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Pyroptosis Induction and Detection

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Abstract

Pyroptosis is a form of programmed pro-inflammatory cell death that plays a protective role in the host response to infection, but can also promote pathogenic inflammation. Pyroptosis is mediated by the cysteine protease, caspase-1. Caspase-1 cleaves gasdermin D, releasing the N-terminal pore-forming domain, which inserts into the plasma membrane and drives osmotic lysis. Caspase-1 also proteolytically activates the inflammatory cytokines interleukin 1 β (IL-1 β) and IL-18. This unit describes methods for stimulating pyroptosis and assessing subsequent loss of plasma membrane integrity. We also describe an ELISA to quantify released IL-1 β . These methods can be applied to many different types of experiments.

Keywords

pyroptosis; caspase-1; inflammasome; cell death

INTRODUCTION

Pyroptosis is a form of programmed pro-inflammatory cell death that plays a protective role in the host response to infection (Jorgensen & Miao, 2015), but can also drive pathogenic inflammation (Bergsbaken, Fink, & Cookson, 2009). Pyroptosis is defined by the requirement for proteases in the caspase-1 family, which cleave specific cellular substrates to produce the features associated with this form of cell death. Inflammasomes are innate immune signaling platforms that respond to microbial or damage-associated stimuli and activate caspase-1 (Martinon, Mayor, & Tschopp, 2009). Pattern recognition receptors in the NOD-like receptor (NLR) and AIM2-like receptor (ALR) family oligomerize upon detection of these stimuli, and recruit adapter proteins and pro-caspase-1. Caspase-1 becomes active through proximity-induced auto-activation. Activated caspase-1 converts the pro-forms of interleukin 1 β (IL-1 β) and IL-18 to mature inflammatory cytokines. Caspase-1 also cleaves gasdermin D, releasing the N-terminal pore-forming domain, which inserts into the plasma membrane and causes colloid-osmotic lysis (Liu & Lieberman, 2017). Lysis releases cytoplasmic contents including the enzyme lactate dehydrogenase (LDH).

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The protocols described in this unit can be used to activate caspase-1 and stimulate pyroptosis in mouse macrophages via the NLRP3 inflammasome. We also present alternative protocols to simulate pyroptosis via the NLRP1b, AIM2 and NLRC4 inflammasomes. We describe a method to measure cell lysis based on a colorimetric assay for enzymatic activity of released lactate dehydrogenase (LDH). We also present methods to assess loss of membrane integrity on a single cell basis using fluorescence microscopy. Finally, we describe a protocol to measure IL-1 β by ELISA. These techniques can be applied to investigate other stimuli that induce pyroptosis, treatments that alter susceptibility to pyroptosis, and a number of other applications.

BASIC PROTOCOL 1. PYROPTOSIS INDUCTION VIA THE NLRP3 INFLAMMASOME AND QUANTIFICATION OF CYTOPLASMIC LACTATE DEHYDROGENASE (LDH) RELEASE

This protocol describes a method to activate caspase-1 via the NLRP3 inflammasome by treating murine bone marrow macrophages (BMM) with nigericin. Subsequent pyroptotic cell lysis is assessed by measuring release of the cytoplasmic enzyme LDH. This technique can be used with many different caspase-1 activating stimuli and provides a rapid (30 min incubation), population-based quantification of cell lysis. It is critical to use both wild type and caspase-1 deficient macrophages to ensure that the cell lysis observed is dependent on caspase-1.

Materials

BMMs differentiated from bone marrow in the presence of 30% L929-cell conditioned medium for 7 days (additional media added to cells at day 3 or 4) (Swanson & Isberg, 1995; see UNIT 14.1)

DMEM-10 complete with antibiotics and DMEM-5 complete without antibiotics (see recipes)

Ultrapure *Salmonella minnesota* R595 (Re) LPS (see recipe)

CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega cat. no. G1780) or an equivalent cytotoxicity assay kit.

Sterile tissue culture-treated flat-bottom 96-well plate

96-well flat bottom, low binding plate

5 mM nigericin (see recipe)

Cell counter

Centrifuge able to spin plates at 10°C

Tissue culture incubator, humidified, set at 37°C and 5% CO₂

15-ml conical centrifuge tubes

Plate reader

NLRP3 priming and activation in mouse macrophages

1. Differentiated bone marrow macrophages (BMM) obtained using the methods described in Swanson and Isberg, 1995 or in CPI UNIT14.1 are plated in 96 well tissue culture plates at 2×10^4 BMM per 100 μ L of DMEM-5 complete without antibiotics. Centrifuge the plate for 5 min at $200 \times g$ to obtain equal distribution of the cells over the bottom of the well. Seed 9 wells for each treatment (3 spontaneous lysis, 3 total lysis, and 3 for the experimental setup).

Not centrifuging the plate will result in the majority of the cells to be around the edge of the well, while the middle of the well will contain fewer cells.

2. The following day, replace the medium with 50 μ L DMEM-5 complete without antibiotics containing 100 ng/mL LPS and incubate for 3–4 h at 37°C and 5% CO_2 .

During this time, other treatments can also be started. Keep in mind that the treatment should not interfere with the ability of LPS to bind to TLR4 and signaling pathways leading to NLRP3 priming. If this is the case, a different priming agent needs to be used such as PAM3CSK4 or treatment has to be delayed until priming is completed.

3. Following priming, add 50 μ L of DMEM-5 complete without antibiotics + LPS containing 10 μ M nigericin to the experimental wells, 50 μ L of DMEM-5 complete without antibiotics + LPS to the spontaneous wells, and 40 μ L of DMEM-5 complete without antibiotics + LPS to the total lysis wells. Final concentration of nigericin will be 5 μ M. Incubate for 1 h at 37°C and 5% CO_2 .
4. After 30 min add 10 μ L of $10 \times$ lysis buffer (provided with the cytotoxicity kit) to the total lysis control wells. In lieu of the provided lysis buffer, 10 μ L of 9% Triton X-100 can also be added to lyse the macrophages.

During the 1 h incubation, remove the assay buffer and the substrate from the freezer and let them thaw to room temperature while protected from light.

Detection of LDH release using Cytotox96 assay

1. After 1 h exposure to nigericin, centrifuge the plate for 5 min at $500 \times g$ at 10°C to pellet cell debris to the bottom of the well.

During the spin: Add 12 mL of assay buffer to 1 vial of substrate and dissolve by thorough mixing. Incubate for 5 min at room temperature before starting the cytotoxicity assay.

2. Transfer 50 μ L of supernatant from each well to a new, low binding 96 well plate. It is advised to keep the wells in the same order as the experimental plate. Also include three wells of medium only and three wells of medium + lysis buffer to use as blanks for the OD readings.

3. Add 50 μ L of reconstituted substrate to each well and incubate protected from light for 30 min.

It is a good idea to inspect the plate after 15 min to ensure that the reaction will not saturate the detection limit of the plate reader.

4. Add 50 μ L of stop solution to each well and read absorbance at 490 nm within 1 h of adding stop solution. Minimize the generation of bubbles as they will interfere with the OD reading.
5. Subtract the average values of the culture medium background from all values from wells containing cells.
6. Calculate percentage cytotoxicity using the following formula:

$$\text{Percent cytotoxicity} = ((\text{OD-experimental} - \text{OD-spontaneous}) / (\text{OD-total lysis} - \text{OD-spontaneous})) \times 100$$

ALTERNATE PROTOCOL 1. NLRP1b INFLAMMASOME ACTIVATION

This protocol presents an alternative method to activate caspase-1 via stimulation of the NLRP1b inflammasome with recombinant *Bacillus anthracis* lethal toxin. Lethal toxin consists of two protein components: protective antigen and lethal factor (Moayeri, Leppla, Vrentas, Pomerantsev, & Liu, 2015). Unlike, NLRP3, the NLRP1b inflammasome does not require priming to activate caspase-1 and cause pyroptosis. However, transcriptional induction of pro-IL-1 β does require a priming stimulus, if cytokine secretion is to be measured by ELISA. NLRP1b is polymorphic between mouse strains and only macrophages from BALB/c or other susceptible lines will undergo pyroptosis in response to anthrax lethal toxin (Moayeri, Sastalla, & Leppla, 2012). Macrophages from C57BL/6 mice are resistant to lethal toxin-induced pyroptosis.

Additional Materials (also see Basic Protocol)

1 mg/mL anthrax protective antigen (see recipe)

1 mg/mL anthrax lethal factor (see recipe)

NLRP1b activation in mouse macrophages

1. Plate BMM from a susceptible mouse strain after differentiation as described above and adhere overnight.
2. The next day, remove the medium and add 100 μ L of fresh medium containing 1 μ g/mL lethal toxin (1 μ g/ml each protective antigen and lethal factor). As a negative control use protective antigen without the addition of lethal factor, or protective antigen in combination with enzymatically inactive lethal factor.

Note: Any pretreatments to alter signaling pathways or other cellular processes should be done prior to exposure to lethal toxin. Great care needs to be taken that the molecules used do not interfere with binding / entry of lethal toxin to the cell. Pretreatments should be done using 50

μL of media. Following the pretreatment, add 50 μL of media + 2 μg/mL lethal toxin to obtain a final concentration of 1 μg/mL lethal toxin.

3. Incubate for 1 h at 37°C and 5% CO₂.
4. Process the plate, including adding the lysis buffer to the total lysis wells, as described in Basic Protocol 1 (from Step 4 on) to quantify LDH release.

ALTERNATE PROTOCOL 2. AIM2 INFLAMMASOME ACTIVATION

This protocol presents an alternative method to activate caspase-1 using poly(dA:dT) transfection to activate the AIM2 inflammasome (Hornung et al., 2009). Poly(dA:dT) is a synthetic repetitive double-stranded DNA sequence and is delivered intracellularly by complexing with a cationic lipid transfection reagent. We provide a protocol for preparation of poly(dA:dT) complexed with Lipofectamine 2000. As with NLRP1b, AIM2 activation does not require priming to stimulate pyroptosis. However, transcriptional induction of pro-IL-1β does require a priming stimulus, if cytokine secretion is to be measured by ELISA.

Additional Materials (also see Basic Protocol)

1 mg/mL poly(dA:dT) (see recipe)

Lipofectamine 2000 (e.g., Thermo Fisher Scientific)

OptiMEM (e.g., Thermo Fisher Scientific)

1.5 mL tubes

AIM2 activation in mouse macrophages

1. Plate BMM after differentiation as described in Basic Protocol 1 and adhere overnight.
2. The following day, combine 4 μL lipofectamine 2000 with 96 μL OptiMEM in a sterile 1.5 mL tube.
3. Combine 0.8 μL poly(dA:dT) with 99.2 μL OptiMEM in a sterile 1.5 mL tube.
4. Incubate both tubes for 5 min at room temperature.
5. Add diluted poly dA:dT to diluted transfection reagent. Vortex for 1 sec and incubate 20 min at room temperature to allow complex formation.
6. Aspirate media from cells. Replace with 50 μL transfection mix per well. Add 50 μL OptiMEM to the control wells.
7. Incubate for 1 h at 37°C and 5% CO₂.
8. Add 50 μL of DMEM-5 complete without antibiotics per well and continue incubation for an additional 1 hour (2 h total).
9. Process the plate, including adding the lysis buffer to the total lysis wells, as described in Basic Protocol 1 (from step 4 on) to quantify LDH release.

ALTERNATE PROTOCOL 3. NLRC4 INFLAMMASOME ACTIVATION

This protocol presents an alternative method to activate caspase-1 via stimulation of the NLRC4 inflammasome by infection with *Salmonella enterica* serovar Typhimurium (*Salmonella*). NLRC4 activates caspase-1 together with NAIP proteins, which are cytosolic receptors for bacterial flagellin and type III secretion system proteins (Zhao & Shao, 2015). Unlike NLRP3, the NLRC4 inflammasome does not require priming to activate caspase-1 and cause pyroptosis. However, transcriptional induction of pro-IL-1 β does require a priming stimulus if cytokine secretion is to be measured by ELISA.

Additional Materials (also see Basic Protocol)

Salmonella enterica serovar Typhimurium (SL1344) (Obtainable from ATCC)

LB agar plates

LB high salt (0.3 M NaCl) (see recipes)

NLRC4 activation in mouse macrophages

1. Streak *Salmonella* strain SL1344 onto LB agar to isolate single colonies two days before the experiment. Make sure to handle *Salmonella* using appropriate BSL-2 conditions. Incubate the plate overnight at 37°C.
2. One day before the experiment:
 - a. Plate BMM after differentiation as described in Basic Protocol 1 and adhere overnight.
 - b. Pick a single colony from the agar plate and inoculate into 2 mL LB + 0.3M NaCl. Incubate overnight shaking at 37°C
3. On the day of the experiment, dilute overnight cultures 1:15 into 2 mL LB + 0.3M NaCl. Incubate for exactly 3 h shaking at 37°C.
4. Replace the macrophage medium with 50 μ L DMEM-5 complete without antibiotics and continue to incubate at 37°C and 5% CO₂.
5. Harvest 1 mL of the 3 h bacterial culture by centrifuging for 1 min at 16,000 rcf.
6. Wash the bacteria using 1 mL PBS and resuspend in 1 mL PBS. Keep on ice until use, and prepare inoculum as quickly as possible.
7. Dilute *Salmonella* in DMEM-5 complete without antibiotics, as needed to produce an expected multiplicity of infection (MOI) of 10 bacteria to 1 macrophage.

Note: It is recommended to determine the number of bacterial in the 1 mL of PBS prior to performing the actual infection experiment. Follow the steps to start the overnight culture, dilution, and harvesting as would be done for the infection experiment. Plate 10-fold serial dilutions to determine bacterial numbers. Follow these same growth conditions,

including when the overnight culture is started and when the dilution is performed, the day of the experiment.

8. Add 50 μ L of diluted inoculum to the experimental wells.
9. Incubate for 1 h at 37°C and 5% CO₂.
10. Plate 10-fold serial dilutions of the bacteria onto LB agar to determine the actual MOI.
11. Process the plate, including adding the lysis buffer to the total lysis wells, as described in Basic Protocol 1 (from step 4 on) to quantify LDH release.

BASIC PROTOCOL 2. VISUALIZING LOSS OF MEMBRANE INTEGRITY BY FLUORESCENCE MICROSCOPY

One of the drawbacks of using the LDH release assay to quantify cell lysis is that it is a population-based approach. No information can be obtained regarding the fate of a single cell. To study what happens on a single cell level, microscopy-based approaches can be employed in combination with the use of cell-impermeable dyes. The polar structure of these dyes prevents them from crossing an intact plasma membrane. Membrane pores formed by gasdermin D during pyroptosis allow entry of small cell-impermeable dyes, and subsequent lytic membrane rupture releases larger cytoplasmic proteins (DiPeso, Ji, Vance, & Price, 2017; Fink & Cookson, 2006).

The most commonly used dyes have low intrinsic fluorescence and demonstrate fluorescence enhancement on binding cellular nucleic acids, allowing the user to visualize the cells. An example of one such dye is ethidium bromide, but a wide range of molecules have been developed, allowing the user to choose one that fits the excitation and emission spectrum needed for their application. By counterstaining with cell-permeable dyes, the percentage of cells with compromised membranes can be determined. Another option is to use dyes that rely on metabolic properties of the cell so that they only label cells that are viable. Table 1 lists several dyes with their maximal excitation and emission wavelengths and mechanism of labeling. This is not an exhaustive list and other dyes are available. Several companies also produce assay kits that contain both live and dead cell dyes. This protocol describes staining dead cells with ethidium bromide (EtBr). The cells are counterstained with the membrane permeable dye, Syto62, which labels both RNA and DNA.

Materials

BMMs differentiated from bone marrow in the presence of 30% L929-cell conditioned medium for 7 days (additional media added to cells at day 3 or 4) (Swanson & Isberg, 1995)

DMEM and DMEM-5 complete without antibiotics (see recipes)

Sterile flat-bottom 24-well plate

Sterile glass coverslips

Ultrapure *Salmonella minnesota* R595 (Re) LPS (see recipe)

5 mM nigericin (see recipe)

Hanks Balanced Salt Solution (HBSS)

Viable cell dye (e.g., 5 mM Syto62 cat. no. S11344 Thermo Fisher Scientific)

10 mg/mL ethidium bromide solution

Fluorescence microscope

Preparation, priming, and inflammasome activation of macrophages

1. After differentiation as indicated above, plate 2×10^5 BMM using 1 mL of DMEM-5 complete without antibiotics in a 24 well plate containing a sterile glass coverslip.

After seeding, it is recommended to push the coverslip down to the bottom of the well using a sterile pipet tip or sterile forceps to prevent it from floating to the top or from lying slanted in the well. It is also recommended to centrifuge the plate after seeding to achieve uniform distribution of the cells over the bottom of the well.

2. The following day, replace the medium with 500 μ L DMEM-5 complete without antibiotics containing 100 ng/mL LPS and incubate for 3–4 hrs at 37°C and 5% CO₂.

As described in the previous section any treatments can be commenced at this time as long as they do not interfere with the priming process.

3. Remove the medium and add 250 μ L of DMEM-5 complete without antibiotics + LPS containing 5 μ M nigericin. Be sure to include untreated wells in order to determine the level of spontaneous cell death.

Labeling of cells for fluorescence microscopy

1. Remove medium containing nigericin and wash the cells with 1 mL serum-free DMEM. Keep coverslips at 4°C when not in use.
2. Dilute Syto62 to 10 μ M and EtBr to 25 μ g/mL in HBSS. A titration experiment should be performed to determine the optimal dye concentration for each user.
3. Flip coverslips onto a 7 μ L drop of the stain mixture 5 minutes prior to viewing by fluorescence microscopy.

An alternative to this protocol would be to use a chamberslide or glass-bottom plates. These will allow for direct imaging without transferring the coverslips. If the user's microscope has an environmental chamber, the dyes can be added to the culture medium before addition of nigericin and the appearance of dead cells can be monitored over time.

BASIC PROTOCOL 3. ELISA FOR MOUSE IL-1 β

This protocol measures secreted IL-1 β using a simple sandwich ELISA. A variety of commercial ELISA kits for IL-1 β are also available, however we have found this protocol to be more cost effective, and just as reliable and easy to perform. It is important to remember that although the NLRP1b, AIM2, and NLRC4 inflammasomes do not require a priming stimulus for caspase-1 activation and pyroptosis, transcriptional induction of pro-IL-1 β does require priming. Thus, significant IL-1 β secretion in the absence of priming will not be detectable.

Materials

BMMs differentiated from bone marrow in the presence of 30% L929-cell conditioned medium for 7 days (additional media added to cells at day 3 or 4) (Swanson & Isberg, 1995)

DMEM and DMEM-5 complete without antibiotics (see recipes)

Sterile flat-bottom 96-well plate

Ultrapure *Salmonella minnesota* R595 (Re) LPS (see recipe)

5 mM nigericin (see recipe)

Multichannel pipet and disposable pipet tips

Plastic wrap or plate sealer

Immunoassay microplates (e.g. Nunc Maxisorp cat. no. 442404)

Anti-mouse IL-1 β capture antibody (e.g. Biolegend cat. no. 503502)

Carbonate coating buffer (see recipe)

Wash buffer (see recipe)

Blocking buffer (see recipe)

Biotin coupled anti-mouse IL-1 β detection antibody (e.g. eBioscience cat. no. 13-7112-85)

Streptavidin (SA)-horseradish peroxidase (HRP) conjugate (e.g. Biolegend cat. no. 405210)

Chromogenic substrate for detecting HRP activity (e.g. TMB substrate eBioscience cat. no. 00-4201-56)

Recombinant IL-1 β standard (e.g. Biolegend cat. no. 575102)

Recombinant pro-IL-1 β negative control (e.g. eBioscience cat. no. 39-8114-65)

Stop solution appropriate for substrate employed (see recipe)

Plate reader with appropriate filter for substrate employed

Preparation, priming, and inflammasome activation of macrophages

1. After differentiation, plate 5×10^4 BMM using 100 μ L of DMEM-5 complete without antibiotics in a 96 well tissue culture plate. Centrifuge the plate for 5 min at $200 \times g$ to obtain equal distribution of the cells over the bottom of the well.
2. The following day, replace the medium with 75 μ L DMEM-5 complete without antibiotics containing 100 ng/mL LPS and incubate for 3–4 h at 37°C and 5% CO_2 .
3. Following priming, add 75 μ L of DMEM-5 complete without antibiotics + LPS containing 10 μ M nigericin to the experimental wells, for a final nigericin concentration of 5 μ M. Incubate for 1 h at 37°C and 5% CO_2 .
4. Centrifuge the tissue culture plate for 5 min at $500 \times g$ at 10°C to pellet cell debris to the bottom of the well.

Coat plate with monoclonal anti-IL-1 β antibody and block

1. Coat desired wells of an immunoassay microplate with 50 μ L capture Ab diluted 1:500 in Carbonate coating buffer.
2. Seal the plate with plastic wrap or a plate sealer to minimize evaporation and incubate overnight at 4°C .
3. Remove the capture antibody solution and wash the plate three times with wash buffer by filling the wells with 300 μ L. Tap the plate on absorbent towels after the final wash to completely remove residual buffer.
4. Block non-specific binding sites by adding 200 μ L blocking buffer per well. Incubate for 1 h at room temperature.

Capture IL-1 β on antibody-coated plate

1. Dilute recombinant IL-1 β standard to 2000 pg/mL in blocking buffer. Prepare six additional 2-fold serial dilutions in 1.5 mL tubes. Remove well contents from blocked, antibody coated plate.
2. Pipet 100 μ L diluted IL-1 β standard, negative control (diluted pro-IL-1 β), and supernatant samples into wells of the ELISA plate.

Note: Alternatively, 120 μ L of macrophage supernatant can be frozen in snap cap strip tubes or well-sealed 96 well plates at -20°C or -80°C , and assayed at a later date. If using frozen supernatant, allow the samples to come to room temperature before adding them to the ELISA plate.

Note: Depending on experimental conditions, macrophage supernatant samples may require dilution in blocking buffer in order to obtain IL-1 β concentrations within the linear range of the ELISA assay. Appropriate sample dilution should be established by each investigator.

3. Add 100 μ L of blocking buffer to blank wells for determination of background signal.
4. Incubate sealed ELISA plate at 4°C overnight.

Detect captured IL-1 β

1. Remove well contents and wash the ELISA plate three times with wash buffer. Tap the plate on paper towels after the final wash to remove residual buffer.
2. Dilute detection antibody in blocking buffer 1:500 and add 50 μ L to each well. Incubate for 1 h at room temperature.
3. Wash the plate three times with wash buffer. Tap the plate on paper towels after the final wash to remove residual buffer.
4. Add 50 μ L SA-HRP diluted 1:1000 in blocking buffer. Incubate for 1 h at room temperature.
5. Wash the plate three times with wash buffer. Tap the plate on paper towels after the final wash to remove residual buffer.
6. Pipet 50 μ L chromogenic substrate solution to each well and incubate at room temperature. Substrate incubations should be carried out in the dark or as recommended by manufacturer.
7. When color has turned sufficiently blue, add 50 μ L stop buffer to all wells to stop the reaction.
8. Read plates in a plate reader at appropriate wavelength for substrate employed.
9. Construct a standard curve (absorbance versus pg/mL IL-1 β) and calculate the concentration of IL-1 β in the unknown samples using this standard curve.

REAGENTS AND SOLUTIONS

Use MilliQ 0.2 μ m-filtered water in all recipes and protocol steps. For common stock solutions, see appendix.

Anthrax Protective Antigen and Anthrax Lethal Factor, 1 mg/mL

Dissolve anthrax lethal factor and anthrax protective antigen (List Biological Laboratories cat. no. 172B and 171E) each to a final concentration of 1 mg/mL in sterile dH₂O. Aliquot and store at –80°C. Avoid freeze-thaw cycles. Remove one aliquot of each from the freezer 30 min prior to starting the experiment. Allow them to come to room temperature. Enzymatically inactive anthrax lethal factor (List Biological Laboratories cat. no. 176) can be used as a negative control, if desired.

Carbonate Coating Buffer

Dissolve 4.16 g Na₂CO₃•H₂O and 8.4 g NaHCO₃ in 1 L of dH₂O. Bring pH to 9.5. Store at 4°C.

DMEM-10 complete with antibiotics

DMEM – high glucose, no glutamine supplemented with

5 mM HEPES

0.2 mg/ml L-glutamine

0.05 mM 2-mercaptoethanol

50 mg/ml gentamicin sulfate

10,000 U/ml penicillin/streptomycin

10% (v/v) FBS

Store at +4°C

For DMEM-10 complete without antibiotics, omit penicillin/streptomycin and gentamicin sulfate

For DMEM-5 complete without antibiotics, omit pen/strep and gentamicin sulfate, use phenol-red free DMEM and reduce FBS to 5% (v/v). It is necessary to use phenol-red free DMEM for the LDH assay since it would interfere with the colorimetric assay.

ELISA Blocking Buffer

PBS with 5% (v/v) FBS. Keep sterile and store at 4°C. Bring to room temperature before use.

ELISA Wash Buffer

PBS with 0.05% (v/v) Tween-20. Store at 4°C

High Salt LB

Dissolve 25 g of LB powder and 7.5 g of NaCl in 1 L of dH₂O. Sterilize by autoclaving. Store at room temperature.

LPS, 1 mg/ml

Prepare a 1 mg/ml stock of *Salmonella Minnesota* R595 (Re) LPS (e.g. List Biologicals cat. no. 434) in H₂O containing 0.5% triethylamine. Aliquot in 100 µl and store at +4°C.

Nigericin, 5 mM

Add 1.34 ml of 100% ethanol to 5 mg of nigericin sodium salt (e.g. Millipore Sigma cat. no. N7143-5MG) and invert until completely dissolved. Aliquot and store at +4°C protected from light.

Poly(dA:dT), 1 mg/mL

Reconstitute lyophilized poly(dA:dT) (e.g. Invivogen cat. no. ttrl-patn) in sterile endotoxin free water to a concentration of 1 mg/mL. Vortex until completely dissolved. Aliquot and store at –20°C. Avoid freeze-thaw cycles.

Stop buffer $2N\ H_2SO_4$ **COMMENTARY****Background Information**

Pyroptosis is a form of programmed pro-inflammatory cell death described over a decade ago (Fink & Cookson, 2005, 2006). The term derives from the Greek roots “pyro,” relating to fire or fever, and “ptosis” (pronounced “to-sis” with a silent “p”) meaning “falling” (Cookson & Brennan, 2001; Fink & Cookson, 2005). As in apoptosis, “ptosis” connotes a controlled physiologic process of removing individual components of an organism, as leaves falling from a tree (Kerr, Wyllie, & Currie, 1972). Pyroptosis was distinguished from apoptosis, a pathway of programmed cell death that is primarily considered to be non-inflammatory. Many other forms of programmed cell death have now been described, each resulting from specific molecular pathways, which produce the features characteristic of each form of cell death (Galluzzi et al., 2012). Pyroptosis is defined by the molecular requirement for caspase-1 or members of the caspase-1 family of proteases, which includes mouse caspase-11. In addition to pyroptosis, many other forms of cell death culminate in terminal plasma membrane disruption or lysis, but the pre-lethal molecular events that trigger this final outcome are different. Thus, observation of LDH release or dye uptake alone is not specific for pyroptosis, but the use of genetically deficient cells can demonstrate dependence on caspase-1 or inflammasome components.

Activation of the NLRP3 inflammasome generally requires priming from a pattern recognition receptor (signal 1), followed by activation (signal 2) (Elliott & Sutterwala, 2015). In this unit, we describe a simple and widely used method of NLRP3 activation using LPS priming and subsequent treatment with nigericin. A large number of highly diverse triggers including ATP, and particulate stimuli such as urate or silica crystals can also provide signal 2, via (as yet) incompletely understood mechanisms. This unit also includes alternative protocols to simulate pyroptosis via the NLRP1b, AIM2 and NLRC4 inflammasomes. Many other stimuli can be used to activate these and other inflammasomes. For example, pyroptosis can also be triggered by activation of the pyrin inflammasome with purified *Clostridium difficile* toxins TcdA and TcdB (Van Gorp et al., 2016; Xu et al., 2014). LPS transfection activates the non-canonical caspase-11 inflammasome, which is sufficient to trigger pyroptosis, but additionally activates NLRP3 and caspase-1 for secretion of IL-1 β and IL-18 (Kayagaki et al., 2011).

Caspase-1 was initially recognized for its ability to process pro-IL-1 β into its active form and was called interleukin-1-beta converting enzyme (ICE). Cleavage of pro-IL-1 β and other caspase-1 substrates including pro-IL-18 and gasdermin D can be detected by Western blot, as can autoproteolysis of caspase-1 itself (Schneider, Thomas, & Gross, 2013; Zhao, Shi, & Shao, 2018). LDH release and IL-1 β ELISA are easier to perform with a large number of samples, while Western blot provides definitive evidence of substrate proteolysis. Other caspase-1-dependent events that occur during pyroptosis include DNA fragmentation, nuclear condensation and lysosome exocytosis (Bergsbaken, Fink, den Hartigh, Loomis, &

Cookson, 2011; Miao, Rajan, & Aderem, 2011). Formation of the gasdermin D pore directly allows uptake of small membrane impermeant dyes such as EtBr, which can be detected by microscopy as we describe here. Many other membrane impermeant dyes can also be used with a variety of excitation and emission spectra. If instruments capable of live cell analysis are available, dye uptake can also be monitored over time, providing information on the kinetics of membrane disruption.

Critical Parameters and Troubleshooting

Pyroptosis Induction—NLRP3 activation requires a priming stimulus, which induces NLRP3 transcription (Sutterwala, Haasken, & Cassel, 2014) and licenses NLRP3 through non-transcriptional mechanisms involving regulation of ubiquitination and phosphorylation (Juliana et al., 2012; Py, Kim, Vakifahmetoglu-Norberg, & Yuan, 2013; Song et al., 2017; Stutz et al., 2017). Conditions that interfere with these licensing mechanisms may diminish NLRP3 activation. The duration of priming can also influence NLRP3 activation. “Rapid priming” with signals 1 and 2 provided together, is sufficient to activate NLRP3 (Juliana et al., 2012). However, prolonged priming (12 to 24 hours) negatively regulates NLRP3 (Gurung et al., 2015; Hernandez-Cuellar et al., 2012). The concentration and duration of exposure to nigericin or other inflammasome activating stimuli may also contribute to the magnitude of the pyroptotic response.

Activation of the NLRP1b inflammasome with purified *Bacillus anthracis* lethal toxin requires the use of macrophages from susceptible strains of mice (for example BALB/c, but not C57BL/6) (Boyden & Dietrich, 2006). Lethal toxin is comprised of the proteins, protective antigen and lethal factor. Protective antigen binds cell surface receptors and translocates the lethal factor protease into the cytosol (Moayeri et al., 2015). Protective antigen cleavage by cell surface proteases such as furin is required for lethal toxin uptake. Manipulations that interfere with these processes will diminish NLRP1b activation. *Salmonella* must be grown under “SPI-1-inducing” conditions in order to specifically activate the NLRC4 inflammasome (Fink & Cookson, 2007; Mariathasan et al., 2004). Under other infection conditions, *Salmonella* is also capable of stimulating NLRP3 (Broz et al., 2010). It is important to reconstitute lyophilized poly(dA:dT) in sterile endotoxin free water, as transfection of contaminating LPS would activate the caspase-11 inflammasome (Kayagaki et al., 2011).

Assessing Membrane Disruption—The assay for LDH enzymatic activity produces a red formazan product, which is quantified spectrophotometrically. Culture medium often contains phenol red and LDH in FBS, both of which contribute to background absorbance. Thus, absorbance from culture medium alone must be subtracted from measurements from wells containing cells. The signal-to-noise ratio of the assay is optimized by using medium without phenol red and containing a lower percentage of FBS. Control wells should be included in the assay, with untreated cells for spontaneous LDH release and lysed cells for maximal LDH release. When treating cells with small molecules, it may be helpful to include controls with the small molecules and lysed cells to identify potential interference with the chemical reactions to measure LDH activity. Different cell types may contain different amounts of LDH and require optimization of the number of cells to use per well.

Likewise, the incubation time for the LDH assay may vary depending on the number of cells plated. The reaction can be stopped when the lysis wells become deep maroon in color.

Measurement of IL-1 β Release by ELISA—Pro-IL-1 β can be released nonspecifically during plasma membrane disruption, thus ELISA specificity for processed versus pro-IL-1 β is important to consider. Sandwich ELISAs generally do not detect pro-IL-1 β efficiently (Herzyk, Berger, Allen, & Wewers, 1992; Schneider et al., 2013). Testing recombinant pro-IL-1 β is recommended to determine ELISA specificity for mature IL-1 β under user-specific conditions. In some circumstances, IL-1 β may also be processed by inflammasome-independent mechanisms (Netea, van de Veerdonk, van der Meer, Dinarello, & Joosten, 2015); genetically deficient cells can be used to determine the requirement for caspase-1 or inflammasome components for IL-1 β secretion. Supernatant samples may require dilution in blocking buffer to ensure that IL-1 β measurements are within the linear range of the assay. To avoid evaporation or cross contamination between wells, plates should be sealed completely during incubations. Wash buffer should be completely removed, particularly after the final wash in each step, with multiple firm taps on a stack of paper towels or other absorbent surface. Solutions should be added to the wells as soon as wash buffer is removed to minimize drying of the wells. Ensure bubbles are not present prior to reading the plate.

Reporting Results—Precise terminology is recommended when reporting experimental results to describe the specific feature of cell death being measured. For example, “percent LDH release” is preferable to less specific terms such as “percent cytotoxicity” or “percent pyroptosis.” Specific features of cell death result from different molecular pathways and may be differentially susceptible to experimental intervention. For example, during pyroptosis glycine prevents rapid LDH release (Fig. 1), but not gasdermin D-mediated pore formation, small dye uptake or IL-1 β secretion (Evavold et al., 2017; Fink & Cookson, 2006). Furthermore, not all of the features associated with pyroptosis represent terminal events from which a cell cannot recover. For example, certain conditions may allow gasdermin D pore formation and IL-1 β release, without lysis or loss of metabolic function (Evavold et al., 2017; Zanoni et al., 2016). Therefore, caspase-1 activation and gasdermin D-mediated pore formation may not always cause cell death, defined as terminal events from which a cell cannot recover (Galluzzi et al., 2015). However, substantial membrane disruption allowing release of large intracellular enzymes such as LDH represents a lethal event, consistent with cell death.

Understanding the Results

LDH release during pyroptosis—The level of LDH release (Fig. 1) measured following activation of the different inflammasomes will vary depending on the duration the macrophages are exposed to the stimulus, as well as the concentration of the stimulus. Whereas there should be no (or almost no) detectable LDH release from caspase-1 deficient macrophages, wild type macrophages should demonstrate at least 30–40% lysis following exposure to an inflammasome trigger.

Visualizing membrane integrity loss by fluorescence microscopy—During pyroptosis, compromise of plasma membrane integrity will allow uptake of membrane

impermeant dyes such as EtBr (Fig. 2). As with detection of LDH release following exposure to inflammasome stimuli, uptake of membrane impermeable dyes should be dependent on caspase-1 during pyroptosis.

Detection of IL-1 β by ELISA—Activation of the different inflammasomes will trigger processing of pro-IL-1 β into the active form by caspase-1. The level of released IL-1 β (Fig. 3) will depend on the priming conditions and level of caspase-1 activation that is induced, but no processing and secretion should occur in macrophages deficient in caspase-1.

Time Considerations

The measurement of LDH released during pyroptosis is a quick method to determine the level of cell lysis induced. Depending on how many treatments are performed the total time will be around ~6 hours from the start of the experiment. This includes priming, exposure to the inflammasome stimulus, and detection of LDH.

Analysis of macrophages that have undergone pyroptosis will depend on number of coverslips and the type of microscope used, but should be around 5 minutes per coverslip. The experience of the user will also influence the time needed.

Analysis of the level of IL-1 β secreted by sandwich ELISA takes a total of 3 days. However most of this time is hands-off since there are two overnight incubations. The majority of the hands-on time will be on the third day.

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Figure 1.

LDH release following pyroptosis induction. BMMs were treated with nigericin or infected with *Salmonella* as described in basic protocol 1 and alternative protocol 3. Pyroptosis was induced in the presence of 5 mM glycine as indicated. LDH release was measured as described in basic protocol 1. Data are means $\pm$ SD of three replicates.

Figure 2.

EtBr uptake following pyroptosis induction. BMMs were infected with *Salmonella* as described in alternative protocol 3, or treated with PBS as a negative control. After 30 minutes, cells were stained with ethidium bromide (membrane impermeant, red) and Syto62 (membrane permeant, blue) for 5 min before imaging by confocal microscopy as described in basic protocol 2. White arrows in *Salmonella* field point to cells not undergoing pyroptotic cell death.

Figure 3.

Quantification of secreted IL-1 β following pyroptosis induction. BMMs were treated with nigericin, transfected with poly(dA:dT) or infected with *Salmonella* as described in basic protocol 1 and alternative protocols 2 and 3. IL-1 β in the supernatant was measured by ELISA as described in basic protocol 3. Data are means $\pm$ SD of three replicates. ND (none detected)

Table 1

Examples of Fluorescent Dyes

Viable cell dye			
Name	Ex/Em	Binding property	Notes
Syto Series (TF)	Multiple	DNA and RNA	
CFSE	490/520	primary amines	cleaved by cellular esterases dilutes with cell division
Calcein-AM	495/515	cleaved by cellular esterases, non-fluorescent until cleaved	
Live-dye (Enzo)	488/518		Proprietary
Dead cell dye			
Name	Ex/Em	Binding property	Notes
Ethidium Bromide	360/590	DNA/RNA	
Propidium Iodide	536/617	DNA/RNA	
7-AAD	546/647	DNA	
Sytox Series (TF)	Multiple	DNA/RNA	Some only for FC
Zombie Series (BL)	Multiple	primary amines	
Nuc Dead ReadyProbe	Multiple	DNA	TF, 488 and 647 Ex
Green L/D stain	488/530	DNA	ICT

Abbreviations: Ex/Em: excitation/emission; TF: ThermoFisher; FC: Flow cytometry; BL: Biolegend; L/D: Live/Dead; ICT: ImmunoChemistry Technologies.