Methods for Assessing the Effects of LXR agonists on Macrophage Bacterial Infection

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Short title: LXRs and Macrophage Bacterial Infection

Abstract

Macrophages are phagocytic cells that actively engulf and kill microorganisms within a specialized phagolysosomal system. Several pathogenic bacteria, however, actively co-opt host mechanisms and escape from microbial digestion to establish intracellular replication within macrophages. This chapter highlights detailed protocols to measure the effects of the LXR pathway on bacterial infection of murine bone marrow-derived macrophages.

Keywords

LXR, macrophage, bacteria, infection

1. Introduction

Macrophages play essential roles in the immune response against pathogens. Upon recognition of pathogen-associated molecular patterns and/or opsonins, they internalize microorganisms, including bacterial cells, through phagocytosis. After engulfment, macrophages kill and digest the internalized material within the phagolysosomal system ***(1)***. Although a wide range of microorganisms are successfully eliminated by phagocytes, several pathogenic bacteria have developed strategies, including the capability to actively invade host cells and escape from microbial digestion within phagolysosomes, to survive within the host. Paradoxically, despite harbouring an arsenal of microbicidal tools, macrophages represent a cellular compartment in which many pathogens establish for intracellular replication and subsequent dissemination ***(2)***. As an example, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) actively promotes its own uptake by macropinocytosis through the use of a type III secretion system that allows the bacterium to inject effectors that target the host cell cytoskeleton ***(3)***. Later on, the bacterium is able to modify the phagosome transforming it into a Salmonella-containing vacuole that supports bacterial cell survival and replication. Infection and intracellular survival in macrophages is required for full virulence of *S.* Typhimurium *in vivo* ***(4)***.

Nuclear receptors are a family of ligand-activated transcription factors that control many aspects of physiology. Within this family, liver X receptors (LXRs) are activated by specific oxidized forms of cholesterol (oxysterols) and intermediaries of cholesterol biosynthesis to subsequently regulate the expression of genes involved in lipid and glucose homeostasis and in immune responses ***(5, 6)***. Two LXR isoforms have been

described (LXRα and β) and each of them forms heterodimers with retinoid X receptors (RXRs) to positively modulate target gene expression. Recent work from our group has identified a molecular mechanism by which LXR agonists interfere with the capability of *S.* Typhimurium to infect murine macrophages ***(7)***. This mechanism involves transcriptional activation of the NADase CD38, which translates in reduced intracellular NAD+ levels and interferes with pathogen-induced changes in the F-actin cytoskeleton, limiting the capability of non-opsonized Salmonella to infect macrophages.

In this chapter we describe in detail protocols based on the use of flow cytometry and confocal microscopy to measure the effects of the LXR pathway on infection of murine bone marrow-derived macrophages by an invasive *S.* Typhimurium strain.

2. Materials

All the materials must be sterile and endotoxin-free.

1. LXR agonists: T0901317, GW3965 and 25-hydroxycholesterol.
2. RXR agonist: LG100268.
3. Dimethyl sulfoxide (DMSO).
4. High-glucose DMEM with L-glutamine and without sodium pyruvate.
5. Foetal Bovine Serum (FBS).
6. Phosphate-buffered saline (PBS) without calcium/magnesium.
7. Bacterial liquid growth medium 2xYT: 1.6 % Bacto tryptone, 1 % Bacto yeast extract, 0.5 % NaCl, pH 7.

8. Bacterial liquid growth medium Super Optimal Broth (SOB): 2 % Bacto tryptone, 0.5 % Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM MgSO4, pH 7.

9. Ampicillin.

10. Transformation buffer 1: 0.1 M RbCl, 50 mM $MnCl_2{\cdot}4H_2O$, 30 mM KAc, 10 mM $CaCl_2{\cdot}2H_2O$, 10 % glycerol, pH 5.8.

11. Transformation buffer 2: 0.2 M MOPS, 10 mM RbCl, 75 mM $CaCl_2{\cdot}2H_2O$, 12 % glycerol, pH 6.8.

12. 100 mL and 1L Erlenmeyer flasks.

13. Tissue-culture plates (6-well and 24-well).

14. Cell scrapper.

15. 50 mL polypropylene tubes.

16. 1.5 mL polypropylene microtubes.

17. Centrifuge.

18. Hemacytometer.

18. Liquid bath.

19. Incubator at 37 °C.

20. Humidified incubator at 37 °C, 5 % CO_2.

21. Horizontal shaker.

22. Petri dishes containing solid LB-agar.

23. Bunsen burner.

24. L-shaped glass spreader.

25. 75% ethanol.

26. Spectrophotometer.

27. Paraformaldehyde (PFA) 4% in PBS, prepared fresh.

28. Wheat germ agglutinin (WGA) conjugated to Alexa Fluor 488.

29. DAPI (1 μg/ml).

30. Mounting medium.

31. Microscopy slides and coverslips.

32. Milli-Q H_2O.

33. Flow cytometer (equipped with an excitation red laser (561 nm) and fluorescence detector at 610/20 nm)

34. Confocal microscope (equipped with lasers exciting at 405 nm, 488 nm and 561 nm wavelengths).

3. Methods

All the steps before sample processing must be carried out under sterile conditions.

3.1 Macrophage Plating and Culture

1. Murine bone marrow-derived macrophages should be generated as described ***(8)***.

2. Plate macrophages using the following indications:

3.1.1. For flow cytometry:

1. Plate 1.5 x 10^6 macrophages per well in 6-well plates with 2 mL of DMEM-10 % FBS and allow them to attach for 2 h in a humidified incubator at 37 °C, 5 % CO_2.

3.1.2. For confocal microscopy:

1. Plate 2 x 10^5 macrophages per well in 24-well plates containing UV-sterilized coverslips. Cover the cells with 1 mL of DMEM-10 % FBS and allow them to attach for 2 h in a humidified incubator at 37 °C, 5 % CO_2.

3.2 Treatment with LXR/RXR agonists

1. Prepare a stock solution of LXR (or RXR) agonist (1-10 mM in DMSO). Store stock at -80 °C.

2. Once the cells have attached to the plates, add LXR/RXR agonists at 1 µM each for the desired period of time (*see* **Note 1**). Culture cells in a humidified incubator at 37 °C, 5 % CO_2. For control samples, incubate the cells with DMSO (vehicle) at the same dilution than the one in the samples treated with ligands.

3.3 Obtention of bacterial cells expressing red fluorescent protein (RFP)

This protocol shows steps used for the generation of fluorescent *S.* Typhimurium strain SL1344. To avoid contamination by other microorganisms, perform all the steps next to a Bunsen burner or inside a biological safety cabinet with laminar flow.

3.3.1. Prepare competent bacterial cells for heat shock transformation.

1. Inoculate a bacterial colony in a 100 mL Erlenmeyer flask containing 30 mL of SOB.
2. Grow bacteria overnight at 37 °C with horizontal shaking at 250 rpm.
3. Subculture the cells in a 1 L Erlenmeyer flask using a 1:50 dilution in 100 mL of SOB.

4. Grow bacteria at 37 °C with horizontal shaking at 250 rpm until the optical density of the culture at 600 nm (OD_{600}) reaches 0.45 (mid-log phase).

5. Distribute the volume to four 50 mL Falcon tubes and keep them on ice for 15 min.

6. Centrifuge for 15 min at 3000 g, 4 °C to pellet the bacterial cells.

7. Remove supernatants and resuspend bacterial cell pellets in 10 mL of transformation buffer 1.

8. Centrifuge for 15 min at 3000 g, 4 °C.

9. Remove supernatants and resuspend in 4 mL of transformation buffer 2.

10. Prepare 500 µL aliquots and freeze at -80 °C.

3.3.2. Transform *S.* Typhimurium strain SL1344 with pBR.RFP.1 plasmid encoding red fluorescent protein (RFP) ***(9)*** (*see* **Note 2**) by heat shock.

1. Mix 100 µL of competent bacteria with 1-10 ng of supercoiled plasmid DNA. For the negative control, use H_2O instead of plasmid DNA.

2. Keep on ice for 30 min.

3. Perform the heat shock at 42 °C for 1.5 min (in a liquid bath).

4. Put the cells on ice for 5 min.

5. Add 2xYT medium up to 1 mL and grow the transformed bacterial cells in a 1.5 mL microtube for 1 h at 37 °C with horizontal shaking at 250 rpm.

6. Plate 50 µL bacterial growth onto LB-agar plates supplemented with 100 µg/mL ampicillin and allow the colonies to grow for 15 h at 37 °C.

3.4 Bacterial cell growth

1. Pick a bacterial colony with a sterile pipette tip and transfer it to a 50 mL tube containing 10 mL 2xYT medium supplemented with 100 µg/mL ampicillin. To obtain a saturated culture, let the bacteria grow for at least 16 h at 37 °C with horizontal shaking at 250 rpm.

2. Once at saturation (OD_{600} = 2), dilute the bacterial cell culture 1:100 by transferring 100 µL to a new 50 mL tube with 10 mL 2xYT and culture it at 37 °C, 250 rpm for 2-3 h (at this time bacterial growth would be in log phase and bacterial cells would express optimal levels of effectors for invasion).

3. Measure the OD_{600} of the bacterial cell culture using a spectrophotometer and estimate the bacterial cell concentration using a standard curve (*see* **Note 3**).

3.5 Macrophage infection

1. Add bacteria to the macrophage culture at the desired multiplicity of infection (MOI) (*see* **Note 4**). Let the infection occur for 30 min in an incubator at 37 °C, 5 % CO_2. Also include two types of controls: a) negative control (non infected cells) and b) control for attachment without engulfment (macrophages are incubated with the bacteria for 30 min at 4 ^{o}C).

2. Place the plates on ice to stop infection and bacterial replication. Remove culture media and wash the cells three times with 2mL of ice-cold PBS to remove non internalized bacteria.

3.6 Process the cells for the analysis of infection:

3.6.1. Analysis by flow cytometry

1. Scrape cells in 1 mL PBS and transfer them to 1,5 mL microtubes.

2. Centrifuge for 5 min at 0.2 g, 4 °C to pellet the cells.

3. Remove supernatant and fix the cells in 150 µL 4 % PFA for 30 min at room temperature.

4. To measure infection, analyse RFP fluorescence in macrophages by flow cytometry using a 561 nm laser for excitation and a 610/20 nm filter for the detection of emission. Check bacterial cell fluorescence by comparing it to a negative control (bacterial cells not transformed with an RFP encoding plasmid).

3.6.2. Analysis by confocal microscopy

1. Fix cells on coverslips (inside the cell culture plates) with 300 µL 4% PFA for 30 min at room temperature.

2. Wash cells with 1 mL PBS.

3. Stain cell membranes with 2.5 µg/mL fluorescent WGA for 30 min at room temperature.

4. Stain nuclei with 1 µg/ml DAPI for 5 min at room temperature.

5. Wash cells twice for 5 min with 1 mL PBS.

6. Wash cells with 1 mL mQ H_2O.

7. Plate coverslips on microscopy slides with a drop of mounting medium (follow recommendations for the specific mounting medium).

8. Store samples in the dark at 4 °C until subsequent analysis by confocal microscopy (*see* **Note 5**).

9. For each fluorochrome, collect serial 1 µm z-axis optical images from whole cells using a 63X objective. Use the following lasers: 405 nm (DAPI, nuclei), 488 nm (WGA-Alexa Fluor 488, membranes) and 561 nm (RFP, bacteria).

3.6 Estimation of MOI after the infection

1. Make dilutions of bacterial culture in 1.5 mL microtubes with 2xYT (*see* **Note 6**).

2. Dispense 100 µL of each dilution on LB-agar plates (in duplicates) and incubate overnight at 37 °C.

3. Count colonies to calculate the exact MOI used in the experiment.

4 Notes

1. LXRs form heterodimers with RXRs to activate transcription of their target genes. Agonists for both nuclear receptors can be used (1µM each) to obtain synergistic effects on induction of gene expression.

2. Transformation with a plasmid which, in addition to fluorescence, confers antibiotic resistance enables the selection of bacteria of interest and avoids contamination by other bacteria. When using antibiotic-resistant colonies, antibiotics should be added to the solid LB-agar plates and to the bacterial growth medium.

3. To obtain a standard curve make serial dilutions of a saturated bacterial cell culture ranging from the original concentration to 1:100 dilution (e.g. 1:2, 1:4, 1:10, 1:100). Measure OD600 and dispense 100 µL of each dilution on solid LB-agar plates. Incubate the plates with bacteria overnight at 37 °C. Then count colonies to calculate the bacterial cell concentration and generate the standard curve by plotting cell concentration vs absorbance.

4. At MOI 5-10, invasive *Salmonella* will infect 10-40% macrophages within 30 min and a reduction of infection by LXR agonists can be detected.

5. For optimal results, analysis of infection by confocal microscopy should be performed no longer than 2 weeks after sample preparation.

6. Despite the fact that an estimation of the bacterial MOI is performed before the infection based on the OD_{600} of the bacterial growth, it is recommended to calculate the exact MOI used in each experiment by plating bacterial cell dilutions on LB-agar plates. Usually, dilutions 10^{-6}-10^{-7} work best for subsequent counting of viable bacterial colonies on plates.

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7 References

1. Flannagan RS, Cosío G, and Grinstein S (2009) Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. Nat Rev Microbiol 7:355–366
2. Price JV and Vance RE (2014) The Macrophage Paradox. Immunity 41:685–693
3. Guiney DG and Lesnick M (2005) Targeting of the actin cytoskeleton during infection by Salmonella strains. Clin Immunol 114:248–55
4. Haraga A, Ohlson MB, and Miller SI (2008) Salmonellae interplay with host cells. Nat Rev Microbiol 6:53–66
5. Hong C and Tontonoz P (2014) Liver X receptors in lipid metabolism: opportunities for drug discovery. Nat Rev Drug Discov 13:433–44
6. Pascual-García M and Valledor AF (2012) Biological roles of liver x receptors in immune cells. Arch Immunol Ther Exp (Warsz) 60
7. Matalonga J, Glaria E, Bresque M, et al (2017) The Nuclear Receptor LXR Limits Bacterial Infection of Host Macrophages through a Mechanism that Impacts Cellular NAD Metabolism. Cell Rep 18
8. Valledor AF, Comalada M, Xaus J, et al (2000) The differential time-course of extracellular-regulated kinase activity correlates with the macrophage response toward proliferation or activation. J Biol Chem 275:7403–7409

9. Birmingham CL, Smith AC, Bakowski MA, et al (2006) Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. J Biol Chem 281:11374–83