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Growing and handling of *Mycobacterium tuberculosis* for macrophage infection assays

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Summary

Macrophage survival assays are a critical component of any *Mycobacterium tuberculosis* research program. Here we describe the methods that we use routinely for infection of macrophages of various origins. The protocols are efficient, relatively simple and are accepted widely. We provide users with methods for the infection of small numbers of macrophages--more suitable for microscopy; and for larger numbers of macrophages--for flow cytometry analysis or extraction for biochemical characterization.

Keywords

phagocytosis; macrophage; *Mycobacterium tuberculosis*; infection

1. Introduction

Although we have learned a lot about biology of *Mycobacterium* spp. that are cultured in broth rich with nutrients, there is a critical need to understand how the pathogen adapts to more natural environments – such as its host macrophage. Macrophages are among the very first cells that will encounter *Mycobacterium tuberculosis* (Mtb) following its inhalation into the lung and they play crucial role in the outcome of infection. Therefore *in vitro* models of macrophage infection represent a powerful tool for studying host-pathogen interaction. However it is important to remember that this bacterium is a human pathogen and must be handled under biosafety level 3 containment (see Note 1). Using this model the dynamics of transcriptional response of Mtb to the host environment has been successfully probed (1–3). Through the use of fluorescently labeled reporter Mtb we have been able to look at the localization of bacteria, and the availability of host lipids within the macrophage (4). Flow cytometry and functional physiological assays have enabled us to assess impact of infection on relative phagosomal function and metabolic state (4). Finally we have performed an extensive empirical screen for small molecules capable of impairing the survival of Mtb in its host macrophage and identified many inhibitors that blocked growth in intracellular Mtb

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[1]*Mycobacterium tuberculosis* is a human pathogen and must be handled under Biosafety Level 3 containment by trained personnel. Such facilities have to be maintained under registered institutional safety and employee health monitoring programs. Some investigators use non-pathogenic *Mycobacterium* spp., such as *Mycobacterium smegmatis,* as a surrogate but the relevance of data generated with non-pathogenic organisms is open to obvious concerns regarding validity.

but had no effecto on bacteria in rich broth (5). These experimental data all support the contention that macrophage infection and survival assays are critical to expanding our understanding of the biology of the infectious agent as well as its host.

Here we describe the methods that we use for efficient infection of macrophages of differing host origins. One of the biggest technical challenges for these infection assays is the extremely hydrophobic nature of the bacterial cell surface due to its high lipid content. To counteract this we use gelatin-containing infection medium to break up clumps of bacteria without lysing macrophages in addition to the multiple passaging of bacterial suspensions through syringe needles.

We include protocols for both small and large scale infections. The small scale protocols are intended for analysis by methods such as confocal microscopy. Whereas the large scale infection protocols are designed for isolation of macrophages for lipid or protein extraction, bacterial and host RNA and DNA isolation, electron microscopy, flow cytometry or isolation of bacteria after macrophage infection. It is intended that these methods may be modified to accommodate the specific needs of your assay and appropriate scale of choice.

2. Materials

2.1. Cells, Reagents, and Buffers

1. Macrophages: Bone marrow–derived murine macrophages (BMMØ) are our cells of choice for these assays. BMMØ are derived from the bone marrow extracted from the femur, tibia, and ilium of euthanized mice and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% L-cell conditioned media (BMMØ media). J774 cells (available from the American Type Culture Collection, Rockville, MD) are maintained in DMEM supplemented with 10% FBS and 2 mM L-glutamine + 1 mM sodium pyruvate.
2. **For small-scale protocol**: Sterile glass slide with lid and with chambers of preferred format. Typically, 8-well multi-chamber slide with glass coverslip (ibidi, etc.) bottom and with 1 cm^2 growth area per well is used if infected macrophages will be examined by confocal microscopy.
3. **For large-scale protocol**: Sterile 75 cm^2 polystyrene tissue culture flask with filtered cap. Sterile 150 cm^2 polystyrene tissue culture flask with filtered cap also can be used (both from Corning etc) (*see* Note 2).
4. BMDM medium: DMEM supplemented with 10% fetal calf serum, 10% L-cell conditioned medium, 2 mM L-glutamine, 1 mM sodium pyruvate. Filter-sterilize. Store at 4°C for no longer than 2 months.
5. HMDM medium: DMEM supplemented with 10% human serum, 2 mM L-glutamine, 1 mM sodium pyruvate. Filter-sterilize. Store at 4°C for no longer than 2 months.

[2]In case of 150 cm^2 tissue culture flasks double all the amounts described for 75 cm^2 tissue culture flask.

6. Antibiotics: 100 U/mL penicillin and 100 μg/mL streptomycin.
7. 25 cm^2 and/or 75 cm^2 polystyrene tissue culture flask with filtered cap or any sterile flask covered with filtered cap that allows to grow up to 10 ml and 40 ml of bacterial culture, respectively, with approximately 50 ml and 230 ml of free space inside the flask.
8. Middlebrook 7H9 OADC medium: 4.7 g of the Middlebrook 7H9 Broth dehydrated base powder, 2 ml glycerol, 0.05% tyloxapol, 100 mL of Middlebrook OADC Enrichment, 900 mL of distilled water. Filter-sterilize. Store at room temperature.
9. 15 ml conical tube(s).
10. Basal uptake buffer (BUB): 2.25 g glucose, 2.5 g bovine serum albumin, 0.5 ml gelatin, 50 mg $CaCl_2$, 50 mg $MgCl_2$, 500 ml phosphate-buffered saline (PBS). Filter-sterilize, store at 4°C for no longer than 6 months (*see* Note 3).
11. Sterile 1 ml tuberculin syringe with 25 gauge needle.

2.2. Instruments

1. CO_2 incubator.
2. Spectrophotometer with ability to measure absorbance at 600 nm.
3. Centrifuge that allows to spin 15 ml-conical tubes at 3,300 × g.

3. Methods

3.1. Macrophage preparation

3.1.1. Small scale, for microscopy

1. Seed macrophages into glass slide with chambers of the preferred format at about 80% confluency in macrophage media without antibiotics. Typically, 8-well multi-chamber slide with glass coverslip bottom is used to seed 1.8×10^5 murine BMDM in 0.2 ml of macrophage media per well (*see* Note 4).
2. Incubate at 37°C and 7% CO_2 overnight. Since murine BMDM can still slowly replicate, you need to assume that there is 2×10^5 cells per well on the day of infection.

3.1.2. Larger scale: 75 cm^2 or 150 cm^2 tissue culture flasks—Here we give an example how to perform an infection of macrophages seeded in one 75 cm^2 tissue culture flask. Double all the amounts if you wish to use 150 cm^2 tissue culture flask.

1. Seed 1.5×10^7 macrophages in 20 ml of macrophage media without antibiotics per flask.

[3]If gelatin becomes solidified, pre-warm it at 55–65 °C overnight. Due to gelatin high viscosity, syringe would be the easiest way to measure it out.
[4]If you are using human monocyte-derived macrophages, seed 3×10^5 monocytes per well in media containing antibiotics 7–10 days before infection, change for media without antibiotics on day 7 of incubation.

2. Incubate at 37°C and 7% CO_2 overnight.

3.2. Preparation of bacterial cultures

1. Inoculate 8 ml Middlebrook 7H9 OADC medium in a 25 cm^2 tissue culture flask with approximately 0.1–0.2 ml of frozen *M. tuberculosis* glycerol stock.
2. Incubate at 37°C in standing culture for 7–10 days, until culture reaches logarithmic phase (*see* Note 5).
3. Pass into required volume of Middlebrook 7H9 OADC medium depending on scale of infection at starting optical density at 600 nm (OD_{600}) of about 0.05. Use 25 cm^2 flasks for volumes not more than 10 ml, and 75 cm^2 flasks for volumes up to 40 ml (*see* Note 6).
4. Incubate at 37°C in standing culture for 7 days, culture should reach OD_{600} of approximately 0.6 (*see* Note 7).

3.3. Infection Protocol

3.3.1. Small scale, for microscopy

1. Measure OD600 of bacterial culture. Spin down equivalent of 2ml of culture with OD600=0.6 in 15 ml conical tube at 3,300 × g for 10 minutes. For example, if your culture has an OD600 of 0.5, you need to spin down 2.4 ml (0.6*2ml/0.5=2.4ml).
2. Assuming that OD600=0.6 gives 10^8 bacteria/ml, you will have 2×10^8 bacteria in the pellet.
3. Resuspend bacterial pellet in 2 ml of BUB, so that you have 1×10^8 bacteria per ml. Place this suspension in a new 15 ml centrifuge tube (*see* Note 8).
4. Using a 1ml tuberculin syringe with 25 gauge needle insert the syringe into the Falcon tube with extreme care and pass the bacterial suspension in and out of the syringe 12–15 times (*see* Note 9). Proceed to the next step immediately, so that bacteria don't have chance to clump (*see* Note 10).
5. Add required volume of bacterial suspension to the macrophages to achieve desired multiplicity of infection (MOI) (Table 1) (*see* Note 11). In order to

[5]Cultures can be grown shaking to avoid the generation of hypoxic conditions. In that case, the rate of growth will be twice as fast, so adjust timing accordingly. We prefer to use standing cultures so that the surface glycocalyx of Mtb is less disturbed during culture.
[6]It's important to pass macrophages after inoculum culture had grown, since the latter quite often contains clumps of bacteria. Therefore avoid transferring these clumps directly into the culture when you perform this procedure.
[7]We recommend using cultures at logarithmic stage for infection, since they are more homogeneous.
[8]Try not to touch sides of the new tube with any part of pipette for safety reasons.
[9]It's important to use 15 ml conical, since tuberculin syringe fits perfectly inside of it without touching the bottom. When attaching needle to the syringe be careful not to touch the main part of syringe below the plunger to avoid contamination of bacterial suspension. Keep the syringe in the tube throughout this entire process to minimize the potential for self-inoculation. This process can be performed with blunt syringe needles.
[10]If you wish to perform infection with different strains of Mtb, you shouldn't syringe more than 4 bacterial suspensions before proceeding to the next step to avoid re-clumping of bacteria.
[11]We have noticed that different strains of Mtb behave differently in macrophage infection and induce differing levels of cytotoxicity. When infecting murine BMDM, CDC1551 can be used at higher MOI such as 5 and 10 for 7–10 days of infection without eliminating

minimize dilution or avoid over-filling the well it is advisable not exceed 40 μl of BUB. If volume is too small to pipette, dilute bacterial suspension as necessary.

6. Mix bacteria with macrophage media in the well by gentle pipetting up and down 5 times (*see* Note 12).
7. Incubate at 37°C and 7% CO_2 for 2–5 hours.
8. Replace media used for infection with 0.22 ml of fresh macrophage media without antibiotics. If assay will exceed two days, change media every other day.

3.3.2. Larger scale: 75 cm^2 tissue culture flask: Our calculations are designed to generate an MOI of 4:1. Adjust amounts accordingly if you need to use different MOI.

1. Measure OD of bacterial culture. Spin down equivalent of 1ml of culture with OD600=0.6 in 15 ml conical tube at 3,300 × g for 10 minutes. For example, if your culture has an OD600 of 0.5, you need to spin down 1.2 ml (0.6*1ml/0.5=1.2ml).
2. Assuming that OD600=0.6 gives 10^8 bacteria/ml, you will have 1×10^8 bacteria in the pellet.
3. Resuspend bacterial pellet in 1.5 ml of BUB. Place this suspension in a new 15 ml Falcon tube in an appropriate holder (*see* Note 8).
4. Using a 1ml tuberculin syringe with 25 gauge needle insert the syringe into the Falcon tube with extreme care and pass the bacterial suspension in and out of the syringe 12–15 times (*see* Note 9). Proceed to the next step immediately, so that bacteria don't have chance to clump (*see* Note 10).
5. Add 3.5 ml of BUB, mix well. Now you have 2×10^7 bacteria per ml.
6. Transfer 3ml of bacterial suspension into 75 cm^2 flask containing macrophages in 20 ml of macrophage media. Mix well by either pipetting up and down multiple times or gentle shaking (*see* Note 13). Use Table 2 to perform infections at desired MOI.
7. Incubate at 37°C and 7% CO_2 for 2–5 hours.
8. Replace media used for infection with 20 ml of fresh macrophage media without antibiotics. If assay will exceed two days, change media every other day.

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host cells, whereas H37Rv and Erdman shouldn't be used at MOI higher than 5, otherwise macrophages will be killed within 3–5 days. In our experience HMDM are less tolerant of Mtb than murine BMDM, so we usually use MOI for CDC1551 not higher than 5 (0.5–3 is preferred), and for Erdman and H37Rv not higher than 1 to maintain infected macrophage monolayers for at least 5 days.

[12]Make sure that you don't introduce any bubbles in media while pipetting to avoid escape of media due to capillary motion.

[13]If you are infecting multiple flasks, you can mix macrophage media with bacterial BUB-suspension at the same ratios in the separate large flask for all of the samples. Then replace the media in each macrophage-containing flask with this bacterial BUB-macrophage media suspension. Handling the suspension in this manner provides greater consistency between samples.

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Table 1

Calculations of bacterial amounts needed to infect macrophages at desired MOI.

	MOI 1:1	MOI 2:1	MOI 4:1	MOI 5:1	MOI 10:1
Amount of bacteria needed to be added to 2×10^5 cells per well	2×10^5	4×10^5	8×10^5	1×10^6	2×10^6
Volume of bacterial suspension needed to be added to 2×10^5 cells per well, μl	2	4	8	10	20

Table 2

Calculations of bacterial amounts needed to infect macrophages at desired MOI.

	MOI 1:1	MOI 2:1	MOI 4:1	MOI 5:1	MOI 10:1
Amount of bacteria needed to be added to 1.5×10^7 cells in 75 cm^2 flask	1.5×10^7	3×10^7	6×10^7	7.5×10^7	1.5×10^8
Original volume of bacteria at OD600 0.6 needed to be pelleted, ml	0.5	0.5	1	1	2
Total volume of BUB that needs to be added to bacterial pellet, ml	10	5	5	4	4
Concentration of bacteria in BUB suspension, bacteria/ml	5×10^6	1×10^7	2×10^7	2.5×10^7	5×10^7
Volume of bacterial suspension needed to be added to 1.5×10^7 cells in 75 cm^2 flask, ml	3	3	3	3	3