Chapter 14

In Vitro Measurement of Phagocytosis and Killing of *Cryptococcus neoformans* by Macrophages

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Abstract

Macrophages are pivotal cells in immunity against a wide range of pathogens. Their most important property, as suggested by their name, is to ingest pathogens, leading to their killing, the release of inflammatory mediators and antigen processing. On the other hand, macrophages can also be exploited by microbes as a niche for survival in the host, as exemplified by *Cryptococcus neoformans*. This encapsulated yeast is an important cause of meningoencephalitis in immunocompromised people, particularly those with AIDS. Using culture and microscopy techniques, we present here methods that can be used to quantify phagocytosis of *C. neoformans* and its killing by macrophages, as well as the viability of the phagocyte after interaction.

Key words: Macrophage, J774 cells, Phagocytosis, Microscopy, *Cryptococcus neoformans*, Giemsa, Trypan blue, Colony forming units

1. Introduction

Macrophages are cells of the immune system that specialize in clearing self and non-self material via ingestion (phagocytosis) and degradation. In addition to this innate immunity effector role, macrophages also have two additional important functions. They can secrete many cytokines and chemokines that modulate the immune response and present antigens via MHC II, thus making this cell a crucial link between innate and adaptive immunity.

Macrophages play a central role in immunity against several microbial pathogens. In this chapter, we focus on one of them, *Cryptococcus neoformans*, an opportunistic pathogen that causes about 600,000 deaths/year (1). *Cryptococcus neoformans* cells are surrounded by a polysaccharide capsule, which is their most important virulence attribute (2). The presence of the capsule hinders

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access to structures on the surface of the yeast cell that are recognized by macrophage phagocytic receptors, blocking its ingestion by phagocytic cells unless opsonins are present (3). This fact permits detailed studies about phagocytosis mediated by different means, such as antibodies or complement.

The interaction between macrophages and *C. neoformans* can be studied with a wide range of different experiments, each addressing a different aspect (4). All of them start with infection of macrophages with *C. neoformans* in tissue culture plates, detailed in Subheading 3.1. The kinetics of phagocytosis is quantified by the phagocytosis assay (Subheading 3.2), in which the number of macrophages with internalized *C. neoformans* cells is counted on a microscope after staining. The fate of the phagocytosed fungal cells is measured by plating them and counting colony forming units (CFUs) using the *C. neoformans* killing assay (Subheading 3.3), whereas the effects of such interaction on the macrophage itself are evaluated by trypan blue staining followed by microscopy on the macrophage viability assay (Subheading 3.4).

2. Materials

1. Tissue culture medium: Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented with 10% fetal calf serum, 10% NCTC-109 (Gibco/Invitrogen, Grand Island, NY), 1× MEM nonessential amino acids, 100 IU/mL penicillin and 100 μg/mL streptomycin.
2. IFN-γ: Recombinant murine interferon-γ, produced in *Escherichia coli* (Roche, Mannheim, Germany). Prepare small aliquots and keep frozen at −80°C for prolonged storage. Individual aliquots may be kept at −20°C for immediate use.
3. Lipopolysaccharide (LPS) diluted in PBS to a concentration of 1 mg/mL. Aliquot and store at −20°C.
4. Cold methanol: Store an aliquot of methanol in an explosion-proof −20°C freezer until needed.
5. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.5 mM Na_2HPO_4.
6. Giemsa stain: Purchased as concentrated stock (Ricca Chemical Company, Arlington, TX). Dilute 20 times in water and, if necessary, filter the diluted dye to remove precipitates before addition to the plate.
7. Sabouraud dextrose broth and agar.
8. Trypan blue 0.4% solution.

9. Mouse serum complement (Pel-Freez Biological, Rogers, AR): aliquot in microcentrifuge tubes and stock at –80°C. Remove aliquots immediately before use and discard leftovers, as complement proteins are unstable.
10. Cellstripper solution (Mediatech Inc, Manassas, VA).

3. Methods

3.1. Infection of Macrophages with Cryptococcus neoformans

This protocol describes the preparation of 96-well plates containing macrophages and their infection with *C. neoformans*. These plates will be used in all three experiments described later.

1. Grow J774 cells (see Note 1) in tissue culture medium; 1–2 days prior to the experiment, split the cells so that they will be confluent at the day of the experiment. Use plastic Petri dishes that have not been tissue culture treated so that the cells can be more easily detached.
2. Maintain a stock of *C. neoformans* isolate H99 (see Note 2) in glycerol at –80°C. In the week prior to starting the experiment, inoculate a Sabouraud dextrose agar plate with *C. neoformans* from the stock. Grow for 2 days at 30°C and maintain at 4°C until used.
3. Day 1: Macrophages are plated to achieve a final density of 50,000 cells per well at the time of infection and activated if necessary. To achieve this, one volume of a macrophage suspension with 25,000 cells is mixed with one volume of medium that is supplemented with two times the final concentration of IFN-γ and LPS.
4. Remove the medium from the Petri dish containing J774 cells. Add 5 mL of fresh tissue culture medium into the dish and pipette up and down to dislodge the cells.
5. Count a 1:10 dilution of the cell suspension on a hemocytometer. One full Petri dish usually yields about 10^7 cells. Resuspend them at 5×10^5 cells/mL in feeding medium. You will use 50 μL of this cell suspension per well.
6. In a separate tube, prepare tissue culture medium that has been warmed to 37°C and supplemented with 200 U/mL recombinant IFN-γ and 1 μg/mL LPS. You will use 50 μL of this supplemented medium per well.
7. In a 96-well tissue culture treated plate, add 50 μL of the supplemented medium to each well that will be used (see Note 3). Add 50 μL of the cell suspension and mix by tapping gently against the side of the plate. This will result in wells with 2.5×10^4 J774 cells in 100 μL of medium containing 100 U/mL IFN-γ and 500 ng/ml LPS (see Note 4).

8. Prepare one additional row in the 96-well plate with 50 μL of the supplemented medium and 50 μL of the cell suspension. These wells will be used as controls (see Note 5).
9. Incubate at 37°C with 10% CO_2 for 24 h.
10. Prepare the *C. neoformans* culture by inoculating a single colony in 10 mL of Sabouraud dextrose broth. Grow at 37°C with 150 rpm shaking.
11. Day 2: Prepare a tube with feeding medium supplemented with 200 U/mL IFN-γ, 1 μg/mL LPS and an opsonin at four times the final concentration (see Note 6). You will use 50 μL of this medium per well.
12. Collect 1 mL of the overnight *C. neoformans* culture into a microcentrifuge tube. Wash twice by centrifuging for 2 min at 400 × *g*, removing the supernatant and resuspending in tissue culture medium.
13. Count a 1:100 dilution of the cells and dilute to 10^6 cells/mL. You will use 50 μL of this cell suspension per well.
14. Add 50 μL of the supplemented medium to each well containing J774 cells on the 96-well plate. Add 50 μL of the *C. neoformans* suspension to each well. This will result in infection of the J774 cells at 1:1 effector-to-target ratio (see Note 7).
15. Return to the tissue culture incubator for phagocytosis to happen. The incubation time will depend on which experiment is to be performed (see Subheadings 3.2–3.4 below).

3.2. Phagocytosis Assay

1. Day 1: Prepare a 96-well plate with macrophages and *C. neoformans* cells as described above.
2. Day 2: Incubate the *C. neoformans* and J774 cells prepared using the protocol in Subheading 3.1 for 2 h (see Note 8).
3. To fix the cells, remove the medium and add 200 μL of ice-cold methanol into each well. Incubate at room temperature for 30 min. In the meantime, prepare the staining solution by diluting the concentrated Giemsa stain 20 times in distilled water.
4. Remove the methanol and wash the wells twice with 200 μL of PBS. The washes should be gentle to avoid detaching the macrophages from the plate.
5. Add 100 μL of the diluted Giemsa stain to each well. Incubate at 4°C for at least 2 h, but preferentially overnight.
6. Remove the stain and wash the wells again twice with PBS. Use an inverted microscope to observe the cells (Fig. 1) and count the proportion of macrophages that have internalized *C. neoformans* or the phagocytic index (see Notes 9 and 10).

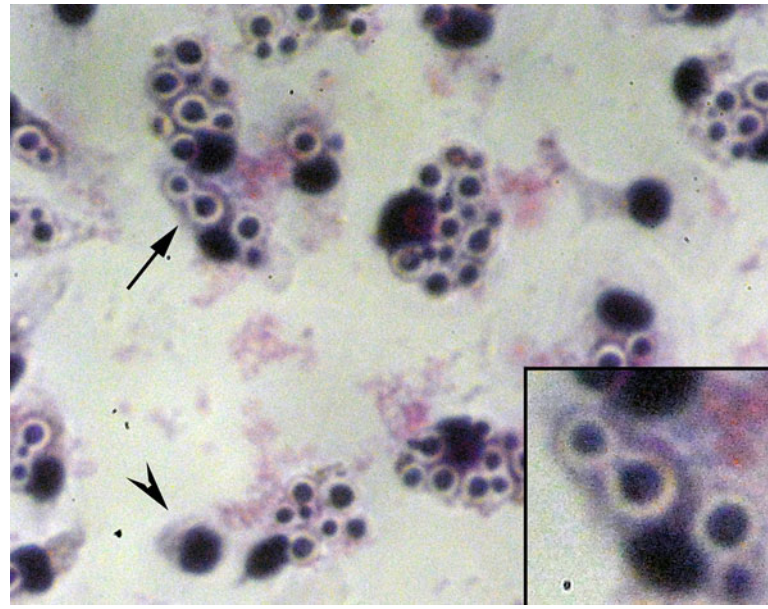

Fig. 1. Photomicrograph of a phagocytosis assay done with primary murine bone marrow-derived macrophages and *Cryptococcus neoformans* isolate 24067. The Giemsa stain results in *light staining* of the macrophage cytoplasm and *dark purple* staining of the macrophage nucleus and *C. neoformans* cells. The capsule is not stained but can be observed as a *white halo* surrounding the ingested fungi, as in the cell pointed with an *arrow* and magnified in the *inset*. The *arrowhead* indicates a macrophage that has not phagocytosed any yeast cell.

3.3. Cryptococcus neoformans Killing Assay

Prepare at least one plate of Sabouraud dextrose agar and one microcentrifuge tube with 400 µL of sterile PBS for each well on the experiment. Also, prepare Sabouraud dextrose agar plates to count the number of CFUs on the inoculum (see Note 11).

1. Day 1: Prepare a 96-well plate with macrophages and a *C. neoformans* cells as described in Subheading 3.1.
2. Day 2: Incubate the plates containing *C. neoformans* and J774 cells between 2 and 24 h (see Note 12).
3. In the meantime, prepare the agar plates to count CFUs on the inoculum. Into three microcentrifuge tubes, pipette 950 µL of sterile PBS. Add 50 µL of the fungal suspension used to infect the macrophages to each one. Plate 5 µL of each dilution on a separate Sabouraud dextrose agar plate. Incubate the plates at 30°C for 2 days.
4. After the desired incubation time, collect the 200 µL of supernatant from each well of the 96-well plate into a labeled microcentrifuge tube that already has 400 µL of sterile PBS. Add 200 µL of sterile distilled water into each well and lyse the macrophages for 30–40 min at 37°C.

5. Vigorously pipette up and down to collect every *C. neoformans cell* on the well and add the liquid to the respective microcentrifuge tube. Rinse the well with 200 μL sterile PBS and add to the respective microcentrifuge tube. The tubes should now have a final volume of 1 mL and contain all of the *C. neoformans* cells from each well.
6. Plate 5 μL of the suspension in each microcentrifuge tube in a Sabouraud dextrose agar plate and incubate at 30°C for 2 days to count CFUs (see Note 13).

3.4. Macrophage Viability Assay

1. Day 1: Prepare a 96-well plate with macrophages and a *C. neoformans* cells as described in Subheading 3.1.
2. Day 2: Incubate the plates containing *C. neoformans* and J774 cells between 2 and 24 h.
3. After the desired incubation time, remove and discard the medium and add 100 μL of warm Cellstripper solution to each well. Return to the tissue culture incubator for 5 min.
4. Pipette up and down to detach the macrophages and transfer to a microcentrifuge tube. Add 100 μL of the trypan blue solution and count cells on a microscope using a hemocytometer in up to 5 min (see Note 14). Dead macrophages will be stained blue, whereas live ones will be colorless.

4. Notes

1. In this chapter, we describe experiments using the J774A.1 macrophage-like cell line (5) (ATCC number TIB-67). This line, derived from a murine reticular sarcoma, has been used extensively with *C. neoformans* and other pathogens and is very easy to handle and maintain. Other types of macrophages such as RAW 264.7 murine and THP-1 human monocytic cell lines can be used, as can primary macrophages. The only important alteration to keep in mind is that the number of macrophage cells to be plated on day 1 (Subheading 3.1) has to be adjusted according to the replication rates of the specific macrophage, such that on day 2 there are 50,000 macrophages per well.
2. We use isolate H99 (ATCC number 208821). Other *C. neoformans* isolates can be used with no additional alterations. The methods described here can also be used with other microorganisms, albeit adaptations might be necessary.
3. The number of wells with macrophages is dependent on the experimental design and objectives. It is desirable to have at least three individual wells for each condition to be tested plus the control wells. Because cells have to be counted rapidly in

trypan blue, it is advisable to keep the number of conditions tested in a macrophage viability assay as low as possible.

4. To activate the macrophages, we use the Th1 cytokine IFN-γ and the toll-like receptor agonist LPS. Whether or not to activate the cells should ideally be decided after pilot experiments are done in both conditions. Moreover, the cytokine, concentrations and time stated in the protocol should be used as a guide only. For example, primary murine peritoneal macrophages do not need activation, whereas primary bone marrow-derived macrophages do. Activation can even have deleterious effects: in our hands, the fungistatic activity of J774 cells as measured in the *C. neoformans* killing assay was often reduced.
5. Several controls are essential in order to properly interpret the results from these experiments:
   - Phagocytosis assay: (a) wells having macrophages only and (b) wells having macrophages and *C. neoformans*, but no opsonin. In (b), less than 2% of the macrophages should have ingested *C. neoformans* cells.
   - *Cryptococcus neoformans* killing assay: (a) contamination control with wells having macrophages only, which should have no colonies at all after plating for CFUs and (b) wells with *C. neoformans* only, which show how much the fungal cells grow at the particular conditions.
   - Macrophage viability assay—wells with macrophages only. The viability of these cells should be higher than 90%.
6. Commonly used opsonin are IgG antibodies that bind to the capsule, used at a final concentration of 10 μg/mL, and mouse serum, at a final concentration of 20%.
7. The effector-to-target ratio (proportion of macrophage to *C. neoformans* cells) has to be carefully planned according to the experimental objectives and can range between 1:10 and 10:1. Higher ratios tend to result in more efficient phagocytosis and *C. neoformans* killing, whereas lower ratios tend to increase the impact on macrophage viability.
8. In presence of opsonins, macrophages phagocytose *C. neoformans* within minutes. The proportion of macrophages with internalized fungal cells increases during the first 2 h of interaction, when a plateau is reached (6).
9. The addition of a film with square divisions to the eyepiece can help counting the macrophages with internalized fungi. Alternatively, photographing the wells with a digital camera attached to the microscope and then counting on a computer screen can be easier. In any case, at least 100 macrophages from two or more fields should be analyzed.

10. There are two ways to quantify phagocytosis; the choice of which one to use will depend on the experimental objectives. The most straightforward is by scoring macrophages as either having or not internalized fungi, which gives the percent phagocytosis. Alternatively, the number of ingested fungal cells can be counted as well to determine the phagocytic index, which is defined by the number of internalized fungal cells divided by the total number of macrophages.
11. Having the plates and tubes labeled and organized prior to starting the experiment saves time and greatly decreases the chance of mistakes, especially in large experiments with many different conditions being tested simultaneously.
12. The incubation time for *C. neoformans* killing assays depends on the properties you want to test. Phagosomal acidification and fusion with lysosomes happen as soon as 15 min after phagocytosis and *C. neoformans* replicates approximately every 3 h. Thus, 2 h incubations are better to evaluate the macrophage's fungicidal activity, whereas 24 h incubations evaluate fungistatic activity.
13. The volume of the fungal suspension to be plated varies with the conditions being tested. In experiments done in the presence of fungicidal drugs, for instance, it is necessary to plate a larger volume; on the other hand, the control wells with *C. neoformans* only in 24 h experiments usually have about ten times more *C. neoformans* cells than the inoculum, so it is necessary to dilute the cell suspension prior to plating.
14. Trypan blue is a vital stain, but it is also toxic to cells. Prolonged exposure to trypan blue leads to artificially low viability measurements, so it is very important to only add the dye immediately before analyzing on the microscope and count the cells within at most 5 min.

Acknowledgments

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