In vitro Inflammasome Assay

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[Abstract] Innate immune cells sense pathogen and danger-associated molecular patterns (PAMPs and DAMPs) through a range of innate immune pattern recognition receptors (PRRs). One type of PRRs are the Nod-like receptors (NLRs), which form inflammasomes; a molecular platform required for the recruitment and activation of Caspase-1, which in turn cleaves and activates IL-1β, IL-18. Examples of inflammasome forming NLRs are NLRP3, NLRP1, NAIP and NLRC4. We can easily identify new inflammasome activators by performing the following protocol.

Materials and Reagents

1. Mice (*e.g.* C57B/6)
2. DMEM (Life Technologies, Gibco®, catalog number: 10566-024)
3. 1% Penicillin-Streptomycin (10,000 U/ml) (Life Technologies, Gibco®, catalog number: 15140148)
4. 1x PBS
5. Accutase (PAA Laboratories GmbH, catalog number: L11-007)
6. Ultrapure *Escherichia coli* (*E.coli*) K12 LPS (Life Technologies, Invitrogen™, catalog number: tlrl-peklps)
7. IL-1β ELISA (eBioscience, catalog number: 88-7013-88)
8. Primary antibodies against IL-1β (*e.g.* R&D System, catalog number: AF-401-NA) and Caspase-1 (*e.g.* Aidpogen International, catalog number: AG-20B-0042-C100) (suitable for western blot)
9. HRP-conjugated secondary antibody (*e.g.* Cell Signaling anti-mouse HRP, Cell Signaling Technology, catalog number: 7076)
10. Nitrocellulose membrane (0.45 µm) (GE Healthcare, Hybond, catalog number: 95038-402)
11. 5% sodium azide in water (Sigma-Aldrich, catalog number: 26628-22-8)
12. Skim milk powder (Sigma-Aldrich, or your local grocer)

13. ECL solution (Pierce, catalog number: 34095 or GE Healthcare, catalog number: RPN2133)
14. 1 M dithiothreitol (DTT) (Sigma-Aldrich, catalog number: D0632)
15. Stimulus: *e.g.* 1 M ATP (Sigma-Aldrich, catalog number: A26209), 10 mM nigericin (Sigma-Aldrich, catalog number: N7143), 300 µg/ml monosodium urate (Michigan State University, catalog number: U2875)
16. Flushing medium (see Recipes)
17. Red blood cell lysis solution (see Recipes)
18. Bone-marrow-derived macrophage (BMM) culture medium (see Recipes)
19. Ponceau staining solution (see Recipes)
20. 3x western blot sample buffer (see Recipes)
21. Blocking buffer (see Recipes)
22. Running buffer (see Recipes)
23. Blotting buffer (see Recipes)

Equipment

1. Razor blade
2. Laminar flow hood
3. Bench top centrifuge with 96-well plate adaptors
4. 10 ml syringe
5. 22 G gauge needles
6. 18 G blunt needles
7. 100-µm cells strainers
8. 10 cm cell-culture treated petri dishes
9. MaxiSorb ELISA plates (Nunc®)
10. 96-well tissue culture plate
11. ELISA plate reader
12. Western blot equipment (protean mini 1 mm) (Bio-Rad Laboratories)
13. Film, developer, dark room or equivalent development equipment
14. Parafilm or plastic

Software

1. ELISA analysis software

Procedure

A. Bone marrow isolation and cell culture
   1. Sacrifice mice by CO_2 inhalation and cervical dislocation.
   2. Remove the fibia and tibia and place in flushing medium on ice.
   3. Under a laminar flow hood clean bones, removing excess muscle by scraping with razor blade, and cut ends of bones.
   4. Flush bones with flushing medium, using a 10 ml syringe and a fine-gauge needle.
   5. Resuspend clumps from bone by passing through the syringe with a 18 G blunt needle.
   6. Filter through 100 µM cell strainer.
   7. Pellet cells (5 min at 1,200 rpm).
   8. Resuspend in 1 to 5 ml RBC lysis buffer, incubate for 5 min at room temperature.
   9. Add 14 ml PBS, pellet cells (5 min at 1,200 rpm).
   10. Count and wash with PBS.
   11. Resuspend cells in BMM medium at a density of 10^6/ml, plate 10 ml per 10 cm Petri dish.
   12. Culture cells for 6 to 7 days at 37 °C, 5% CO_2, topping up medium on day 3 and 6 with 5 ml BMM medium.
   13. After 6-7 days remove the culture supernatant, wash petri dish once with 5 ml PBS. Adherent macrophages appear flat, with round cell body and extending dendrites. Flow cytometry analysis for macrophage markers, such as F480/CD11b can be used to determine macrophage purity
   14. Remove adherent BMMs with 5 ml accutase, leaving petri dishes to incubate at room temperature until cells become rounded and start to lift (around 10 min).
   15. Wash cells with PBS.
   16. Count and resuspend in BMM medium at 10^6/ml, and plate 200 µl/well in a flat-bottom 96-well tissue culture plate.
   17. Incubate overnight at 37 °C to enable cells to become adherent.

B. Stimulation
   1. Prime cells: remove cell supernatant and add 200 µl fresh BMM medium with 20 ng/ml ultrapure LPS, incubate at 37 °C for 3 h. For a negative control add medium without LPS. BMMs derived from Caspase1/11-deficient mice can also be used as a negative control.
      Note: BMMs require a priming step in order to induce expression of inflammasome components, such as Nlrp3 (Some stimuli may activate NF-κB pathway and induce priming without the need for LPS-priming.).
   2. Add stimuli to cells in 50 µl BMM medium, without removing medium with LPS (You can also remove medium with LPS if desired.).

3. Incubate at 37 °C, time depending on the stimulus, *e.g.* 1 M ATP or 10 mM nigericin for 30 min to 1 h, 300 µg/ml MSU for 6 h.
4. *Note: For Helminth inflammasome activation we used HES (5 or 50 µg/ml), pyrogen-free HES (P.HES) (5 or 50 µg/ml) or homogenized Heligmosomoides polygyru (H. polygyrus) L5 parasite (HPL5) (100 µg/ml), and incubated overnight*.
5. Spin down the plate (1,400 rpm for 3 min), collect the cell supernatants for ELISA or WB analysis, freeze until use.
   Note: Aim to collect about 20 -50 µl less than the initial volume you put in.
6. Wash cells 1x with cold PBS and add 40 µl 1x western blot sample buffer supplemented with fresh 10% 1 M DTT directly to cells (can freeze until use or proceed to preparation for WB, below).

C. Measurement

1. Perform IL-1β ELISA with 50 - 100 µl of cell supernatant as per manufacturers' instructions.
2. Proceed to western blot with samples you want to test further.
3. For cell extracts, pool each triplicate into 1 tube.
4. For cell supernatant, take an aliquot of supernatant and add to 2 parts of 3x western blot sample buffer (with 10% fresh 1 M DTT). Load 20 to 30 µl of the supernatant.
5. Option: concentrate protein from the supernatant:
   a. Add equal volumes of supernatant and methanol, and add chloroform 1/8 of the volume of supernatant, mix well. (*e.g.* 100 µl supernatant + 100 µl methanol + 12.5 µl chloroform)
   b. Centrifuge for 3 min at top speed (in bench-top eppendorf centrifuge).
   c. Remove as much liquid as possible without disturbing the pellet at the interface.
   d. Add another volume of methanol (same as initial), mix well.
   e. Centrifuge at top speed for 3 min.
   f. Remove supernatant, careful not to disrupt protein pellet.
   g. Air dry for approx. 20 min.
   h. Add 20 to 40 µl 1x western blot sample buffer (with 10% fresh 1 M DTT), depending on how concentrated you want it.
6. Boil samples at 95 °C for 5 min, then cool on ice.
7. Subject 20-30 µl sample to polyacrylamide gel electrophoresis using a 15% gel and running buffer.
8. Blot the proteins onto a nitrocellulose membrane using the blot buffer.

9. To ensure loading was even, stain membrane in ponceau red solution for approx. 2 min, wash with distilled water until excess stain is removed, make a copy of the blot for your documentation.
10. Block the membrane by incubating in blocking buffer for at least 5 min at room temperature with mild shaking.
11. Add your antibody, anti-IL-1β or caspase-1 (which detect pro and cleaved forms) (1:1,000 to 1:2,000 in blocking buffer containing 0.05% azide) and incubate overnight at 4 °C with mild shaking (keep antibody source at -20 °C for repeated use).
12. Wash 3 x 5 min with PBS-tween (0.5%) at room temperature with mild shaking.
13. Add HRP-conjugated secondary antibody 1:5,000-1:10,000 in blocking buffer and incubate for 1 h at room temperature with mild shaking.
14. Wash for at least 20 min in PBS-tween (changing the buffer at least 4 times) at room temperature with mild shaking.
15. Dry the membrane on a tissue, immediately lay it over a 1-ml drop of regular or high fidelity ECL solution (on a piece of parafilm or plastic) for 1 min, remove excess solution on a tissue.
16. Develop the blot using standard techniques and equipment (Detection of cleaved IL-1β and caspase-1 in the cell supernatant indicates inflammasome activation.).
17. Repeat steps 9-15 with additional antibodies (*e.g.* tubulin for loading control).

Recipes

1. Flushing medium
   DMEM
   1% Penicillin-Streptomycin
2. Red blood cell lysis solution
   155 mM NH_4Cl
   10 mM $KHCO_3$
   1 mM EDTA
3. BMM culture medium
   DMEM
   10% FCS
   20% supernatant from L929 cell culture
   1% Penicillin-Streptomycin
4. 3x western blot sample buffer
   187.5 mM Tris-HCl (pH 6.8)
   6% w/v SDS

0.03% w/v phenol red

30% w/v glycerol (adjust to pH 6.8)

5. Ponceau staining solution (for 500 ml)

   0.05 % Ponceau S 250 mg

   3% tricholacetic acid 15 g

6. Blocking buffer

   5% skim milk in PBS-tween

7. Running buffer (for 5 L)

   Tris base 75 g

   Glycine 360 g

   SDS (20%) 125 ml

8. Blotting buffer (for 20 L)

   Tris base 50 g

   Glycine 238 g

   Ethanol 3.3 L

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References

1. Zaiss, M. M., Maslowski, K. M., Mosconi, I., Guenat, N., Marsland, B. J. and Harris, N. L. (2013). IL-1beta suppresses innate IL-25 and IL-33 production and maintains helminth chronicity. *PLoS Pathog* 9(8): e1003531.

