Video Article

Establishment of an *In vitro* System to Study Intracellular Behavior of *Candida glabrata* in Human THP-1 Macrophages

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Abstract

A cell culture model system, if a close mimic of host environmental conditions, can serve as an inexpensive, reproducible and easily manipulatable alternative to animal model systems for the study of a specific step of microbial pathogen infection. A human monocytic cell line THP-1 which, upon phorbol ester treatment, is differentiated into macrophages, has previously been used to study virulence strategies of many intracellular pathogens including *Mycobacterium tuberculosis*. Here, we discuss a protocol to enact an *in vitro* cell culture model system using THP-1 macrophages to delineate the interaction of an opportunistic human yeast pathogen *Candida glabrata* with host phagocytic cells. This model system is simple, fast, amenable to high-throughput mutant screens, and requires no sophisticated equipment. A typical THP-1 macrophage infection experiment takes approximately 24 hr with an additional 24-48 hr to allow recovered intracellular yeast to grow on rich medium for colony forming unit-based viability analysis. Like other *in vitro* model systems, a possible limitation of this approach is difficulty in extrapolating the results obtained to a highly complex immune cell circuitry existing in the human host. However, despite this, the current protocol is very useful to elucidate the strategies that a fungal pathogen may employ to evade/counteract antimicrobial response and survive, adapt, and proliferate in the nutrient-poor environment of host immune cells.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50625/>

Introduction

Candida species are the leading cause of life-threatening invasive fungal infections in immunocompromised patients¹. *Candida glabrata*, an emerging nosocomial pathogen, is the second or third most frequently isolated *Candida* species from Intensive Care Unit patients depending upon the geographical location¹⁻³. Phylogenetically, *C. glabrata*, a haploid budding yeast, is more closely related to the non pathogenic model yeast *Saccharomyces cerevisiae* than to pathogenic *Candida* spp. including *C. albicans*⁴. Consistent with this, *C. glabrata* lacks some key fungal virulence traits including mating, secreted proteolytic activity and morphological plasticity⁴⁻⁵.

Although *C. glabrata* does not form hyphae, it can survive and replicate in murine and human macrophages⁶⁻⁸ suggesting that it has developed unique pathogenesis mechanisms. Limited information is available about the strategies that *C. glabrata* employs to survive nutrient-poor intracellular macrophage environment and counteract oxidative and nonoxidative host responses mounted by host immune cells⁵. A pertinent macrophage model system is a prerequisite to delineate the interaction of *C. glabrata* with host phagocytic cells via functional genomic and proteomic approaches. Peripheral blood mononuclear cells (PBMCs) and bone marrow-derived macrophages (BMDMs) of human and murine origin, respectively, have earlier been used to study the interaction of *C. glabrata* with host immune cells^{7,9}. However, difficulty in obtaining PBMCs and BMDMs, their limited life span and intrinsic variation among different mammalian donors restrict the utilization of these cells as versatile model systems.

Here, we describe a method for establishment of an *in vitro* system to study the intracellular behavior of *C. glabrata* cells in macrophages derived from human monocytic cell line THP-1. The overall goal of this protocol was to enact a simple, inexpensive, quick, and reproducible cell culture model system that can be easily manipulated to study different aspects of host-fungal pathogen interaction.

THP-1 cells have previously been used to decipher the host immune response against a wide range of pathogens including bacteria, viruses, and fungi¹⁰⁻¹². Monocytic THP-1 cells are easy to maintain and can be differentiated, upon phorbol ester treatment, to macrophages which mimic monocyte-derived macrophages of human and express appropriate macrophage markers¹³. The main advantages of THP-1 macrophage model system are the ease-of-use and the lack of sophisticated equipment requirement.

The protocol presented here is easily adaptable to study the interaction of other human fungal pathogens with host immune cells. The current procedure can also be employed to identify virulence factors for the pathogen of interest using high throughput mutant screens. This proof-of-concept was exemplified by the successful use of THP-1 culture model system to identify a set of 56 genes that are required for survival of *C. glabrata* in human macrophages⁸.

Protocol

It is recommended to perform *C. glabrata* infection experiments in a laboratory with biosafety containment level 2 (BSL-2).

1. Preparation of THP-1 macrophage monolayer.
2. Preparation of *C. glabrata* cell suspension.
3. Infection of THP-1 macrophages with *C. glabrata* cells.
4. Measurement of phagocytosis rate and intracellular replication via colony forming unit assay.
5. Monitoring of intracellular replication using confocal laser scanning microscopy.

1. Preparation of THP-1 Macrophage Monolayer

1. THP-1 is a human monocytic cell line derived from the peripheral blood of a 1-year-old male child suffering from acute monocytic leukemia¹⁴. THP-1, a suspension cell line, is routinely maintained at 37 °C in humidified 5% CO₂ in RPMI-1640 culture medium supplemented with 10% FBS (fetal bovine serum). Treatment with phorbol 12-myristate 13-acetate (PMA, a phorbol ester) results in terminal differentiation of THP-1 monocytic cells into macrophages. Importantly, PMA treatment does not affect cell viability and differentiated cells do not divide.
2. Seed approximately 1.5×10^6 THP-1 monocytes in 100 mm culture dishes in RPMI-1640 complete medium (RPMI-1640 medium supplemented with 10% serum, 2 mM glutamine and 1x antibiotics; 10 ml/dish) and let cells grow at 37 °C in 5% CO₂ for 2 days.
3. Once THP-1 cells have reached a density of 8×10^5 cells/ml, transfer the culture dish to the cell culture laminar flow hood and gently swirl it to resuspend settled cells.
4. Pipette the cell suspension out, place into a 15 ml sterile tube and centrifuge at 1,000 rpm (130 x g) for 4 min. Discard supernatant and suspend THP-1 cell pellet in 5 ml fresh, prewarmed, RPMI-1640 complete medium.
5. Enumerate cells with a hemocytometer and dilute the cell suspension to a final density of 10^6 cells/ml with prewarmed RPMI-1640 complete medium.
6. Add 1 µl of 160 mM PMA stock (phorbol 12-myristate 13-acetate solution prepared in dimethyl sulfoxide) to 10 ml THP-1 cell suspension (final concentration 16 nM) and mix well. Dispense 1 ml cell suspension into each well of a 24-well tissue culture plate and incubate plate in the incubator maintained at 37 °C with 5% CO₂ for 12 hr.
7. Remove old medium, add 1 ml prewarmed RPMI-1640 complete medium and allow cells to recover for 12 hr at 37°C in 5% CO₂.
8. Observe cells under an inverted microscope to ensure that oval-shaped THP-1 monocytic cells in suspension have been differentiated into flattened, spindle-shaped and adherent macrophages. These differentiated cells are now ready to conduct *C. glabrata* infection studies.

2. Preparation of *C. glabrata* Cell Suspension

C. glabrata wild-type strain Bg2 will be used to infect THP-1 macrophages. *C. glabrata* cells are routinely cultured in liquid YPD (Yeast extract (1%)-Peptone (2%)-Dextrose (2%)) medium. Solid YPD medium is prepared by adding 2% agar before medium autoclaving.

1. To prepare a culture of *C. glabrata*, pick a single colony up from the agar plate with a sterile, disposable loop and inoculate into 10 ml YPD liquid medium and incubate for 14-16 hr with shaking (200 rpm) at 30 °C.
2. Centrifuge this culture (1 ml) at 4,000 rpm (1,800 x g) for 5 min in a table top microcentrifuge, wash cells thrice with sterile PBS (phosphate-buffered saline, pH 7.4) and suspend cell pellet in 1 ml PBS.
3. Measure OD₆₀₀ of *C. glabrata* cell suspension and dilute with an appropriate volume of sterile PBS to get a density of 2×10^6 cells/ml (OD₆₀₀ = 0.1). Alternatively, the desired cell density can be achieved by counting yeast cells using a hemocytometer.

3. Infection of THP-1 Macrophages with *C. glabrata* Cells

1. Add 50 µl *C. glabrata* cell suspension to THP-1 macrophages (10^6 cells seeded per well of a 24-well culture plate) to obtain a MOI (multiplicity of infection) of 0.1 and incubate plate at 37 °C with 5% CO₂.
2. To determine viable yeast counts, dilute 50 µl *C. glabrata* cell suspension 100-fold in sterile PBS and plate 100 µl on YPD solid medium. Incubate plates at 30 °C and count manually the number of yeast colonies appearing after 1-2 days. These numbers will, henceforth, be referred as 0 hr *C. glabrata* CFUs (colony forming units).
3. After 2 hr coculturing of THP-1 cells with *C. glabrata* cells, discard culture medium by inverting 24-well tissue culture plate in a reservoir and wash gently thrice with prewarmed sterile PBS to remove nonphagocytosed extracellular *C. glabrata* cells. Gentle washes with PBS are absolutely essential to accomplish complete removal of all extracellular yeast without causing any damage to the THP-1 macrophage monolayer.

4. Measurement of Phagocytosis Rate and Intracellular Replication via Colony Forming Unit Assay

1. Lyse *C. glabrata* infected THP-1 macrophages in 1 ml sterile H₂O for 2 min, scrape gently to remove the macrophage debris from the well and collect cell lysate in a microcentrifuge tube. Microscopic verification of complete macrophage lysis is pivotal for the recovery of all internalized yeast.
2. Prepare a 100-fold dilution of lysate in sterile PBS, plate 100 µl on YPD solid medium and incubate plates at 30 °C.
3. After 1-2 days, count manually the number of *C. glabrata* colonies that appeared on YPD medium, multiply with dilution factor (2 hr CFUs) and calculate phagocytosis rate which refers to the percent of *C. glabrata* cells that are ingested by THP-1 macrophages after 2 hr coincubation using the following formula.

$$\% \text{ phagocytosis} = [(CFUs \text{ at } 2 \text{ h}) / (CFUs \text{ at } 0 \text{ h})] \times 100$$

4. To measure the rate of intracellular replication of *C. glabrata* cells in THP-1 macrophages, collect intracellular yeast at different time points post infection *i.e.* 4, 6, 8, 10, 12, and 24 hr, by repeating steps 3.3-4.3.
5. Calculate fold survival/replication of *C. glabrata* cells in THP-1 macrophages by dividing total CFUs at any given time point with those at 2 hr (phagocytosed yeast).

5. Monitoring of Intracellular Replication Using Confocal Laser Scanning Microscopy

1. Following the steps described in Section 1, seed PMA-treated 5 x 10⁵ THP-1 cells in each well of two four-well chamber slides and incubate at 37 °C with 5% CO₂ for 12 hr.
2. Replace old medium with prewarmed RPMI-1640 complete medium and allow cells to recover from PMA treatment for 12 hr.
3. Prepare GFP (green fluorescent protein)-tagged *C. glabrata* cell suspension as described in Section 2 and infect to THP-1 macrophages to a MOI of 1. If *C. glabrata* strains expressing GFP are not available, yeast cells labeled with FITC (fluorescein isothiocyanate) can be used to monitor early events post infection *viz.* phagocytosis rate and phagolysosomal maturation at 2 hr.
4. Incubate slides for 2 hr at 37 °C with 5% CO₂, invert them carefully to discard medium and wash 3x with sterile, prewarmed PBS.
5. Add 500 µl prewarmed RPMI-1640 complete medium to each chamber of one slide and incubate it at 37 °C with 5% CO₂ for 22 hr.
6. To fix 2 hr *C. glabrata*-infected THP-1 macrophages, add 500 µl of 3.7% formaldehyde (prepared in PBS) to each chamber of the other slide and incubate it at room temperature for 20 min in the dark.
7. Wash slide thrice with PBS, add 500 µl Triton-X (0.7%), and incubate at room temperature for 5 min in dark.
8. Wash slide 3x with PBS, remove chamber from culture slide and air dry it for 3-5 min in the dark.
9. Carefully mount a coverslip using Vectashield mounting medium containing DAPI (4',6-diamidino-2-phenylindole) on slide avoiding bubble formation. Remove gently the excess liquid with Kimwipe and seal coverslip edges with fingernail polish. Store slide in the dark at 4°C until use.
10. Repeat steps 5.4 and 5.6-5.9 to process THP-1 cells 24 hr post infection.
11. Image cells using a laser scanning confocal microscope (60X oil-immersion objective, excitation at 405 nm and 488 nm for DAPI and GFP stain, respectively).

Representative Results

Infection analyses of PMA-treated THP-1 macrophages with *C. glabrata* wild type (*wt*) cells revealed that *wt* cells were phagocytosed by macrophages at a rate of 55-65% after 2 hr coincubation. Further, *C. glabrata* cells were able to resist killing by THP-1 macrophages and underwent a moderate 5- to 7-fold increase in CFUs after 24 hr of coculturing with THP-1 macrophages⁸. Intracellular replication of wild type cells, transformed with GFP-expressing plasmid, in THP-1 macrophages was also verified with confocal fluorescence microscopy wherein the number of intracellular yeast cells per THP-1 macrophage increased from one or two to seven to twelve over a period of 24 hr (**Figure 1**).

Figure 1. A confocal image of formaldehyde fixed, GFP-tagged *C. glabrata*-infected THP-1 macrophages displaying intracellular replication with THP-1 cells harboring 1-2 and 5-8 yeast at 2 hr and 24 hr, respectively. Nuclei are stained with DAPI. Bar = 10 μ m.

Figure 2. Schematic representation of the *C. glabrata* mutant library screen for altered survival profiles in THP-1 macrophages via signature-tagged mutagenesis (STM) approach. Red and green circles on hybridized membranes denote reduced and elevated representation of the tags, respectively, in output samples compared to input samples.

Discussion

Innate immune system plays an important role in the control of opportunistic fungal infections. Macrophages contribute to antifungal defense by ingestion and destruction of the fungal pathogen. Thus, elucidation of factors that are required for survival and/or counteracting the antimicrobial functions of macrophages will advance our understanding of fungal virulence strategies. In this context, we have established an *in vitro* cell culture model system using macrophages derived from a human monocytic cell line THP-1 to characterize the interaction of a human opportunistic fungal pathogen *C. glabrata* with host phagocytic cells. Although this THP-1 macrophage model system has successfully been used to identify *C. glabrata* mutants with reduced survival which displayed attenuated virulence in a murine model of systemic candidiasis⁸, a plausible limitation of this system is its isolated context and, thus, results obtained may not always be applicable to the complex mammalian host

immune system. Further, this system, unlike live cell-imaging methods, is unable to account for yeast cells that are either not taken up by THP1-macrophages or killed during phagocytosis.

The significance of this protocol lies in its simplicity, reproducibility and scalability. The method can be scaled down to a 96-well plate and scaled up to a 150 cm² tissue culture flask. The critical steps in this protocol are selection of an appropriate MOI and extensive PBS washes to minimize extracellular replication and plating of appropriate lysate dilution to obtain 100-200 yeast colonies per plate. A MOI of 0.1 is ideally suited to monitor intracellular replication over a period of 24 hr as the number of extracellular yeast cells remains minimal during this prolonged coculturing of THP-1 macrophages with *C. glabrata* cells. Further, a MOI of 1.0 is optimal for visualizing the maximal number of *C. glabrata* infected-macrophages per field to analyze early events of *C. glabrata* infection microscopically without any effect on intracellular replication profiles of yeast cells. Notably, extracellular *C. glabrata* cells, including nonphagocytosed yeast that remain attached to macrophage membrane, can be differentiated from internalized yeast cells by inside-out staining⁸.

To specifically enumerate intracellular survival/replication, CFU comparison should be made between the number of internalized yeast at 2 hr and those at later time points. Comparison with 0 hr CFUs will skew the results if initial attachment and phagocytosis rates are different for different strains. It is noteworthy here that the number of intracellular yeast cells during the infection period can also be measured by flow cytometry and confocal/live cell imaging microscopy approaches.

Additionally, intracellular yeast recovered at different time points post infection using this protocol can be used for several analyses including microscopy, reactive oxygen species (ROS) accumulation, chromatin extraction, and RNA and protein isolation to discern the epigenetic, transcriptional, and metabolic response of *C. glabrata* cells to the macrophage internal milieu. It is important to completely eliminate the extracellular yeast and the macrophage debris before performing any biochemical analysis on macrophage-internalized yeast.

Another application of this *in vitro* cell culture model system is its adaptability to screen mutants for altered survival profiles, either individually or multiplexed in a pool of 96 strains via signature-tagged mutagenesis (STM) approach. An advantage of the STM strategy is the parallel screening of hundreds of mutants in a single experiment. This approach has recently been used to screen a *C. glabrata* mutant library which is comprised of 18,350 random Tn7 insertion mutants and assembled in a total of 192 pools wherein each pool is composed of 96 uniquely oligonucleotide-tagged mutants^{8,15}. **Figure 2** pictorially illustrates the outline of the STM screen methodology which consists of three main steps. First, an overnight-grown pool of 96 *C. glabrata* mutants is cultured either in rich medium (input) or infected to THP-1 macrophages in a 24-well tissue culture plate. After 2 hr infection, extracellular yeast cells are removed by PBS washes and infected THP-1 cells are incubated at 37 °C. 24 hr post infection, intracellular yeast cells (output) are recovered by osmolysis of THP-1 cells. The second step involves extraction of genomic DNA from input and output samples followed by amplification of unique signature tags with ³²P-labeled dCTP using primers complementary to the invariant region flanking each unique oligonucleotide sequence. Third, radiolabeled tags from input and output pools are hybridized to a Hybond-Nylon membrane which contains 96 plasmids each carrying a unique signature tag. Hybridization signal for a unique oligonucleotide sequence reflects the abundance of the mutant strain, carrying that particular tag, in a pool of 96 mutants. Output (Op) to input (Ip) ratio for each tag is calculated by dividing the output signal intensity by the input signal intensity. Mutants displaying at least 6-fold higher and 10-fold lower survival can be considered as 'up' (Op/Ip = 6.0, increased survival) and 'down' (Op/Ip = 0.1, reduced survival) mutants, respectively (**Figure 2**). Alternatively, fluorescently-labeled probe-dependent DNA microarrays can be used to determine any variation in the signal intensity of the tag in the input and the output sample which mirrors the intracellular behavior of the mutant.

This method can also be employed to study ROS and cytokine response and phagolysosomal acidification of phagocytic cells upon infection with fungal pathogens. Lastly, owing to the adaptability of this procedure to both, various immune cell types including primary cells as well as fungal organisms, this protocol is suitable to address several aspects of host-fungus interaction.

Disclosures

The authors declare that they have no competing financial interests.

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