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Methods for Detection of Pyrin Inflammasome Assembly in Macrophages Infected with *Yersinia* spp.

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Abstract

The *Yersinia* effector proteins YopE and YopT are important bacterial virulence factors that are secreted into infected host cells and can inactivate Rho GTPases, like RhoA, Rac1, and Cdc42. In order to compensate for the consequences of this effect, the host cell can sense RhoA modifications and trigger a proinflammatory reaction to control the infection. This host response, known as pyrin inflammasome assembly, is normally prevented by another important effector, YopM, allowing *Yersinia* to counteract this conserved innate immune response. Once assembled, the pyrin inflammasome can activate caspase-1 via proteolysis, leading to IL-1 β secretion and cell death through pyroptosis. Here we describe how to measure pyrin inflammasome assembly, in response to YopE or YopT activities, when macrophages are infected with *yopM* mutant *Yersinia*. Using primary mouse macrophages as host cells, we show how to detect this host response through the downstream events of pyrin dephosphorylation, caspase-1 proteolysis, IL-1 β release, and pyroptosis.

Keywords

Yersinia; Effectors; Toxins; Inflammasome; Pyrin; Inflammation; Rho GTPases

1. Introduction

The genus *Yersinia* consists of 17 species of which three are known to be human pathogens: *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* [1]. Although the pathogenicity levels among those three species are consistently different, they all share a similar trait: the presence of a plasmid-encoded Type III Secretion System (T3SS) as a remarkable virulence factor [2]. This secretion system consists of a syringe-like mechanism responsible for injecting effector proteins into the cytosol of host cells upon close contact. Those effector proteins, known as Yops, have various functions that together will succeed in altering host physiology in order for *Yersinia* to evade the immune system and persist [3].

So far, seven effector Yops are known to be secreted inside cells and among them: Not sure if colon belongs here. YopE and YopT are Rho-modifying toxins that utilize different mechanisms to control cellular processes and consequently alter homeostasis [3]. YopE mimics Rho GTPase-Activating Proteins (GAPs), keeping RhoA in an inactive state [4]. YopT is a cysteine-protease that cleaves the C-terminus of RhoA, resulting in the release of this protein from the cellular membrane and inactivation [5]. Although the YopE and YopT effectors have different mechanisms to modify Rho GTPases, they both disrupt the actin cytoskeleton, leading to changes in cell morphology, blockage of phagocytosis, and cell mobility [3].

Besides the intentional change in host cell morphology, inactivation of RhoA by YopE or YopT or other bacterial toxins can trigger assembly of the pyrin inflammasome as a compensatory innate immune response [6, 7, 8]. Pyrin is a pattern recognition receptor (PRR) that is expressed in macrophages, cytokine-activated monocytes, granulocytes, and serosal and synovial fibroblasts [9]. RhoA-activated kinases PRK1 (PKN1) and PRK2 (PKN2) normally phosphorylate pyrin at serines 205 and 241 in mice and Serines 208 and 242 in humans [10, 11, 12]. Phosphorylated pyrin is maintained as an inactive protein by the binding of protein 14-3-3. Upon inactivation of RhoA by bacterial effectors or toxins, phosphorylation of pyrin is lost and protein 14-3-3 is released [10, 11, 12]. Consequently, pyrin becomes active and can bind to adaptor protein ASC, which recruits the cysteine-protease pro-caspase-1, forming the multiprotein complex known as inflammasome (Fig. 1) [13]. This assembly can mediate auto-proteolysis of pro-caspase-1 into its mature form, which is an important step that leads to the generation and release of active IL-1 β and IL-18 cytokines and also cleavage of a cellular protein, gasdermin D (GSDMD) [14]. The generated GSDMD N-terminus fragments relocalizes in the plasma membrane, where it oligomerizes with other N-terminus fragments to form small pores. These pores allow the passage of the cleaved cytokines and also lead to a unique form of cell death, known as pyroptosis [14, 15]. The secretion of proinflammatory cytokines recruits other immune cells to the site of the infection, helping in the clearance of the pathogen. In addition, pyroptotic cell death traps intracellular bacteria and prevents infection of additional cells [16]. Thus, inflammasome function is important for the host to eliminate pathogens [17].

The pyrin inflammasome protects mice against systemic infection by specific pathogens that produce RhoA-inactivating effectors or toxins [7, 8, 18]. The host-adapted pathogen *Yersinia* evolved the effector YopM in order to inhibit YopE- or YopT-triggered pyrin inflammasome formation [7, 8]. YopM counteracts pyrin by hijacking the kinases responsible for keeping this PRR inactivated [7]. Once bound to PRK, YopM increases this kinase's ability to phosphorylate pyrin, consequently negatively regulating the formation of the inflammasome [7]. This leads to immune evasion and persistence of *Yersinia* in the host [7, 8].

To study pyrin inflammasome assembly and function upon host cell infection with *Yersinia*, we optimized different methods that can be used with primary murine macrophages and *yopM* mutant strains. Those methods, used to measure pyrin dephosphorylation, caspase-1 proteolysis, IL-1 β release, and pyroptosis, are described below.

2. Materials

Prepare all macrophage cell media in a biosafety hood. Macrophage cell media needs to be filter-sterilized before use, while bacterial culture media needs to be autoclaved before use. Keep sterile conditions while performing all experiments and infections, to avoid contamination with undesired microorganisms. For media preparation, we suggest the volumes described below. If larger volumes are necessary, adjust accordingly. The material brands described below are suggestions only.

2.1. Bone Marrow-Derived Macrophage (BMDM) Media for Harvest and Infection

1. Heat-inactivated Fetal Bovine Serum (FBS): Thaw FBS bottle completely overnight at room temperature (rt). On the next day, place FBS in a 56 °C preheated water bath for 30 min. While having the FBS in the water bath, swirl the contents in the bottle every 5–10 min. At the end of 30 min, place bottle on ice for 1 h (*see Note 1*). Aliquot in small bottles and store at –20 °C (*see Note 2*).
2. L929 conditioned media: Mix 500 mL Minimum Essential Media (MEM), 50 mL heat-inactivated FBS, 5 mL 1 M HEPES, 5 mL 100 mM sodium pyruvate and 5 mL 100× non-essential amino-acids. Do not filter-sterilize this media before using. Add to each 500 mL of complete media, 4×10^6 L929 cells. Divide 50–56 mL into 175 cm² vented caps flasks and incubate at 37 °C with 5% CO₂. After 10–12 days of growth, remove media and filter. Aliquot in small bottles and store at –20 °C (*see Note 3*).
3. Warm and cold sterile 1× PBS for tissue culture.
4. Nontissue culture-treated petri dishes (100mm).
5. Forceps (autoclaved).
6. Scissors (autoclaved).
7. No. 10 round blade scalpels.
8. 5 mL syringe with 23-gauge (G) needle.
9. Wash Media: In a 50 mL Falcon tube, mix 47.5 mL Dulbecco's Modified Eagle Medium (DMEM) + Glutamax (Gibco®), 2.5 mL heat-inactivated FBS, and 0.5 mL penicillin-streptomycin. Homogenize by inverting the tube 4–6 times (*see Note 4*).
10. Macrophage Growth Media 10/30 (MGM 10/30): For 500 mL of media, mix 290 mL DMEM + Glutamax, 5 mL 1 M HEPES (for 10 mM final concentration), 5 mL 100 mM sodium pyruvate (for 1 mM final concentration), 50 mL heat-inactivated FBS (10% total volume of media), and 150 mL of L929 conditioned medium (30% of total volume of media) (*see Note 5*) [7].
11. cDMEM media: For 500 mL of media, mix 440 mL of DMEM + Glutamax, 5 mL 1 M HEPES, 5 mL 100 mM sodium pyruvate, and 50 mL heat-inactivated FBS (*see Note 5*) [7].

12. MGM 10/10 (infection media): Mix one part of MGM 10/30 to two parts of cDMEM. Make the necessary volume for the experiment (*see* Note 5).
13. O26:B6 *Escherichia coli* LPS (Sigma®): Dilute 1 mg LPS lyophilized powder in 10 mL of sterile 1× phosphate-buffered saline (PBS) to obtain a stock solution of 100 µg/mL. Keep 1 mL working stock at 4 °C and store the other 9 mL (divided into 1.5 mL tubes) at –20 °C [19] (*see* Note 6).
14. Cold Hank's Balanced Salt Solution (HBSS) for cell culture.

2.2. Bacterial Media, Strains, and Intoxication Conditions

1. Luria broth: For 1 L of media, add 10 g Bacto™ tryptone, 5 g Bacto™ yeast-extract, 10 g sodium chloride (NaCl). Add one sodium hydroxide (NaOH) pellet to adjust media to a pH around 7–8 and then autoclave to sterilize.
2. 0.2 M Sodium Oxide (NaOx): For 1 L, dissolve 26.8 g NaOx into 500 mL double-distilled water. Bring the volume up to 1 L. Divide into 100 mL aliquots and autoclave.
3. 0.2 M Magnesium Chloride (MgCl₂): For 1 L, dissolve 40.66 g into 500 mL double-distilled water. Bring the volume up to 1 L. Divide into 100 mL aliquots and autoclave.
4. *Y. pseudotuberculosis yopM* mutant strains [7]: 32777 *yopM* (YopE⁺, YopT⁺ and YopM[–]), 32777 *yopE*^{R144A} *yopM* (YopE[–], YopT⁺ and YopM[–]), 32777 *yopT*^{C139A} *yopM* (YopE⁺, YopT[–] and YopM[–]). For negative controls: 32777 WT and 32777 *yopE*^{R144A} *yopT*^{C139A} *yopM* (YopE[–], YopT[–] and YopM[–]) (*see* Note 7).
5. *Clostridium difficile* toxin B (TcdB) (List Biologicals®): Spin down toxin tube for 30–45 s at 20,000 × *g*. Reconstitute 20 µg of TcdB into 100 µL of sterile double-distilled water to obtain a 200 µg/mL stock solution. Store at 2–8 °C (*see* Note 5).
6. Phase-contrast light microscope.

2.3. Cell Lysate Analysis

1. Radioimmunoprecipitation assay (RIPA) lysis buffer: For 10 mL of RIPA, mix 500 µL 1 M Tris-HCl pH 8.0 (50 mM final concentration), 1.5 mL 1 M NaCl solution (150 mM final concentration), 100 µL NP-40, 0.05 g deoxycholic acid (DOC) and 0.01 g sodium dodecyl sulfate (SDS). Dissolve all components. Complete volume up to 10 mL. Aliquot and store at –20 °C (*see* Note 8).
2. Quantification of protein by Bicinchoninic acid (BCA) assay: Follow BCA Protein Assay Kit protocol recommendations. To create standard reagents, dilute Bovine Serum Albumin (BSA) into RIPA for more accurate results. Follow the microplate procedure for cell lysate analysis. Sample cell lysates can be diluted up to 1:8 in RIPA for the experiment. Do standard BSA curve and samples dilution in duplicate (*see* Note 9).

3. 10% SDS-Polyacrylamide gel: To make resolving gel, add 3.3 mL 30% acrylamide mix, 2.5 mL 1.5 M Tris pH 8.8, 100 μ L 10% SDS, 100 μ L 10% Ammonium Persulfate (APS), 4 μ L Tetraacetythylenediamine (TEMED). Add 4 mL of double-distilled sterile water to complete a 10 mL final volume. To make stacking gel, add 670 μ L 30% acrylamide mix, 500 μ L 1.5 M Tris pH 6.8, 40 μ L 10% SDS, 40 μ L 10% APS, 4 μ L TEMED. Add 2.7 mL of double-distilled sterile water to complete a 4 mL final volume.
4. PVDF membranes (*see* Note 10).
5. 4–12% SDS-Polyacrylamide gradient gel.
6. Running buffer: To make a 10 $\times$ solution, add 30.3 g Tris Base, 144.2 g Glycine, and 10 g SDS to 300 mL of double-distilled water. After dissolving the components completely, add 700 mL of water to make 1 L. Before performing electrophoresis, do a 1:10 dilution in water to make 1 $\times$ running buffer.
7. 1 $\times$ 4-Morpholineethane sulfonic acid (MES) running buffer: To make a 500 mL 1 $\times$ MES running buffer, mix 25 mL NuPAGE™ 20 $\times$ MES SDS running buffer with 475 mL double-distilled water. After assembling running apparatus, add 500 μ L of NuPAGE™ antioxidant (Thermo Fisher Scientific) into middle chamber.
8. Transfer buffer: To make a 10 $\times$ solution, add 145 g Glycine and 29 g Tris base to 300 mL of double-distilled water. After dissolving the components completely, add 700 mL of water to make 1 L. Before performing protein transfer to membrane, make 1 $\times$ transfer buffer by adding 100 mL 10 $\times$ transfer buffer, 700 mL double distilled water, and 200 mL methanol (*see* Note 11).
9. Plate shaker.
10. Tris Buffered Saline-Tween (TBS): To prepare 10 $\times$ solution, add 26.5 g Tris Base, 44.4 g Tris-HCl, 80 g NaCl, and 2 g potassium chloride (KCl) to 300 mL of double-distilled water. After dissolving all components, add 700 mL of water to complete 1 L. To make 1 $\times$ TBS-T solution, mix 100 mL of 10 $\times$ TBS with 900 mL of water (1 L). Add 1 mL of Tween®–20 for a final concentration of 0.1% (*see* Note 12).
11. Blocking solution: In a 50 mL conical tube add 4 g powdered milk to 50 mL 1 $\times$ TBS-T. Mix until the clumps dissolve. Keep it at 4 °C.
12. Amersham ECL detection blotting reagent kit (GE Healthcare®) (*see* Note 13).

2.4. Primary and Secondary Antibodies

1. Rabbit monoclonal antimouse total pyrin antibody (Abcam®—ab195975): Divide 100 μ L into 10 μ L aliquot and store at –20 °C for long term (*see* Notes 14 and 15).
2. Rabbit monoclonal antimouse S205 phosphorylated pyrin antibody (Abcam®—ab201784): Divide 100 μ L into 10 μ L aliquot and store at –20 °C for long term (*see* Notes 14 and 15).

3. Rabbit polyclonal antimouse β -actin antibody (Cell signaling®—#4967): Aliquoting is not necessary. Store at -20°C for long term.
4. Rabbit polyclonal antimouse caspase-1 antibody: Antibody provided by Dr. Gabriel Nuñez. Use as previously described [7]. Store at -80°C for long term. Keep a working stock at -20°C for short term (see Notes 14 and 16).
5. Polyclonal goat antirabbit IgG (H + R) antibody conjugated with horseradish peroxidase (HRP): Rehydrate antibody with indicated volume of water and glycerol (1:1), for a 50% final glycerol volume. Aliquot and store at -20°C for long-term usage (see Notes 14 and 17).

2.5. Cell Supernatant Analysis

1. Quantikine ELISA Mouse IL-1 β /IL-1F2 Immunoassay (R&D Systems®)—Use this kit to measure IL-1 β concentrations in the cell culture supernatant. Follow kit instructions carefully (see Note 18).
2. 96-well microplate for LDH assay.
3. CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega®)—Use this kit to measure lactate dehydrogenase (LDH) release. Follow kit instructions carefully (see Note 18).
4. Microplate reader (see Note 19).
5. Plate shaker.

3. Methods

3.1. BMDMs Isolation and Culture Conditions

1. The isolation of BMDMs should be performed as previously reported [7]. Remove the femurs from 8-week-old WT C57BL/6 mice and clean off muscle tissue as completely as possible using scalpel. Cut ends of the bones and flush them with Wash Media (5 mL per femur) using a 5 mL syringe with a 23 G needle, in a petri dish. Break up clumps of bone marrow gently by pipetting up and down. The solution should become turbid.
2. Transfer the bone marrow cells in Wash Media into a 15 mL conical tube and in a centrifuge spin for 10 min at $112 \times g$ (rt). This is considered the first wash. Once the centrifugation is done, discard the supernatant and resuspend the pellet into 10 mL of Wash Media by pipetting gently up and down. Spin for 5 min at $112 \times g$. This is the second wash. Repeat the last wash one more time, for a total of three washes.
3. After washing, resuspend the pellet with 10 mL of the media MGM 10/30 (5 mL for each femur). Split the resuspension into four 100 mm non-tissue culture dishes (2.5 mL each) and add 7.5 mL of fresh MGM 10/30 to complete a total of 10 mL per dish.

4. Incubate the cells at 37 °C and 5% CO₂ for 4 days. On day 4, check cells for viability and contamination. The cells should be adhered to the bottom and have macrophage-like morphology. Remove old media and add 10 mL of fresh MGM 10/30. Return to incubator until day 7.
5. On day 7, after checking for viability, aspirate media and add 5 mL of cold PBS to each dish to remove debris. Shake it slowly and aspirate immediately. Add 10 mL of cold PBS to each dish and incubate them on ice for 10–25 min. Do not incubate the cells on ice for more than 30 min.
6. After incubation, dislodge BMDMs from dish by pipetting up and down. The solution should turn opaque due to the presence of the cells. Once the bottom of the dish is clean, transfer the suspended cells into a 50 mL conical tube and spin (rt) on a centrifuge for 5 min at $112 \times g$.
7. Gently aspirate PBS away from the pellet and add 20 mL of media MGM 10/10 (5 mL per dish) and break up all the clumps. Use a hemocytometer to count the cells and plate the BMDMs as needed. The density on a 6-well plate should be 0.8×10^6 cells/well in a total volume of 3 mL/well and in a 24-well plate should be 1.5×10^5 cells/well in a total volume of 1 mL/well. Incubate cells at 37 °C with 5% CO₂.
8. One hour after cell plating, add *Escherichia coli* LPS to a final concentration of 100 ng/mL to prime the cells and incubate as in Subheading 3.1, **step 7** until the next day (day of infection) (see Note 6).

3.2. BMDM Infections

1. Inoculate LB broth with non-italics *Y. pseudotuberculosis yopM* mutant strains and controls. Allow them to grow overnight at 28 °C (see Note 7).
2. The following day, dilute overnight cultures 1:40 in LB broth containing 20 mM sodium oxalate and 20 mM of MgCl₂ (final concentration). Grow them for 1 h at 28 °C, and then shift the cultures to 37 °C for 2 h, to allow production of the T3SS and secretion of the Yops.
3. Take 1.5 mL of the cultures, spin down (rt) in a microcentrifuge tube at $20,000 \times g$ for 5 min, and aspirate the supernatant. Resuspend the pellet with warm 1.5 mL $1 \times$ PBS and take 800 μ L to measure the OD₆₀₀ (OD₆₀₀ of 1 = 1.778×10^9 bacteria/mL). Dilute the bacteria into warm serum-free non-italics MGM 10/10 at a Multiplicity of Infection (MOI) of 30 using 15 mL conical tubes for each infection [7].
4. Use a phase-contrast light microscope to check for macrophage LPS-induced activation and sterility. Remove MGM 10/10 containing LPS from macrophages and wash with 1 mL of warm $1 \times$ PBS. Remove the PBS wash immediately and add 3 mL of media containing bacteria from **step 3** in appropriate wells. At least two wells should be incubated with serum-free MGM 10/10 only for uninfected control and for maximum LDH release control (described on Subheading 3.6).

For a positive control, use 0.2 µg/mL final concentration of purified *C. difficile* TcdB in serum-free MGM 10/10 (*see* Note 5).

5. After addition of media with bacteria to LPS-primed BMDMs, spin plates for 5 min at $112 \times g$ (rt) to facilitate the contact of *Y. pseudotuberculosis* with BMDMs. Incubate plates for 90 min at 37 °C with 5% CO₂ [7].
6. After 90 min, check BMDMs by phase-contrast light microscopy to confirm that the bacterial infection has resulted in YopE- and YopT-mediated cell rounding and collect 1.5 mL supernatant from each well, with the exception of the LDH control. Spin supernatant in microcentrifuge tube for 10 min at $20,000 \times g$ (4 °C) and transfer 1 mL to a new tube. Store at –20 °C.
7. Remove the remaining culture media from plates and wash the BMDMs once with 1 mL of cold HBSS. Add 100 µL of RIPA lysis buffer with protease inhibitor cocktail to coat each well and incubate plates on ice for 10–15 min (*see* Note 20).
8. Remove the plates from ice and use a pipet tip to scrape the bottom of plates to suspend remaining cell debris. Collect the buffer with lysed cells and spin in a microcentrifuge tube for 20 min at $20,000 \times g$ (4 °C). Transfer cell lysate supernatant to a new tube and store it at –20 °C.
9. The LDH control well should be kept at –80 °C until used (*see* Note 21).

3.3. Western Blot for Detection of Total and Phosphorylated Pyrin

1. Calculate total protein concentration in cell lysate by using a protein assay standard curve (described in Subheading 2.3, **item 2**).
2. Resolve the samples on a 10% SDS-PAGE gel at 200 V. Load between 3 and 15 µg for best resolution and visualization of proteins.
3. After electrophoresis, transfer proteins to a PVDF membrane for 1 h at 350 mA.
4. Block membrane for 1 h in Blocking solution.
5. Probe membrane overnight by shaking it at 4 °C with rabbit monoclonal antibody against mouse pyrin in Blocking solution in a 1:1000 dilution for detection of total pyrin (*see* Notes 15 and 20).
6. For detection of phosphorylated (inactive) pyrin, probe membrane overnight by shaking it at 4 °C with rabbit monoclonal antibody against mouse S205 phosphorylated pyrin, in Blocking solution in a 1:1000 dilution (*see* Notes 15 and 20).
7. Wash excess primary antibody from membrane with 1× TBS-T four times and probe with secondary goat antirabbit antibody conjugated to HRP, in a 1:10,000 dilution. This secondary antibody can be used for detection of both S205 phosphorylated pyrin and total pyrin.
8. After 1 h, remove secondary antibody and wash membrane with 1× TBS-T four times. Visualize desired proteins by using ECL chemiluminescent detection

reagent. Signal visualization can be obtained by exposure to X-ray film or through use of a Western Blot Imager. Bands for both phosphorylated (inactive) and total pyrin should be visualized between 100 and 110 kb.

9. As a loading control, a primary rabbit monoclonal antibody against mouse β -actin can be used on the same membranes. Use an antirabbit secondary antibody conjugated to an HRP for visualization, as described in **step 6**.

3.4. Western Blot for Detection of Caspase-1 Proteolysis

1. Using the total protein concentration obtained in Subheading 3.1, **step 1**, load between 3 and 15 μ g of protein into a 4–12% gradient gel. Resolve the samples by running the gel in 1 $\times$ MES running buffer at 175 V and transfer to a PVDF membrane as described above.
2. Block membrane for 1 h in Blocking solution and probe overnight for caspase-1 by shaking at 4 $^{\circ}$ C using anti caspase-1 rabbit polyclonal antibody [7] in Blocking solution in a 1:10,000 dilution.
3. Wash excess primary antibody from membrane with 1 $\times$ TBS-T three times and probe with secondary polyclonal goat antirabbit IgG (H + R) + HRP in a 1:20,000 dilution. Wash excess secondary antibody with 1 $\times$ TBS-T four times. Signal visualization can be obtained as in Subheading 3.3, **step 6**. Using the gradient gel and the antibody described in **step 2** enables the visualization of both procaspase-1 and cleaved caspase-1 (*see* Note 16).
4. In this case, β -actin can also be used as a loading control. Follow the same steps as summarized on Subheading 3.3, **step 7**.

3.5. Supernatant Analysis for IL-1 β Release: ELISA Kit

1. Thaw frozen supernatants at room temperature. Spin samples at 20,000 $\times g$ in a microcentrifuge (rt). Prepare reagents according to the commercial kit protocol. Prepare the Mouse IL-1 β standard according to vial label. This generates an 800 pg/mL stock solution, which can be used to produce twofold dilutions series from 800 to 0 pg/mL. Those dilutions will be part of the standard curve.
2. The kit provides 96-well microplates with strips of wells. Remove excess wells and save for future experiments.
3. Follow the kit protocol. A microplate reader that can be set between 450 and 570 nm is necessary for results collection. Analyze through a software that generates graphs and compares different data sets (Excel, GraphPad Prism, etc.). The standard curve should generate an equation based on absorbance $\times$ concentration: this formula should be used to calculate samples concentration (*see* Note 22). Graph needs to compare IL-1 β release in the different samples.
4. For biological relevance, collect data from three independent experiments, and analyze and compare them through a statistical test (one-way ANOVA, two-way ANOVA, Mann-Whitney, etc.), according to the experiment goals.

3.6. Supernatant Analysis for Cell Death Through Pyroptosis: LDH Release Assay Kit

1. Thaw frozen supernatants at room temperature as described in Subheading 3.5, step 1.
2. Thaw LDH control well, collect media, and spin in a microcentrifuge tube for 20 min at $20,000 \times g$ (4 °C). Have a no-cell control, containing only fresh MGM 10/10 as a negative control.
3. Set up a 96-well flat bottom plate and add 50 μ L aliquots from controls and samples. Follow kit protocol. In this case, a microplate reader that analyzes 490 or 492 nm is necessary.
4. Calculate the results according to kit protocol and analyze data through graph-generating software, as explained in Subheading 3.5, **step 4**. In this case, the graph is comparing LDH release percentage in the different samples.
5. For biological relevance, collect data from three independent experiments, and analyze and compare them through a statistical test (one-way ANOVA, two-way ANOVA, Mann-Whitney, etc.), according to the experiment goals.

4. Notes

1. Water level in water bath should be sufficient to cover at least half of the bottle of FBS. Ice level should be enough to be above serum level during 1 h.
2. Constant thaw–freeze cycles are not recommended for FBS. Make sure to aliquot necessary amounts for one experiment. Test new batches of FBS before using to make sure it supports BMDMs growth and differentiation.
3. Before starting L929 cell culturing, check for cracks or broken 175 cm² flasks. Also, make sure to have enough flasks for the whole experiment (10–20 flasks). By 10–12 days, L929 cells should be attached to bottom of 175 cm² flasks. In case the cells start to detach, remove media immediately, transfer to 50 mL conical tubes, and spin down in a centrifuge at $112 \times g$ (rt) before filtering, to avoid clogging. Thaw–freeze cycles are not recommended for L929 conditioned media. Make sure to aliquot necessary amounts for one experiment.
4. Wash media mixture should be at room temperature for best bone marrow harvest. Sterilization is not required for this step.
5. FBS is essential for BMDM growth and differentiation. However, during intoxication of cells with TcdB, serum-free media is required. FBS and TcdB compete for the same cell receptors and toxin effects will not be observed [10]. Thus, we recommend that for infections using TcdB as a positive control, **step 4** from Subheading 3.2 is followed. To generate serum-free media, follow same steps for media preparation (*see* Subheading 2.1, **items 4–6**); however, *do not add FBS*. Adjust DMEM volume accordingly. Do not change other components concentrations. Absence of FBS on *Yersinia* spp. infections should not affect results. Serum-free media and FBS-containing media should be warmed to 37 °C before application.

6. The LPS stock generated (100 µg/mL) will allow a 1:1000 dilution for a final concentration of 100 ng/mL. Due to the small volume necessary to prime the cells in each well (3 µL of LPS if using 3 mL/well), we recommend a 1:10 dilution of the LPS in PBS as a first step. From this dilution, use a volume equivalent to 1:100 dilution into the wells, to have a 100 ng/mL final concentration of LPS.
7. Pyrin inflammasome is triggered in the absence of YopM, but in the presence of YopE and YopT. It is important to use mutants lacking *yopM* in order to observe the inflammasome effects. Although the described protocol was established in *Y. pseudotuberculosis*, other pathogenic *Yersinia* species that can produce the T3SS, such as *Y. pestis* [19] and *Y. enterocolitica*, are suitable for those experiments.
8. Other types of cell lysis buffers, such as NP-40 lysis buffer [7], can be used for those experiments. The cell lysis buffer should be carefully chosen depending on the protein analyzed and its cellular location. RIPA thaw–freeze cycles are not recommended. Make sure to create aliquots that have enough lysis buffer for one experiment.
9. BSA for standard curve and samples should be diluted in the cell lysis buffer used to generate cell lysate. Dilutions of 1:8 are recommended for analysis of cell lysate samples. In case 1:8 sample dilutions have absorbance values lower than absorbance value of lowest standard curve dilution, repeat experiment by making less diluted samples (1:6, 1:4, 1:2, etc.). Although BCA protein assay is explained in this protocol, other protein quantification assays, such as Bradford, can be used.
10. PVDF membranes need to be activated prior to protein transfer. To do that, place membrane into Methanol. The volume should be enough to cover the membrane. Leave it for at least 10 min. Wash three times with double-distilled water and assemble transfer apparatus.
11. While preparing 1× Transfer buffer, add the methanol last. This will prevent precipitation of buffer components.
12. 1× TBS-T should have Tween concentrations between 0.05% and 0.1% for best results.
13. The ECL reagent kit comes with two reagents, A and B. Mix them in a 1:1 dilution immediately before using film or Western Blot Imager. The reagent is light-sensitive and might lose effectiveness if mixed in advance. For membranes of size 7 cm × 10 cm or smaller, no more than 500 µL ECL mix reagent is necessary to visualize proteins. If the membrane is bigger than the sizes specified, increase volume of reagent.
14. Upon arrival of antibodies, spin down tubes in microcentrifuge tube at 20,000 × *g* (rt) before rehydration and/or aliquoting. Avoid thaw–freeze cycles.
15. Antibody against total pyrin binds pyrin specifically between amino acids 50–450. This allows detection of both active and inactive pyrans. Antibody against

S205 phosphorylated pyrin binds only pyrin that is specifically phosphorylated at serine 205. This allows detection of inactive pyrin only.

16. Antibody against caspase-1 can bind to precursor procaspase-1 (50 kDa) and cleaved caspase-1 at p20 subunit (20 kDa).
17. Rehydration of secondary antibody can be made in water only as well. For extended storage in this case, store at -80°C .
18. Note that thawing and freezing cycles of cell supernatants should be avoided. It is recommended that both ELISA and LDH assays are performed in the same day.
19. Microplate reader needs to be capable of recording absorbances 490 or 492 nm (LDH assay) and 450 and 540 or 570 nm (ELISA).
20. If using the antibodies described in Subheading 2.4, **items 1 and 2**, avoid the addition of phosphatase inhibitors to the cell lysis buffer. The phosphatase inhibitor treatment can change the binding properties of the total pyrin antibody, leading to suboptimal results. If phosphatase inhibitor treatment is required, use a rabbit monoclonal antibody against mouse total that recognizes amino acids 500–750 (Abcam® ab 214772).
21. The freezing and thawing of macrophages generates maximum cell death and can be used as a positive control during the LDH release analysis.
22. For accurate results, make sure that the standard curve has R^2 equivalent or higher than 0.95.

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Fig. 1.

Yersinia spp. infection triggers the pyrin inflammasome in the absence of YopM. (1) A *Yersinia* bacterium lacking YopM secretes the other six Yop effectors into host cell cytoplasm. (2) YopE and YopT inactivate RhoA by GAP and C-terminal cleavage activities, respectively. (3) The inactivation of RhoA leads to the generation of dephosphorylated pyrin. (4) Dephosphorylated pyrin is active and can recruit Asc and procaspase-1 to form the inflammasome. (5) The inflammasome leads to the generation of mature caspase-1. (6) Caspase-1 can cleave gasdermin-D (GSDMD) to generate GSDMD N-terminal fragments and (7) can also generate mature IL-1β. (8) GSDMD N-terminal fragments can form pores in the membrane, (9) allowing the secretion of IL-1β. (10) GSDMD pores also cause cell death by pyroptosis