gently. Then insert the needle (gently at first) at the top end of the marrow and squirt media. Pull back the needle and insert again. Squirt with very short, high pressure pushes. Push the needle in further until the epiphysis is crossed and media comes out at the bottom end of the bone. Continue flushing a couple more times; when the bone is white, discard it.

Troubleshooting

15. Repeat Steps 11–13 for the femur, cutting off at the hip joint just as the tibia was cut at the ankle joint and using the same 50 mL tube as was used for the tibia for collection.
16. Replace the 25-gauge needle with an 18-gauge needle on the 10 mL syringe. Aspirate the marrow and media from the 50 mL tube up and down 3 times, rinsing the sides of the tube each time, to disperse the marrow.
17. Add an additional 20 mL of BMDM culture media to the 50 mL tube.
18. (Optional) Pass the cell suspension through a 70 μ m cell strainer to remove any remaining bone or muscle fragments. If performing this step, ensure that cells are thoroughly resuspended before removing the liquid from the 50 mL tube, as cells will be lost if not thoroughly resuspended.

(PAUSE POINT) Bone marrow cells can be cryopreserved at this stage by centrifuging at $200 \times g$ at 4 °C for 5 minutes, removing the supernatant, and resuspending the cells in 40 mL freezing media (90% vol/vol FBS and 10% vol/vol DMSO). Cryovials can be filled with 1 mL each and stored at –80 °C for 24 hours before being transferred to liquid nitrogen for long-term storage. Once cryopreserved, bone marrow cells can be stored in liquid nitrogen for up to 5 years. See an example protocol from Marim et al.⁷⁶ for additional details.

19. Collect the cell suspension from the tube and add 10 mL (or $\sim 20 \times 10^6$ cells) to each of the three 150 mm tissue culture dishes. Then add an additional 10 mL BMDM culture media to each dish. Incubate in a humidified incubator at 37 °C.

(Optional): BMDM differentiation—(Timing 7 days)

(CRITICAL—If using cryopreserved bone marrow (see PAUSE POINT in Step 17), once cells are thawed and plated [see an example protocol from Marim et al.⁷⁶] the protocol can be followed from this stage.)

20. On day 3 following plating of bone marrow progenitor cells in Step 18, add an additional 5 mL of BMDM culture media to each dish. The cells should begin to look large and round with enlarged cytoplasm when viewed under a microscope.
21. On day 5, add an additional 5 mL of BMDM culture media to each dish. The cells will be elongating and have irregular shape consistent with classic macrophage morphology when viewed under a microscope.
22. On day 6, remove the media and wash with 10 mL ice cold PBS once. The cells should have a more homogenous morphology than on previous days when viewed under a microscope.

Troubleshooting

23. Add 10 mL ice cold PBS to each dish and incubate for 5 minutes.
24. Gently scrape the cells with a cell scraper and collect cells from all 3 dishes into a single 50 mL tube.

25. Centrifuge at $270 \times g$ at 4°C for 5 minutes and discard the supernatant.
26. Resuspend the pellet in 20 mL BMDM culture media and count the cells. The expected yield is $60\text{--}100 \times 10^6$ cells per healthy 6–8 week old mouse
 Troubleshooting
 (Critical step—Cells must be thoroughly resuspended before being used to count. Clumping will lead to inaccurate numbers and inconsistencies between the number of cells plated for downstream analyses, making the results invalid.)
27. Plan out the 12-well plate layout for your desired *in vitro* inflammasome stimulation assay. At least 4 plates are required for each plate layout of stimulations/conditions to be tested (1 for real-time cell death monitoring, 1 for separate supernatant and protein lysate collection, 1 for combined supernatant and protein lysate collection, and 1 to fix for microscopy) and 1×10^6 cells per well. For each planned stimulation, include 3 biological replicates on each plate for each desired timepoint.
28. Place coverslips in the empty wells of the plate that will be used for microscopy. The other plates do not need any additional items.
29. Plate 1×10^6 cells in 1 mL BMDM culture media per well onto 12-well plates (if proceeding with bacterial stimulation, use antibiotic-free BMDM culture media).
30. Culture overnight in a humidified incubator at 37°C before proceeding to inflammasome activation.
31. On day 7 (the day of inflammasome activation), wash the cells once with warm PBS, then incubate them in 500 μL BMDM stimulation media (for non-bacterial stimulations) or BMDM culture media without antibiotics (for bacterial stimulations) for 2 hours before proceeding with *in vitro* stimulation/infection.

(Optional): *In vitro* inflammasome activation

32. Stimulate the BMDMs to activate the inflammasome of interest (see Table 1). For investigation of inflammasome activation *in vitro* in response to ligand-based triggers, follow options A–G. For investigation of inflammasome activation *in vitro* in response to bacteria, follow options H–J. For investigation of inflammasome activation *in vitro* in response to virus, follow options K–L. For investigation of inflammasome activation *in vitro* in response to fungi, follow options M–N.
 (CRITICAL—Throughout the *in vitro* stimulations, cells should be regularly examined under the microscope to determine whether pyroptosis has already taken place by looking for the visual signs of cell ballooning and lifting.)
 (CRITICAL Using a time course when establishing these methods in your lab for the first time is recommended.)
  - A. LPS + ATP
 (Timing ~4.5 h depending upon the experimental question.)

- i. Remove media from BMDMs and add 495 μ L of 100 ng/mL LPS in BMDM stimulation media (with 0.2 μ L [20 nM] SYTOX Green for 1 of the plates that does not contain the coverslips) per well.
- ii. Incubate in a humidified incubator at 37 °C for 3.5 hours.
- iii. Add 5 μ L of 0.5 M stock ATP (final concentration, 5 mM per well) to each well.
- iv. Place the plate containing SYTOX Green in the IncuCyte system for real-time cell death analysis according to Steps 32–35. Place the other 3 plates in a humidified incubator. Incubate at 37 °C for 30 minutes.

B. LPS + nigericin

(Timing ~4.5 h depending upon the experimental question.)

(CRITICAL Using a time course when establishing this method in your lab for the first time is recommended.)

- i. Remove media from BMDMs and add 498 μ L of 100 ng/mL LPS in BMDM stimulation media (with 0.2 μ L [20 nM] SYTOX Green for 1 of the plates that does not contain the coverslips) per well.
- ii. Incubate in a humidified incubator at 37 °C for 3.5 hours.
- iii. Add 2 μ L of 5 mM stock nigericin (final concentration, 20 μ M per well) to each well.
- iv. Place the plate containing SYTOX Green in the IncuCyte system for real-time cell death analysis according to Steps 32–35. Place the other 3 plates in a humidified incubator. Incubate at 37 °C for 45 minutes.

C. LPS transfection

(Timing 3.5–4.5 h)

- i. Remove media from BMDMs and add 400 μ L pre-warmed Opti-MEM to each well. Incubate in a humidified incubator at 37 °C for approximately 15 minutes while preparing the transfection mix:

Reagent	Amount
Xfect polymer	0.3 μ L/well
LPS	1 μ g/well
Xfect buffer	99.5 μ L/well

- ii. Take 25% of the total transfection mix and supplement with 0.2 μ L/well SYTOX Green.
- iii. Add 100 μ L of the transfection mix (+ 0.2 μ L [20 nM] SYTOX Green for 1 of the plates that does not contain the coverslips) to the wall of each well and swirl the plate to mix.
- iv. Place the plate containing SYTOX Green in the IncuCyte system for real-time cell death analysis according to Steps 32–35. Place the other 3 plates in a humidified incubator. Incubate at 37 °C for 3–4 hours.

D. NAIP-dependent NLRC4 inflammasome activation

(Timing 3.25 h [flagellin] or 6.25 h [rod or needle protein])

- i. Remove media from BMDMs and add 460 μ L pre-warmed Opti-MEM to each well. Incubate in a humidified incubator at 37 °C for approximately 1 hour while preparing the transfection mix:

Reagent	Amount
DOTAP	20 μ L/well
PBS	19 μ L/well
One of the following:	
- rod protein (PrgJ; NAIP1-dependent activation)	1 μ g/well
- needle protein (PrgI; NAIP2-dependent activation)	1 μ g/well
- flagellin protein (NAIP5/6-dependent activation)	0.5 μ g/well

- ii. Take 25% of the total transfection mix and supplement with 0.2 μ L/well SYTOX Green.
- iii. Add 40 μ L of the transfection mix (+ 0.2 μ L [20 nM] SYTOX Green for 1 of the plates that does not contain the coverslips) to the wall of each well and swirl the plate to mix.
- iv. Place the plate containing SYTOX Green in the IncuCyte system for real-time cell death analysis according to Steps 32–35. Place the other 3 plates in a humidified incubator. Incubate at 37 °C for 5 hours (rod and needle proteins) or 2 hours (flagellin).

E. *Clostridium difficile* toxin

(Timing 16.5 h)

- i. Remove media from BMDMs and add 500 μ L of BMDM stimulation media containing 100 μ L filtered bacterial broth (and 0.2 μ L [20 nM] SYTOX Green for 1 of the plates that

does not contain the coverslips) per well. Alternatively, 50 ng of commercial purified TcdA or TcdB can be added in place of the filtered bacterial broth.

- ii. Place the plate containing SYTOX Green in the IncuCyte system for real-time cell death analysis according to Steps 32–35. Place the other 3 plates in a humidified incubator. Incubate at 37 °C for 16 hours.

F. *Bacillus anthracis* protective antigen and lethal factor

(Timing 4.5 h)

(CRITICAL—to use this stimulation, BMDMs from BALB/c or transgenic C57BL/6J mice should be used, as wild type C57BL/6J mice do not express functional NLRP1b)

- i. Remove media from BMDMs and add 500 µL of BMDM stimulation media containing 1 µg protective antigen and 1 µg lethal factor (and 0.2 µL [20 nM] SYTOX Green for 1 of the plates that does not contain the coverslips) per well.
- ii. Place the plate containing SYTOX Green in the IncuCyte system for real-time cell death analysis according to Steps 32–35. Place the other 3 plates in a humidified incubator. Incubate at 37 °C for 4 hours.

G. Poly(dA:dT) transfection

(Timing 4.5 h)

- i. Remove media from BMDMs and add 450 µL pre-warmed Opti-MEM to each well. Incubate in a humidified incubator at 37 °C for approximately 15 minutes while preparing the transfection mix:

Reagent	Amount
Xfect polymer	0.25 µL/well
poly(dA:dT)	2 µg/well
Xfect buffer	47.7 µL/well

- ii. Take 25% of the total transfection mix and supplement with 0.2 µL/well SYTOX Green.
- iii. Add 50 µL of the transfection mix (+ 0.2 µL [20 nM] SYTOX Green for 1 of the plates that does not contain the coverslips) to the wall of each well and swirl the plate to mix.
- iv. Place the plate containing SYTOX Green in the IncuCyte system for real-time cell death analysis according to Steps 32–

35. Place the other 3 plates in a humidified incubator. Incubate at 37 °C for 4 hours. Monitor for cell death every hour.

H. Noncanonical NLRP3 inflammasome activation with Gram negative bacteria (*Escherichia coli*, *Citrobacter rodentium*, *Pseudomonas aeruginosa* popB, or *Burkholderia thailandensis*)

(CRITICAL—*Burkholderia thailandensis* can be used for NLRP3 or NLRC4 inflammasome activation. At early timepoints, NLRC4 inflammasome activation is predominant, but at later timepoints the NLRP3 inflammasome takes over.)

(Timing 20.5 h)

- i. Remove media from BMDMs and add 500 µL of diluted bacteria (with 0.2 µL [20 nM] SYTOX Green for 1 of the plates that does not contain the coverslips) per well. *E. coli*, *C. rodentium*, *P. aeruginosa* popB, or *B. thailandensis* bacteria can be used at an MOI of 1, 10, or 20 (see Reagent Setup).
- ii. Place the plate containing SYTOX Green in the IncuCyte system for real-time cell death analysis according to Steps 32–35. Place the other 3 plates in a humidified incubator. Incubate at 37 °C for 4 hours at 37 °C.
- iii. Remove the media and wash the cells with 500 µL warm PBS three times.
- iv. Add 500 µL BMDM stimulation media with 50 µg/mL gentamicin (and 20 nM SYTOX Green for the plate used in the IncuCyte) to each well.
- v. Place the plate containing SYTOX Green back in the IncuCyte system for real-time cell death analysis according to Steps 32–35. Place the other 3 plates in a humidified incubator. Incubate at 37 °C overnight (16 hours).

I. NAIP-NLRC4 inflammasome activation with Gram negative bacteria (*Burkholderia thailandensis*, *Salmonella enterica* serovar Typhimurium, or *Pseudomonas aeruginosa*)

(CRITICAL—*Burkholderia thailandensis* can be used for NLRP3 or NLRC4 inflammasome activation. At early timepoints, NLRC4 inflammasome activation is predominant, but at later timepoints NLRP3 inflammasome takes over.)

(Timing 2.5–3.5 h)

- i. Remove media from BMDMs and add 500 µL of diluted bacteria (and 0.2 µL [20 nM] SYTOX Green for 1 of the plates that does not contain the coverslips) per well. Bacteria can be used at an MOI of 5 or 10.

- ii. Place the plate containing SYTOX Green in the IncuCyte system for real-time cell death analysis according to Steps 32–35. Place the other 3 plates in a humidified incubator. Incubate at 37 °C for 2–3 hours.

J. *Francisella novicida*

(Timing 20.5 h)

- i. Remove media from BMDMs and add 500 µL of diluted bacteria (with 0.2 µL [20 nM] SYTOX Green for 1 of the plates that does not contain the coverslips) per well. Bacteria can be used at an MOI of 20, 50, or 100 (see Reagent Setup).
- ii. Place the plate containing SYTOX Green in the IncuCyte system for real-time cell death analysis according to Steps 32–35. Place the other 3 plates in a humidified incubator. Incubate at 37 °C for 4 hours.
- iii. Remove the media and wash the cells with 500 µL warm PBS three times.
- iv. Add 500 µL BMDM culture media with 50 µg/mL gentamicin (and 0.2 µL [20 nM] SYTOX Green for the plate used in the IncuCyte) to each well.
- v. Place the plate containing SYTOX Green back in the IncuCyte system for real-time cell death analysis according to Steps 32–35. Place the other 3 plates in a humidified incubator. Incubate at 37 °C overnight (16 hours).

K. Influenza A virus (IAV)

(Timing 16.5 h)

- i. Remove media from BMDMs and add 400 µL of high glucose DMEM without FBS containing Influenza A virus (see Reagent Setup) at an MOI of 20 (and 0.2 µL [20 nM] SYTOX Green for 1 of the plates that does not contain the coverslips) to each well.
- ii. Place the plate containing SYTOX Green in the IncuCyte system for real-time cell death analysis according to Steps 32–35. Place the other 3 plates in a humidified incubator. Incubate at 37 °C for 2 hours to allow absorption.
- iii. Supplement cells with 10% (vol/vol) FBS by adding 100 µL of high glucose DMEM containing 50% (vol/vol) FBS and continue incubating in the IncuCyte for real-time cell death analysis according to Steps 32–35. Place the other 3 plates in a humidified incubator as above at 37 °C for a total of 16 hours.

L. Murine cytomegalovirus (MCMV)

(Timing 16.5 h)

- i. Remove media from BMDMs and add 500 μ L of stimulation media containing virus at an MOI of 10 (and 0.2 μ L [20 nM] SYTOX Green for 1 of the plates that does not contain the coverslips) to each well.
- ii. Place the plate containing SYTOX Green in the IncuCyte system for real-time cell death analysis according to Steps 32–35. Place the other 3 plates in a humidified incubator. Incubate at 37 °C for 16 hours.

M. Aspergillus fumigatus

(Timing 12.5–20.5 h)

(CRITICAL—the IncuCyte system cannot be used to monitor overnight *Aspergillus* or *Candida* infections due to overgrowth of fungal hyphae and autofocusing limitations. *Aspergillus* infection works better in BMDCs. We describe the procedure for BMDMs here, but BMDCs can be substituted)

- i. Remove media from BMDMs and add 500 μ L of diluted conidia (and 0.2 μ L [20 nM] SYTOX Green for 1 of the plates that does not contain the coverslips) per well. Conidia can be used at an MOI of 1–20.
- ii. Place the plate containing SYTOX Green in the IncuCyte system for real-time cell death analysis according to Steps 32–35. Place the other 3 plates in a humidified incubator. Incubate at 37 °C for 12–20 hours.

N. Candida albicans

(Timing 12.5–24.5 h)

(CRITICAL—the IncuCyte system cannot be used to monitor overnight *Aspergillus* or *Candida* infections due to overgrowth of fungal hyphae and autofocusing limitations.)

- i. Remove media from BMDMs and add 500 μ L of diluted live *C. albicans* (and 0.2 μ L [20 nM] SYTOX Green for 1 of the plates that does not contain the coverslips) per well. Blastospores can be used at an MOI of 1–5.
- ii. Place the plate containing SYTOX Green in the IncuCyte system for real-time cell death analysis according to Steps 32–35. Place the other 3 plates in a humidified incubator. Incubate at 37 °C for 12–24 hours.

(Optional) Data and sample collection downstream assays

33. Prepare the four plates for downstream inflammasome activation assays. Follow Option A for real-time cell death analysis. Follow Option B for fixing cells for microscopy. Follow Option C for combined supernatant and protein lysate collection for caspase-1 western blotting. Follow Option D for separate supernatant collection for LDH and ELISA assays and protein lysate collection for gasdermin D western blotting.

CRITICAL: All Options should be followed in parallel in order to obtain a comprehensive overview of the inflammasome activation status. The time-points at which the plates are processed are dependent on the *in vitro* inflammasome model chosen in Step 31 and the experimental design.

Option A: Real-time cell death analysis—(Timing will be equal to that of the inflammasome trigger used in step 31)

(CRITICAL—the IncuCyte system cannot be used to monitor overnight *Aspergillus* or *Candida* infections due to overgrowth of fungal hyphae and autofocusing limitations.)

(CRITICAL—The IncuCyte system can be substituted with other live-cell imaging systems or approaches.)

- i. During the incubation step using the inflammasome activation conditions in step 31, place the plate containing 20 nM SYTOX Green in each well in an IncuCyte Zoom incubator imaging system.
- ii. Add 1 µL Nuclear-ID to one well to allow the determination of the total number of cells.
- iii. Image the plate with a 20× objective every hour or half hour (for a time course > 3 hours) or every 5 or 15 minutes (for a time course ≤ 3 hours), capturing 9 images per well.
- iv. Analyze all images and data using the IncuCyte S3 software to generate masks for quantification. Use Basic Analyzer with the TopHat segmentation-based object detection.

Troubleshooting

Option B: Fixing cells for microscopy—(Timing 1.75 h)

- i. Remove the plate containing the coverslips from the incubator after the desired amount of time.
- ii. Aspirate the supernatant and wash the cells 3 times by gently adding and aspirating 500 µL PBS. Take care to avoid dislodging the cells on the coverslip.
- iii. Add 250 µL of the 4% (wt/vol) paraformaldehyde solution and incubate for 15 minutes at room temperature.
- iv. Aspirate the paraformaldehyde and wash cells 3 times with 500 µL PBS.

- v. Add 250 μ L permeabilization solution (0.1% [vol/vol] Triton X-100 in PBS) and incubate for 10 minutes.
- vi. Aspirate the permeabilization solution and wash cells 3 times with 500 μ L PBS.
- vii. Block the cells in 10% (vol/vol) normal goat serum with 0.1% (vol/vol) Tween-20 for 1 hour at room temperature. The cells are now ready to be used for staining and ASC speck analysis in Step 33 Option A.
- viii. (Pause point—cells can be stored in blocking solution at 4 °C for up to 1 week.)

Option C: Combined supernatant and protein lysate collection for caspase western blotting—(Timing 0.5 h for processing)

- i. Remove a third plate without coverslips from the incubator after the desired amount of time. Remove 150 μ L of supernatant. (This supernatant can also be used for additional analyses in Step 33 Options B-E and stored if processed as described in Step 32 Option D.iii-iv.) Do not remove the remaining supernatant.
- ii. Combine 50 μ L caspase lysis buffer + 150 μ L 4 $\times$ SDS buffer per well, and then add 200 μ L of the mix to each well.
- iii. Collect lysed cells and supernatant from each individual well by pipetting the liquid up and down while scraping the bottom of the well with the pipette tip. Place the protein lysate into labeled 1.5 mL tubes.

Troubleshooting

- iv. Heat the tubes to 100 °C for 12 minutes.
- v. Centrifuge the tubes at 14,500 $\times$ g for 30 seconds at room temperature.
(Critical step—Insoluble proteins can interfere with downstream analyses and lead to inaccurate results. The combined supernatant and protein lysates are now ready for caspase-1 western blotting in Step 33 Option E.
(Pause point—combined supernatant and protein lysates can be stored at –20 °C or –80 °C until ready to use.)

Option D: Separate supernatant collection for LDH and ELISA assays and protein lysate collection for gasdermin D western blotting—(Timing 0.5 h for processing)

- i. Remove a second plate without coverslips from the incubator after the desired amount of time.
- ii. Collect supernatant (150 μ L per timepoint) at desired timepoints throughout the incubation in individual, labeled 1.5 mL tubes.
- iii. Once supernatant has been collected, centrifuge the tubes at 14,500 $\times$ g at 4 °C for 5 minutes.
- iv. (Critical step—Insoluble proteins can interfere with downstream analyses and lead to inaccurate results.)

- v. Transfer the supernatant to a fresh, labeled 1.5 mL tube and save the cell pellet on ice to pool with the corresponding lysate in Step vii.
(Pause point— If supernatants are not being used immediately for further analysis in Step 33 Options B-E, they can be stored at -80°C until ready to use, although using fresh supernatants for analysis is preferable for ELISA assay and is absolutely required for the LDH assay.)
- vi. Add 1 mL ice cold PBS to each well, then aspirate the PBS to wash the cells.
- vii. Add 150 μL $1\times$ RIPA buffer and 50 μL $4\times$ SDS buffer.
- viii. Collect the cell lysate from each individual well by pipetting the liquid up and down while scraping the bottom of the well with the pipette tip. Place the cell lysate into the labeled 1.5 mL tubes that contain the corresponding cell pellets obtained in step iv.

Troubleshooting

- ix. Heat the tubes to 100°C for 12 minutes.
- x. Centrifuge the tubes at $14,500 \times g$ for 30 seconds at room temperature.
(Critical step—Insoluble proteins can interfere with downstream analyses and lead to inaccurate results.) The protein lysates are now ready for gasdermin D western blotting in Step 33 Option E.
(Pause point—protein lysates can be stored at -20°C or -80°C until ready to use.)
(CRITICAL STEP—when cell types other than BMDMs are used, protein concentrations in supernatants may be too low for downstream analyses. In these cases, the supernatants can be precipitated prior to use using trichloroacetic acid precipitation [see an example protocol from Link and LaBaer¹²²].)

Downstream assays to quantify inflammasome activation

- 34. Use the samples collected in the *in vivo* inflammasome activation assays (Step 1) and the plates prepared after *in vitro* inflammasome activation assays (Step 32 Options B-D) for downstream assays to quantify the inflammasome activation status. Follow Option A for ASC speck analysis, Option B for LDH assays, Option C for Caspase-1 and IL-18 sandwich ELISAs, Option D for IL-1 β multiplex ELISA, and Option E for Caspase-1 and gasdermin D western blot.

CRITICAL: In order to obtain a comprehensive overview of the inflammasome activation status, we recommend following all five Options.

Option A: ASC speck analysis—(Timing 4 or 18 h)

(CRITICAL—while the ASC speck analysis method included here is optimized for use following cell fixation in the *in vitro* inflammasome activation approach described in Step

32 Option A, it can be used on tissue samples obtained from the *in vivo* inflammasome activation methods listed in step 1.)

- i. (Optional) When using tissue samples collected in Step 1, tissue should be embedded in OCT and frozen at -80°C , then sectioned ($\sim 5\text{ }\mu\text{m}$ thick) using a cryostat and collected on a slide. Then fix the tissue section in 4% (wt/vol) paraformaldehyde solution at room temperature for 15 minutes, permeabilize in 0.1% [vol/vol] Triton X-100 in PBS at room temperature for 10 minutes, and block in 10% (vol/vol) normal goat serum with 0.1% (vol/vol) Tween-20 for 1 hour. For a detailed description of tissue sample preparation for microscopy, see the protocol by Kusumbe et al.¹²³.
- ii. Using the coverslips that have been blocked in 10% (vol/vol) normal goat serum with 0.1% (vol/vol) Tween-20 (from Step i for *in vivo* samples, from Step 32 Option B.vii for *in vitro* samples), aspirate the blocking solution.
- iii. Add 250 μL of the anti-ASC primary antibody diluted in blocking solution for 2 hours at room temperature or overnight (16 hours) at 4°C .
- iv. Remove the antibody and wash the coverslip 3 times in 500 μL PBS with 0.1% (vol/vol) Tween-20.
- v. Add 250 μL of the secondary antibody diluted in blocking solution for 1 hour at room temperature. Keep the plate protected from light from this point on to avoid degradation of fluorescence. The secondary antibody used should align with the fluorescence wavelengths that can be detected by your microscope. We typically use Alexa Fluor 568-conjugated anti-rabbit IgG or Alexa Fluor 488-conjugated anti-rabbit IgG.
- vi. Remove the antibody and wash the coverslips 3 times in 500 μL PBS with 0.1% (vol/vol) Tween-20.
- vii. Add 10 μL of the DAPI mounting medium to a glass slide.
- viii. Using forceps, carefully remove the coverslip from the well and dry the non-cell-containing side with a Kimwipe.
- ix. Invert the coverslip (so that the cell-containing side is down toward the slide) and place the coverslip on the drop of DAPI mounting medium on the glass slide.
- x. (Optional) Let the slides dry overnight at 4°C in the dark to allow the mounting medium to harden..
- xi. The cells with ASC specks can now be visualized using a fluorescence microscope. ASC speck quantification can be performed as previously described¹²⁴.

(PAUSE POINT) Slides can be stored at 4°C for up to 1 week or at 20°C for up to 2 months.

Troubleshooting

Option B: LDH assay—(Timing 1 h)

(CRITICAL—while the procedure in this section is optimized for use following supernatant collection in the *in vitro* inflammasome activation methods in Step 32 Option D.iv, it can also be performed with serum samples obtained from the *in vivo* inflammasome activation methods listed in step 1.)

(CRITICAL—This assay can be performed with a multichannel pipette to increase efficiency.)

- i. Take 50 µl of each freshly collected supernatant from the *in vitro* assays (Step 32 Option D.iv) or 50 µl of each of the serum samples collected from the *in vivo* assays in Step 1 and add them to a flat clear bottom 96-well plate.

(Critical step—Avoid freezing supernatants and serum samples before the LDH assay, as this will degrade the catalytic function of the protein that is necessary for the assay readout.)

CRITICAL STEP Each sample should be added to at least 2 wells to provide technical replicates within the same plate, and all 3 biological replicates should be included. Include a maximum LDH release positive control using supernatant from cells that have been treated with the CytoTox96 kit's lysis solution for 45 minutes. Serum-containing media can affect the LDH concentration in the supernatant. Include a BMDM culture media-only control to correct for this effect.

- ii. Add 50 µL of the CytoTox96 Reagent from the kit to each well.
- iii. Cover the plate to protect it from light and incubate for 30 minutes at room temperature.
- iv. Remove the cover from the plate and add 50 µL of the stop solution from the kit to each well.
- v. Check the plate for bubbles and pop any large bubbles with a needle.
- vi. Use a spectrophotometer to measure the absorbance of each well at 490 nm. This should be done within 1 hour of adding the stop solution.
- vii. Subtract the average value of the readings obtained from the BMDM culture media alone negative control from each of the absorbance readings to generate the corrected OD₄₉₀.
- viii. Calculate the percent cytotoxicity in each well using this formula:

$$\text{Percent cytotoxicity } 100 \times = \frac{\text{Experimental Sample Corrected OD}_{490}}{\text{Maximum LDH Release Control Corrected OD}_{490}}$$

Option C: Caspase-1 and IL-18 sandwich ELISAs—(Timing 5.5 h for caspase-1 ELISA; 5–5.5 h for IL-18 ELISA)

(CRITICAL—while the procedure in this section is optimized for use following supernatant collection in the *in vitro* inflammasome activation methods in Step 32 Option D.iv, it can also be performed with serum or tissue samples obtained from the *in vivo* inflammasome activation methods listed in step 1.)

(CRITICAL—In our method, we use a multiplex cytokine ELISA to measure IL-1 β levels, but this can also be done with a standard IL-1 β sandwich ELISA, such as the IL-1 β mouse ELISA kit from Invitrogen [cat. no. BMS6002], following the general steps in this protocol.)

(CRITICAL—This assay can be performed with a multichannel pipette to increase efficiency.)

i. (Optional) When analyzing tissues collected in Step 1, first create tissue homogenates by grinding the tissue by hand or using a power-driven tissue homogenizer. For a detailed description of tissue homogenate preparation, see the protocol by Simpson¹²⁵. ...

ii. Take 210 μ L of the tissue homogenate, serum (from Step 1), or supernatant collected from cells (from Step 32 Option D.iv). If samples are frozen, thaw them on ice and centrifuge the samples at $14,500 \times g$ for 30 seconds at 4 °C before use.

(Critical step—Avoid multiple freeze-thaw cycles, as this will degrade the proteins. Fresh samples are highly preferable.)

iii. (Optional) When using the IL-18 ELISA, first wash the microwell strips included with the IL-18 ELISA kit with 400 μ L of the wash buffer. Aspirate the liquid from the wells, and then wash again with 400 μ L of the wash buffer. Aspirate the liquid from the wells and tap gently on paper towel to remove residual liquid.

(Critical step—This step should be used for the IL-18 ELISA but is not necessary for the caspase-1 ELISA based on the manufacturer's recommended protocol.)

iv. Add 100 μ L of each of the standards to the appropriate well. Each standard should be added to at least 2 wells to provide technical replicates within the same ELISA.

CRITICAL STEP Ensure that each of the standards is used, including the 0 ng/mL negative control that will be essential for correcting for any background fluorescence in the assay.

v. (Optional) When using the IL-18 ELISA, add 50 μ L of sample diluent to each of the wells where you plan to add experimental samples.

(Critical step—This step should be used for the IL-18 ELISA but is not necessary for the caspase-1 ELISA based on the manufacturer's recommended protocol.)

vi. Add 100 μ L of each sample from Step ii (caspase-1 ELISA) or 50 μ L of each sample from Step ii and 50 μ L of the Biotin-Conjugate (IL-18 ELISA) to the

appropriate well. Each sample should be added to at least 2 wells to provide technical replicates within the same ELISA, and all 3 biological replicates for each treatment condition should be included.

CRITICAL STEP The use of biological and technical replicates is important to confirm that the results are accurate. Large variation between technical replicates provides an indication that there was an error in the assay.

- vii.** Cover the plate with the manufacturer supplied plastic film and incubate on a plate shaker at room temperature for 2 hours. When using the IL-18 ELISA, directly proceed to Step xii, when using the caspase-1 ELISA, first perform Steps viii-xi.
- viii.** (Optional) Remove the liquid from the wells.
- ix.** (Optional) Wash each well 3 times with 300–400 μ L of the wash buffer. For each wash, add the wash buffer to all wells, then swirl the plate by hand 5 times before inverting to dump out the liquid. Ensure that all liquid is removed from each well after the last wash by blotting the inverted plate on paper towel several times, until no more liquid is transferred to the paper towel.
- x.** (Optional) Add 100 μ L of the diluted detection antibody to each well.
- xi.** (Optional) Cover the plate with the manufacturer supplied plastic film and incubate on a plate shaker at room temperature for 1 hour.
- xii.** Remove the liquid from the wells.
- xiii.** Wash each well 3 times with 300–400 μ L of the wash buffer. Ensure that all liquid is removed from each well after the last wash.

(Critical step—Leaving residual liquid in the wells can lead to nonspecific antibody retention and inconclusive results.)
- xiv.** Add 100 μ L of the diluted STREP-HRP to each well.
- xv.** Cover the plate with the manufacturer supplied plastic film and incubate on a plate shaker at room temperature for 30 minutes (caspase-1 ELISA) or 1 hour (IL-18 ELISA).
- xvi.** Remove the liquid from the wells.
- xvii.** Wash each well 3 times with 300–400 μ L of the wash buffer. Ensure that all liquid is removed from each well after the last wash.

(Critical step—Leaving residual liquid in the wells can lead to nonspecific signal and inconclusive results.)
- xviii.** Add 100 μ L of the TMB substrate solution to each well.
- xix.** Incubate the plate in the dark at room temperature for 15 minutes (caspase-1 ELISA) or 10 minutes (IL-18 ELISA).

(Critical step—The incubation time with TMB substrate may vary depending on the concentrations of protein present, so this reaction should be visually checked

to avoid over-development, which can lead to precipitation of the substrate and inaccurate results.)

xx. Add 50 μL (caspase-1 ELISA) or 100 μL (IL-18 ELISA) of the stop solution to each well and gently tap the plate to ensure mixing.

xxi. Use a spectrophotometer to measure the absorbance of each well at 450 nm.

Troubleshooting

xxii. Calculate the average reading for each of the standards from the duplicate readings and subtract the average value obtained from the 0 ng/mL samples from each.

xxiii. Generate a standard curve by plotting absorbance (OD_{450}) on the x-axis and concentration on the y-axis.

Troubleshooting

xxiv. Calculate the caspase-1 or IL-18 concentrations of the experimental samples using the equation that fits the standard curve generated.

Option D: IL-1 β multiplex ELISA—(Timing 6 or 20 h)

(CRITICAL—while the procedure in this section is optimized for use following supernatant collection in the *in vitro* inflammasome activation methods in Step 32 Option D.iv, it can also be performed with serum or tissue samples obtained from the *in vivo* inflammasome activation methods listed in step 1.)

(CRITICAL—In our method, we use a multiplex cytokine ELISA to measure IL-1 β levels, but this can also be done with a standard IL-1 β sandwich ELISA. The multiplex will allow the simultaneous quantification of several other cytokines, which may be beneficial when used with serum or tissue samples in conjunction with the *in vivo* methods from Step 1.)

(CRITICAL—This assay can be performed with a multichannel pipette to increase efficiency.)

- i.** (Optional) When analyzing tissues collected in Step 1, first create tissue homogenates by grinding the tissue by hand or using a power-driven tissue homogenizer. For a detailed description of tissue homogenate preparation, see the protocol by Simpson¹²⁵
- ii.** Take 55 μL of the tissue homogenate, serum (from Step 1), or supernatant collected from cells (from Step 32 Option D.iv). If samples are frozen, thaw them on ice and centrifuge the samples at $14,500 \times g$ for 30 seconds at 4 °C before use.

(Critical step—Avoid multiple freeze-thaw cycles, as this will degrade the proteins. Fresh samples are highly preferable.)

- iii.** Add 200 μL of wash buffer to each well of the plate.

- iv.** Cover the plate with the manufacturer supplied plastic film and incubate at room temperature for 10 minutes.
- v.** Remove the liquid from the wells and ensure there is no residual liquid by tapping vigorously upside down on paper towels.
- vi.** Add 25 μ L of each of the standards to the appropriate well. Each standard should be added to at least 2 wells to provide technical replicates within the same plate.
- vii.** Add 25 μ L of BMDM culture media to each of the standards' wells.
- viii.** Add 25 μ L of assay buffer to each of the wells where you plan to add experimental samples.
- ix.** Add 25 μ L of each sample from Step ii to the appropriate well. Each sample should be added to at least 2 wells to provide technical replicates within the same plate, and all 3 biological replicates should be included.
- x.** Sonicate the pre-mixed bead solution for 30 seconds. Then vortex for 1 minute.
- xi.** Add 25 μ L of the pre-mixed bead solution to each well. Be sure to shake the bottle of bead solution intermittently during this process to prevent the beads from settling.
- xii.** Cover the plate with the manufacturer supplied plastic film and cover with aluminum foil. Incubate on a plate shaker at room temperature for 2 hours or overnight (16 hours) at 4 °C (overnight incubation allows for optimal assay results).
- xiii.** Place the plate in the magnetic separation block and incubate for 1 minute to allow beads to settle.

(Critical step—The beads must be allowed to be pulled toward the magnet to avoid loss of beads during all wash step.)
- xiv.** While keeping the plate in the magnetic separation block, remove the liquid from the wells by pouring it out and then gently tapping the plate on paper towels to remove any residual liquid.
- xv.** Remove the plate from the magnetic separation block and add 200 μ L of the wash buffer.
- xvi.** Incubate the plate on a plate shaker for 30 seconds.
- xvii.** Place the plate in the magnetic separation block and incubate for 1 minute to allow beads to settle.
- xviii.** While keeping the plate in the magnetic separation block, remove the liquid from the wells by pouring it out and then gently tapping the plate on paper towels to remove any residual liquid.
- xix.** Repeat steps xiii-xvi to wash again.
- xx.** Remove the plate from the magnetic separation block and add 25 μ L of detection antibody to each well.

- xxi.** Cover the plate with the manufacturer supplied plastic film and cover with aluminum foil. Incubate on a plate shaker at room temperature for 1 hour.
  - xxii.** Add 25 μ L of streptavidin-phycoerythrin to each well.
  - xxiii.** Cover the plate with the manufacturer supplied plastic film and cover with aluminum foil. Incubate on a plate shaker at room temperature for 30 minutes.
  - xxiv.** Place the plate in the magnetic separation block and incubate for 1 minute to allow beads to settle.
  - xxv.** While keeping the plate in the magnetic separation block, remove the liquid from the wells by pouring it out and then gently tapping the plate on paper towels to remove any residual liquid.
  - xxvi.** Repeat steps xiii-xvi to wash the plate twice.
  - xxvii.** Remove the plate from the magnetic separation block and add 150 μ L of sheath fluid to each well.
  - xxviii.** Incubate the plate on a plate shaker for 5 minutes.
  - xxix.** Place the plate in a Luminex 200 to read the plate using xPONENT software.
- Troubleshooting
- xxx.** Analyze the median fluorescence intensity of the IL-1 β standard with a 5-parameter logistic or spline curve-fitting method to create a standard curve.
- Troubleshooting
- xxxi.** Calculate the IL-1 β concentrations of the experimental samples using the equation that fits the standard curve generated.

Option E: Caspase-1 and gasdermin D western blot—(Timing 8 or 22 h for caspase-1 and 7.5 or 21.5 h for gasdermin D)

(CRITICAL—while the procedure in this section is optimized for use with combined supernatant and protein lysates (Step 32 Option C,v for caspase-1) and cell lysates (Step 32 Option D.ix, for gasdermin D) in the *in vitro* inflammasome activation methods, it can also be performed with tissue samples obtained from the *in vivo* inflammasome activation methods listed in step 1.)

(CRITICAL—Western blotting for other proteins, such as IL-1 β can also be performed using the combined supernatant and protein lysate sample collected in Step 32 Option C,v (recommended for IL-1 β) or the cell lysate collected in Step 32 Option D.ix.)

- i.** (Optional) When analyzing tissues collected in Step 1, first create tissue homogenates by grinding the tissue by hand or using a power-driven tissue homogenizer. For a detailed description of tissue homogenate preparation, see the protocol by Simpson¹²⁵
- ii.** Take 40 μ L of the tissue homogenate from Step i or the combined supernatant and protein lysate samples from Step 32 Option C,v (caspase-1 blot) or 20 μ L tissue

homogenate from Step i or cell lysates from Step 32 Option D.ix (gasdermin D blot). If the samples are frozen, thaw them on ice and centrifuge the samples at $14,500 \times g$ for 30 seconds at 4 °C before use.

(Critical step—The combined supernatant and protein lysate from Step 32 Option C must be used for caspase-1 western blotting to ensure adequate protein concentrations for detection. The combined lysate can also be used for gasdermin D western blotting, but the pro-form of the protein is sometimes weak; therefore, we recommend using the cell lysate from Step 32 Option D.ix for gasdermin D western blotting.)

- iii. Place a 12% (wt/vol) polyacrylamide gel with 10 wells (caspase-1 blot) or a 10% (wt/vol) polyacrylamide gel with 15 wells (gasdermin D blot) in the electrophoresis apparatus.

(Critical step—While both caspase-1 and gasdermin D pro- and cleaved forms can be resolved in some cases on a 12% (wt/vol) gel, in our experience we have found it is best to use different polyacrylamide percentages to ensure proper protein separation within the gel to provide optimal resolution. Additionally, because the combined supernatant and protein lysate [used for caspase-1 blotting] is more dilute than pure protein lysate [used for gasdermin D blotting], more sample should be loaded to ensure the signal is strong; this requires fewer wells to be used per gel.)

- iv. Fill the apparatus with $1\times$ running buffer and remove the comb from the gel.
- v. Slowly load 40 μ L of combined supernatant and protein lysate samples (caspase-1 blot) or 20 μ L of protein lysate (gasdermin D blot) into each lane, being careful not to let any sample spill over into neighboring lanes.

(Critical step—Because the combined supernatant and protein lysate is more dilute than pure protein lysate, more sample should be loaded to ensure the signal is strong.)

- vi. Attach the electrophoresis apparatus to the power source and run the gel at 80 V for 20 minutes and then at 100 V for 45–60 minutes, or until the dye reaches the bottom of the gel but is not eluted out.
- vii. Use the gel releaser to carefully remove the gel from the electrophoresis apparatus.
- viii. Prepare the transfer stack with a PVDF membrane. The PVDF membrane needs to be activated by soaking it in methanol for 1 minute prior to transfer stack assembly. All stack components should be pre-wet in transfer buffer for 5 minutes. The transfer stack should be assembled on the Trans-Blot semi-dry system and include (from bottom platinum anode side to top stainless-steel cathode side): filter paper, PVDF membrane, gel, filter paper. Roll out air bubbles between layers.

(CRITICAL STEP —Nitrocellulose membranes can be used in place of PVDF.)

- ix.** Close the top and secure the safety cover.
- x.** Attach the transfer apparatus to the power source and run the transfer at 25 V for 40 minutes.
- xi.** Remove the membrane and place it in an incubation tray.
- xii.** Add 15 mL of a 5% (wt/vol) skim milk solution and block the membrane on a shaker at room temperature for 1 hour.
- (Pause point—The membrane in blocking solution can be stored at 4 °C overnight.)
- xiii.** Remove the blocking solution and add 10 mL of the diluted antibody solution (anti-caspase-1 antibody or anti-gasdermin D antibody). Incubate on a shaker at room temperature for 2 hours or overnight (16 hours) at 4 °C.
- xiv.** Remove the antibody solution and wash the membrane with 15 mL TBST on a shaker at room temperature for 5 minutes. Discard the TBST.
- xv.** Repeat the wash step a total of 3 times.
- xvi.** Add 10 mL of the diluted secondary HRP-conjugated antibody solution. Incubate on a shaker at room temperature for 1 hour.
- xvii.** Remove the antibody solution and wash the membrane with 15 mL TBST on a shaker at room temperature for 10 minutes. Discard the TBST.
- xviii.** Repeat the wash step a total of 3 times.
- xix.** Add 10 mL of the HRP substrate to the membrane and incubate for 1 minute.
- xx.** Remove the membrane from the substrate and image. This can be done with film or with a chemiluminescence imager.
- Troubleshooting
- xxi.** Add 10 mL of the stripping solution. Incubate on a shaker at room temperature for 5 minutes. Discard the stripping solution.
- xxii.** Wash the membrane with 15 mL PBST on a shaker at room temperature for 10 minutes. Discard the PBST.
- xxiii.** Repeat the wash step a total of 3 times.
- xxiv.** Add 15 mL of a 5% (wt/vol) skim milk solution and block the membrane on a shaker at room temperature for 1 hour.
- (Pause point—The membrane in blocking solution can be stored at 4 °C overnight.)
- xxv.** Add 10 mL of the diluted anti- β -actin antibody solution. Incubate on a shaker at room temperature for 2 hours or overnight (16 hours) at 4 °C.

CRITICAL STEP Including a loading control such as β -actin ensures that equal protein was loaded in each lane to verify that comparisons can be made between samples run on the same blot.

- xxvi.** Remove the antibody solution and wash the membrane with 15 mL TBST on a shaker at room temperature for 5 minutes. Discard the TBST.
- xxvii.** Repeat the wash step a total of 3 times.
- xxviii.** Add 10 mL of the diluted secondary HRP-conjugated antibody solution. Incubate on a shaker at room temperature for 1 hour.
- xxix.** Remove the antibody solution and wash the membrane with 15 mL TBST on a shaker at room temperature for 10 minutes. Discard the TBST.
- xxx.** Repeat the wash step a total of 3 times.
- xxxi.** Add 10 mL of the HRP substrate to the membrane and incubate for 1 minute.
- xxxii.** Remove the membrane from the substrate and image. This can be done with film or with a chemiluminescence imager.

Troubleshooting

Timing

Step 1, *in vivo* inflammasome activation: 6.5 hours–12 months, depending on the model selected

Steps 2–18, bone marrow progenitor cell isolation: 4 hours

Steps 19–21, BMDM differentiation: 6 days

Steps 22–28, seeding BMDMs: 1 hour

Step 29–30, preparing BMDM for stimuli: 20 hours

Step 31, *in vitro* inflammasome activation: 2.5–24.5 hours, depending on the model selected

Steps 32 Option A, real-time cell death analysis: 2.5–24.5 hours, depending on the model selected

Step 32 Option B, fixing cells for microscopy: 1.75 hours

Step 32 Option C, combined supernatant and protein lysate collection: 0.5 hours

Step 32 Option D, separate supernatant and protein lysate collection: 3–25 hours, depending on the model selected

Step 33 Option A, ASC speck staining: 4 or 18 hours

Step 33 Option B, LDH assay: 1 hour

Step 33 Option C, sandwich ELISA: 5.5 hours

Step 33 Option D, IL-1 β multiplex ELISA: 6 or 20 hours

Step 33 Option E, western blot: 8 or 22 hours

Troubleshooting

Troubleshooting guidance is provided in Table 2.

Anticipated results

The *in vivo* inflammasome activation procedures detailed in Step 1 can be used to induce inflammasome activation in various cell types beyond the BMDMs described in Steps 2–30. Following *in vivo* activation, any cell type or tissue of interest can be harvested and subjected to the inflammasome readouts detailed in Step 33. When using BMDMs for *in vitro* inflammasome activation (Steps 2–31), it is anticipated that each mouse will yield 60–100 $\times 10^6$ cells. As each 12-well plate layout to be investigated will require 4 plates and therefore 48 $\times 10^6$ cells, it is important to keep this in mind when selecting the number of mice needed and stimulations planned for a given *in vitro* experiment. It is also important to note that multiple genotypes of mice may be needed in a single experiment to provide the appropriate controls, and BMDMs should be collected from age- and sex-matched mice in parallel for accurate experimental design.

Real-time cell death analysis using the IncuCyte system detailed in Step 32 Option A will provide images of the cells and graphs quantifying cell death over time (Fig. 2). The images allow visualization of the cellular morphology and morphological changes occurring as inflammasome activation takes place and cell death begins. The IncuCyte platform also allows the visualization of other forms of cell death, such as apoptosis and necroptosis, that can be induced downstream of some inflammasome triggers. The extensive crosstalk between pyroptosis, apoptosis and necroptosis has led to the concept of PANoptosis to describe them in a unified mechanism⁸³. Further analyses to follow DNA fragmentation, membrane damage^{49,81}, apoptotic caspase cleavage (western blotting for caspase-8, caspase-3 and caspase-7) and MLKL phosphorylation (a marker of necroptosis) can allow for the assessment of these pathways^{49,53,54,59,61,62,81,82,84}. From the IncuCyte data, the quantification graphs can be displayed as the number of cells undergoing death or as the percentage. We find that using the percentage is generally more informative. Different inflammasome triggers will generate different cell death kinetics. Using the additional plates of BMDMs, supernatants and protein lysates are collected in parallel to allow analysis of the different components of inflammasome activation mirroring the timeline collected using the IncuCyte system.

When inflammasome sensors detect their trigger and begin to nucleate the inflammasome complex, many recruit the adapter protein ASC via PYD-PYD interactions^{1,7}. ASC can act as a bridge between the inflammasome sensor and the effector molecule caspase-1. This process results in the formation of an ASC speck within the cell. Using our protocol, this speck can be visualized by microscopy in activated cells (Step 33 Option A) (Fig. 3). When visualizing ASC, other proteins can be stained as well to determine whether colocalization is occurring or to visualize where within the cell the speck is being formed.

As part of ASC speck formation, caspase-1 molecules are recruited to the complex. This allows dimerization of caspase-1 to facilitate self-processing, cleaving full-length caspase-1 into its active form^{39–41}. This cleavage can be visualized using western blotting as described in Step 33 Option E (Fig. 4). The pro-form of caspase-1 is approximately 45 kDa, while the cleaved form is composed of 20 kDa and 10 kDa subunits. The antibody used in this protocol can detect both the pro- and cleaved forms of caspase-1, so good separation of the distinctly sized forms is imperative during polyacrylamide gel electrophoresis. The conditions described here provide for this separation.

Once caspase-1 has undergone self-processing to form its active form, it can cleave its substrates, including gasdermin D^{42,43}. This cleavage can also be visualized using western blotting as described in Step 33 Option E (Fig. 5). Full-length gasdermin D is approximately 53 kDa, and the cleaved N-terminal portion is 30 kDa. Again, the antibody in this protocol detects both the pro- and cleaved forms of gasdermin D, further emphasizing the importance of having good separation in this molecular weight region during electrophoresis.

The cleaved N-terminal domain of gasdermin D goes on to form pores in the cell membrane, executing pyroptotic cell death^{42,43}. The pores in the membrane allow the release of several molecules from the cell. The proinflammatory cytokines IL-1 β and IL-18, which are cleaved into their active forms by caspase-1 and do not contain specific secretion sequences^{2,44,45}, are released and can be detected using ELISA assays in the supernatant after *in vitro* inflammasome stimulation and in serum after *in vivo* stimulation, as described in Step 33 Options C and D (Fig. 6a,b). Additionally, LDH, a soluble cytoplasmic enzyme that is known to be nearly ubiquitous in mouse cells, can be released from cells with damaged plasma membranes and indicate that cell death is occurring¹²⁶. The release LDH can be detected as described in Step 33 Option B (Fig. 7).

When analyzing data over a time course, it is expected that the kinetics of inflammasome formation (ASC speck formation), caspase-1 cleavage, gasdermin D cleavage, molecule release into the supernatant, and cell death will follow each other, as each of these readouts reflects a distinct step in the same pathway. This allows for multiple lines of evidence to support the activation of the inflammasome. Therefore, our protocol provides a robust methodology for analyzing several points in the pathway, ensuring high quality and reproducible results.

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**Up to three primary research articles where the protocol has been used
and/or developed:**

1. Zheng et al. Cell 2020 (PMID: 32298652) <https://www.sciencedirect.com/science/article/pii/S0092867420303330?via%3Dihub>
2. Samir et al. Nature 2019 (PMID: 31511697) <https://www.nature.com/articles/s41586-019-1551-2>
3. Man et al. Cell 2016 (PMID: 27693356) <https://www.sciencedirect.com/science/article/pii/S0092867416312454?via%3Dihub>

Fig. 1:
Schematic of protocol workflow.

a, *In vivo* stimulation. **b**, Preparation of bone marrow-derived macrophages (BMDMs). Once bone marrow cells are isolated, they are differentiated in culture for 6 days. On day 6, they are counted and seeded into plates for the *in vitro* inflammasome stimulations. For each set of experiments planned, four 12-well plates will be needed (1 to fix for microscopy, 1 for real-time cell death monitoring, 1 for combined supernatant and protein lysate collection, and 1 for separate supernatant and protein lysate collection). **c**, *In vitro* inflammasome activation and cell death assays. Cell images can be obtained, and supernatants can be collected at various timepoints throughout this stimulation as needed.

Fig. 2:

Real-time cell death analysis in BMDMs following inflammasome activation.

a, Real-time analysis of cell death in bone marrow-derived macrophages (BMDMs) isolated from wild type (WT) and *Nlrp3*^{-/-} mice with and without stimulation with LPS + ATP (as in Step 31 Option A), an NLRP3 inflammasome trigger, for the indicated amount of time using the IncuCyte imaging system and SYTOX Green nucleic acid staining (as described in Step 32 Option A). The red outline denotes cells counted as dead in the analysis. Scale bar, 50 μ m. **b**, Quantification of the percent of cells undergoing death based on the data obtained in **a**. Data are displayed as mean $\pm$ SEM. *P* values were determined using two-way ANOVA.

Fig. 3:

ASC speck formation in BMDMs following inflammasome activation.

a,b, Immunofluorescence staining of ASC specks (as described in Step 33 Option A) in bone marrow-derived macrophages (BMDMs) isolated from wild type (WT) and *Nlrp3*^{-/-} mice treated with vehicle (**a**) or LPS + ATP (as in Step 31 Option A), an NLRP3 inflammasome trigger, (**b**) for 4 hours (ATP was added for the last 30 minutes of the 4 hour incubation). Wheat germ agglutinin (WGA) is used as a counterstain. Yellow arrows indicate ASC specks. Scale bar, 5 μ m.

Fig. 4:

Caspase-1 cleavage in BMDMs following inflammasome activation.

Immunoblot analysis of pro- (p45) and cleaved caspase-1 (p20) (as described in Step 33 Option E) in bone marrow-derived macrophages (BMDMs) isolated from wild type (WT) and *Nlrp3*^{-/-} mice with and without stimulation with LPS + ATP (as in Step 31 Option A), an NLRP3 inflammasome trigger, for the indicated amount of time (ATP was added for the last 30 minutes of the stimulation). Cells treated with vehicle did not receive LPS or ATP. Actin is shown as a control.

Fig. 5:

Gasdermin D cleavage in BMDMs following inflammasome activation.

Immunoblot analysis of pro- (p53) and cleaved gasdermin D (p30) (as described in Step 33 Option E) in bone marrow-derived macrophages (BMDMs) isolated from wild type (WT) and *Nlrp3*^{-/-} mice with and without stimulation with LPS + ATP (as in Step 31 Option A), an NLRP3 inflammasome trigger, for the indicated amount of time (ATP was added for the last 30 minutes of the stimulation). Cells treated with vehicle did not receive LPS or ATP. Actin is shown as a control.

Fig. 6:

IL-1 β and IL-18 ELISA results from the supernatant of BMDMs following inflammasome activation.

a,b, ELISA assessment of IL-1 β (as described in Step 33 Option D) (**a**) and IL-18 (as described in Step 33 Option C) (**b**) release from bone marrow-derived macrophages (BMDMs) isolated from wild type (WT) and *Nlrp3*^{-/-} mice with and without stimulation with LPS + ATP (as in Step 31 Option A), an NLRP3 inflammasome trigger, for 4 hours (ATP was added for the last 30 minutes of the 4 hour incubation). Cells treated with vehicle did not receive LPS or ATP. $n = 4$ in each treatment group. Data are displayed as mean $\pm$ SEM. P values were determined using the Student's t -test.

Fig. 7:

LDH release results from the supernatant of BMDMs following inflammasome activation. Quantification of the percent of cells that have undergone death based on the release of lactate dehydrogenase (LDH) (as described in Step 33 Option B) into the supernatants of bone marrow-derived macrophages (BMDMs) isolated from wild type (WT) and *Nlrp3*^{-/-} mice with and without stimulation with LPS + ATP (as in Step 31 Option A), an NLRP3 inflammasome trigger, for 4 hours (ATP was added for the last 30 minutes of the 4 hour incubation). Cells treated with vehicle did not receive LPS or ATP. $n = 4$ in each treatment group. Data are displayed as mean $\pm$ SEM. P values were determined using the Student's t -test.

Table 1.

Overview of the different inflammasome stimulants used in Step 1 (*in vivo*) and Step 31 (*in vitro*).

<i>In vivo</i> inflammasome activation (Step 1)			
Option	Stimulant	Description	Reference
A	<i>Citrobacter rodentium</i>	Bacterial infection given orally.	108
B	<i>Pseudomonas aeruginosa</i>	Bacterial infection given intratracheally.	109
C	<i>Salmonella enterica</i> serovar Typhimurium	Bacterial infection given orally.	35
D	<i>Burkholderia thailandensis</i>	Bacterial infection given intranasally.	35,50
E	<i>Francisella novicida</i>	Bacterial infection given subcutaneously.	46
F	Influenza A virus	Viral infection given intranasally.	53
G	<i>Aspergillus fumigatus</i>	Fungal infection given intranasally. This infection can be performed in an immunocompromised (cyclophosphamide monohydrate and cortisone 21-acetate pre-treated) or immunocompetent mouse. This will determine the infectious dose needed.	56,57,110
H	<i>Candida albicans</i>	Fungal infection given intravenously.	58
I	LPS	A dose of 25 mg/kg LPS can be delivered intraperitoneally in immunocompetent mice to induce a systemic LPS shock syndrome.	59
J	LPS	A dose of 0.5 µg/kg LPS can be delivered intraperitoneally in mice that have TAK1 inhibited to induce a sepsis-like phenotype.	59
K	No external stimulant; <i>Sharpin</i> ^{F^{pdm}} mutation	<i>Sharpin</i> ^{F^{pdm}} mice can be used to study chronic proliferative dermatitis and will spontaneously develop an inflammatory disease characterized by severe dermatitis and inflammation by 10 weeks of age.	60
L	No external stimulant; <i>Pstpip2</i> ^{xmo} mutation	<i>Pstpip2</i> ^{xmo} mice can be used to study chronic recurrent multifocal osteomyelitis and will spontaneously develop a chronic bone disease featuring bone deformity and inflammation by 60–120 days of age.	61
M	Collagen	Collagen emulsion given subcutaneously. This will model the development of arthritis.	63
N	High-fat diet	Mice fed ad libitum on a high-fat diet serve as a model for obesity.	64
O	High-sugar diet	Mice fed ad libitum on a high-sugar diet serve as a model for diabetes and glucose intolerance.	64
P	Western-type diet	Mice fed ad libitum on a Western-type diet serve as a model for atherosclerosis.	111
Q	No external stimulant; Tauopathy-inducing mutations	TPS mice that overexpress human Tau (1N4R) with the P301S mutation or <i>App</i> ^{NL-F} and <i>App</i> ^{NL-G-F} mice can be used to study Tau-related neurodegenerative diseases.	102112113
R	AOM/DSS	AOM delivered intraperitoneally followed by DSS treatment orally induces colorectal tumors.	68
<i>In vitro</i> inflammasome activation (Step 31)			
Option	Stimulant	Description	Reference
A	LPS + ATP	Ligand stimulation to activate the NLRP3 inflammasome.	35
B	LPS + nigericin	Ligand stimulation to activate the NLRP3 inflammasome.	114
C	LPS transfection	Ligand stimulation to activate the non-canonical NLRP3 inflammasome.	35
D	Rod, needle, or flagellin protein	Ligand stimulation to activate the NAIP-dependent NLRC4 inflammasome. Rod, needle, and flagellin proteins will stimulation NAIP1-dependent, NAIP2-dependent, and NAIP5/6-dependent activation, respectively.	35
E	<i>Clostridium difficile</i> toxin	Ligand stimulation to activate the Pyrin inflammasome.	115

F	<i>Bacillus anthracis</i> protective antigen and lethal factor	Ligand stimulation to activate the NLRP1b inflammasome.	116,117
G	Poly(dA:dT) transfection	Ligand stimulation to activate the AIM2 inflammasome.	35
H	Gram negative bacteria (<i>Escherichia coli</i> , <i>Citrobacter rodentium</i> , <i>Pseudomonas aeruginosa</i> popB, or <i>Burkholderia thailandensis</i>)	Bacterial infection to activate the noncanonical NLRP3 inflammasome activation. <i>Burkholderia thailandensis</i> can be used for NLRP3 or NLRC4 inflammasome activation. Use later timepoints to observe NLRP3 inflammasome activation.	35,118
I	Gram negative bacteria (<i>Burkholderia thailandensis</i> , <i>Salmonella enterica</i> serovar Typhimurium, or <i>Pseudomonas aeruginosa</i>)	Bacterial infection to activate the NAIP-NLRC4 inflammasome. <i>Burkholderia thailandensis</i> can be used for NLRP3 or NLRC4 inflammasome activation. Use early timepoints to observe NLRC4 inflammasome activation.	35
J	<i>Francisella novicida</i>	Bacterial infection to activate the AIM2 inflammasome.	35
K	Influenza A virus	Viral infection to activate the ZBP1-NLRP3 inflammasome.	54
L	Murine cytomegalovirus	Viral infection to activate the AIM2 inflammasome	54
M	<i>Aspergillus fumigatus</i>	Fungal infection to activate the NLRP3 and AIM2 inflammasomes.	56,57
N	<i>Candida albicans</i>	Fungal infection to activate the NLRP3 inflammasome.	58,119

Table 2.**Troubleshooting Table**

Step	Problem	Possible reason	Solution
9	Tissue stuck to bone	Tissue does not separate easily or bones break	Sever the tendons connecting the calf to the bone before rubbing with a paper towel
13	Bone does not turn white	Poor removal of marrow cells from bone	Try reinserting the needle at the top of the marrow again and using shorter, more high-pressure bursts of liquid to flush out the cells
19–21	Altered macrophage morphology	Contamination	Ensure that BMDM culture media contains antibiotics and that all work is performed in a laminar flow hood using sterile technique
		Loss of essential genes affected macrophage development or homeostasis	Use bone marrow from cell-specific conditional knockout mice generated using Cre/LoxP technology with an inducible Cre or transduce the fully differentiated floxed macrophages with a lentiviral Cre to delete the gene in differentiated macrophages
25	Clumping cells	Poor resuspension	Thoroughly resuspend the cells in 1 mL media first using a P1000 pipet, then add the additional 19 mL media and mix
	Low cell yield	Poor removal of marrow cells from tibia and femur in step 13	Use higher pressure bursts of liquid to dispel cells from the marrow
		Cells stuck to the sides of the tube in steps 15–18	Ensure the femur is cut above the hip joint and not in the femur itself, as this will result in a loss of marrow and cells Be sure to rinse down the sides of the tube as you disperse the marrow in step 15 and rinse down the sides of the tube again as you add more culture media in step 16
32 Option A	Object masks do not accurately align with SYTOX Green ⁺ cells	Software settings inaccurate	Using an image obtained that contains SYTOX Green ⁺ cells, manually adjust the minimum intensity of fluorescence signals, size of the object, average intensity of an object and/or ellipticity of the object to overlay the mask; we then recommend testing the masks on multiple images from the experiment to avoid overfitting
32 Option D	Low volume of lysate collected	Excess SDS bubbles created	Pipet the liquid slowly to ensure bubbles are not formed; scrape the bottom of the well more thoroughly and pipet gently
33 Option A	Dim fluorescence signal for ASC	Mismatch between primary and secondary antibody	The secondary antibody needs to be an anti-rabbit IgG for the AdipoGen anti-ASC primary antibody in this protocol, but if a different primary antibody is used, be sure the secondary matches the species; also, always include a positive control of wild type cells stimulated with a known activator of the inflammasome of interest (e.g., LPS + ATP for the NLRP3 inflammasome)
	Nonspecific fluorescence signal	Inadequate washing	Keep the coverslip in the PBS wash solution for 5 minutes for each wash and/or increase the number of washes to reduce background
	Few ASC specks in positive control	Poor inflammasome activation	Check cells at a later timepoint to see if more ASC specks are formed
		Cell death has already occurred	Check cells at an earlier timepoint
33 Option C,xxi and D,xxvii	No/low signal	Freeze-thawed supernatant used	Whenever possible, avoid freeze-thaw cycles for supernatants to be used in ELISA
	High signal in all wells	Inadequate washing	Let the wash buffer remain in the wells for 30 seconds during each wash and/or increase the number of washes to reduce background
33 Option C,xxiii and D,xxviii	Poor standard curve	Incorrect standard curve dilutions	Pipette very carefully and double check that the correct volumes are used. Mix each sample by vortexing before taking the volume to create the next dilution
33 Option D,xxvii	No/low signal	Beads lost	Be sure the plate is always on the magnet when inverting to remove liquid during wash steps. Let the plate rest on the magnet for 1 minute before inverting
33 Option E,xx	Incorrect p20 band in the lane directly adjacent to the ladder in caspase-1 blot	Reaction between caspase-1 antibody and ladder	Some ladders react with the caspase-1 antibody at the p20 size; leaving an empty lane between the ladder and your samples can mitigate this reaction

Step	Problem	Possible reason	Solution
	Poor separation between pro- and cleaved form	Insufficient gel run time	Increase the amount of time that the gel is run at 125 V
	Low signal	Low protein amount loaded	Fill the wells in the gel slowly to ensure protein does not spill out from the lane
		Poor transfer	When preparing the transfer stack, ensure that the gel is directly on top of the membrane, with no air bubbles. Check that the transfer apparatus is set to the appropriate voltage and running appropriately
		Insufficient antibody incubation	Incubate the primary antibody overnight
	High background	Inadequate washing	Ensure that Tween-20 has been added to the TBST. Incubate the membrane in the washing solution for 5 minutes during each wash step and/or add additional wash steps
	Poor caspase-1 or gasdermin D cleavage when robust cell death is observed	Cell death is occurring too rapidly	Reduce the MOI for bacterial stimulations, amount of ligand for ligand-based stimulations, and/or amount of time used for stimulation
	Poor caspase-1 or gasdermin D cleavage when poor cell death is observed	Insufficient inflammasome activation	Increase the MOI for bacterial stimulations, amount of ligand for ligand-based stimulations, and/or amount of time used for stimulation
33	Mock-transfected cells are dying	Liposome-induced cell death	Ensure the transfection mix is added to the walls of the well and not in the center of the well
	Poor inflammasome activation and pyroptosis	Toxin-based triggers: insufficient priming	Add LPS priming (see the LPS portion of LpS + ATP activation steps) before the addition of another trigger
		Bacterial triggers: loss of bacteria	Gentamicin can be added directly to the bacteria-containing media instead of washing the cells