In vitro Analysis for Macrophage Binding and Pro-inflammatory Responses to *Candida albicans*

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[Abstract] Macrophage recognition of *Candida albicans* (*C. albicans*) is facilitated by pattern recognition receptors that interact with the fungal pathogen associated molecular patterns (PAMPs). Dectin-1 is the major macrophage receptor that is known to recognize fungal Beta-glucans leading to induction of various immune responses. This receptor is also known to be required for *in vivo* protection against *C. albicans* (Taylor *et al.*, 2007). We recently showed that the Dectin-1 mediated protection *in vivo* is strain-dependent, and that *C. albicans* can adapt to modulate immune recognition by Dectin-1 (Marakalala *et al*., 2013). *In vitro* analysis, however, showed a Dectin-1-dependent and pro-inflammatory responses against all strains tested. This protocol describes in detail the *in vitro* analysis used in the paper. In particular, methods involved in fluorescent labeling of live *C. albicans*, quantification of macrophage binding of the pathogen, and pro-inflammatory responses to yeast and hyphal forms of the fungi are described.

Materials and Reagents

1. *Candida albicans* strains, SC5314 and ATCC18804
2. Mice
   Note: 2x per WT or Dectin-1 KO mice on C57BL/6 background should give sufficient macrophages for a full 24-well plate experiment. Mice were age and sex matched.
3. Thioglycollate-elicited macrophages
   Note: This method has been described in detail in Kerrigan et al. (2012).
4. RPMI 1640 medium (Life Technologies, catalog number: 11875-093)
5. 70% ethanol
6. Sabouraud Dextrose broth (Oxoid Limited, catalog number: CM0147)
7. Rhodamine Green-X (Life Technologies, Invitrogen™, catalog number: R-6113)
8. Difco Thioglycollate Broth (BD Biosciences, catalog number: 225710)
9. Triton-X 100 (Sigma-Aldrich, catalog number: T8787)
10. OptEIA TNF kit (BD Biosciences, catalog number: 555268)
11. Zymosan (Life Technologies, Invitrogen™, catalog number: Z2849)

12. Dulbecco's Phosphate Buffered Saline (PBS) (Sigma-Aldrich, catalog number: D8662)
13. Fetal Bovine/Calf Serum (FCS) (Sigma-Aldrich, catalog number: F6178)
14. EDTA (Sigma-Aldrich, catalog number: E9884)

Equipment

1. Titer-Tek Fluoroskan II (Labsystems)
2. Shaker
   Note: The shaker temperature was controlled at 30 °C and the speed was between 150 to 200 rpm.
3. Centrifuge (Eppendorf, model: 5810R with swing bucket rotor S-4-72)
4. Light microscope
5. Haemocytometer
6. 24-well plates (BD Biosciences)
7. 2 ml Eppendorf tube
8. 96-well plates (black in color for fluorescence measurement) (BD Biosciences)
9. Rubber back of syringe plunger
   Note: The make or brands for above equipment are not important for reproducibility.

Procedure

A. Growth and Labeling of *Candida albicans*

1. Start *C. albicans* cultures by inoculating frozen stocks into 5 ml Sabouraud Dextrose broth and incubate for 16 to 24 h at 30 °C with shaking at 200 rpm.
2. Centrifuge the cells for 5 min at 3,000 rpm and wash three times in 10 ml Dulbecco's PBS.
3. Count the yeast cells under the microscope using a haemocytometer.
4. Adjust the cell density to at least 3.2 x 10^6 yeast/ml, or as desirable in PBS and transfer into a 2 ml eppendorf tube.
5. Add Rhodamine Green-X to a concentration of 200 µg/ml.
6. Cover the tube with foil and incubate with gentle agitation at room temperature for 30 to 45 min.
7. Centrifuge the labelled cells at 3,000 rpm for 5 min and resuspend in PBS.
8. Wash the cells about ten times with 2 ml PBS or until free Rhodamine Green-X is removed.

B. Macrophage binding and pro-inflammatory assays

Notes:

a. *Extraction of macrophages is done in a hood within animal facility.*
b. *The treatment of macrophages with Candida albicans is performed in a hood in a tissue culture BSL2 area.*
c. *The hood surface is sprayed with 70% ethanol to prevent sample contamination.*

1. Inject mice intraperitoneally with 1 ml of thioglycollate broth (Kerrigan *et al.*, 2012). Gently hold the mouse to allow free space on the site of injection. Use a syringe to inject the thioglycollate broth in the lower quadrant of the abdomen; be careful not to prick vital organs such as the bladder or intestines.
2. After four days, isolate peritoneal exudate cells by lavage with ice-cold 5 ml PBS containing 5 mM EDTA.
3. Centrifuge the peritoneal cells at 1,000 rpm for 10 min and resuspend the pellet in 5 ml RPMI medium containing 10% (volume/volume) heat-inactivated FCS (by heating at 60 °C for 30 min).

   Note: All FCS used in the following steps are heat-inactivated.
4. Count macrophages on a haemocytometer, dilute them to 5.0 x 10^5 cells/ml and seed them at a density of 2.5 x 10^5 cells/well in a 24-well plate with RPMI medium containing 10% FCS. Incubate the plate overnight at 37 °C (without any agitation).
5. Aspirate wells and add 0.5 ml fresh RPMI plus 10% FCS to the macrophages that are attached to the wells.
6. Add the Rhodamine Green-X-labelled *C. albicans* at the MOI (multiplicity of infection) of 5:1 or 10:1 to the macrophages.
7. Fluorescein isothiocyanate–labelled zymosan can be used as a control at MOI of 25:1. Zymosan is made of Beta-glucan particles that are recognized by macrophage receptors and induce pro-inflammatory responses; Dectin-1 is a well-studied receptor for these particles (Brown *et al.*, 2003).

   Notes:

   a. *Zymosan is labelled with FITC according to manufacturer's (Molecular Probe) instructions as described also in detail in Kerrigan et al. (2012).*
   b. *The expected results are that Zymosan will bind macrophage and induce proinflammatory responses in a Dectin-1 dependent manner.*
8. Incubate the plate on ice for 30 min to allow the particles to settle, and then a further 30 min incubation at 37 °C, 5% CO_2.
9. Wash the wells three times with 0.5 ml RPMI plus 10% FCS to remove the unbound particles.
10. Add 500 µl of the RPMI plus 10% FCS to the wells and incubate at 37 °C for 3 h, 5% CO_2.

11. Aliquot 150 µl of the supernatant and store at -80 °C for later analysis of pro-inflammatory cytokine production.
12. Wash the wells and lyse the cells by adding 150 µl 3% Triton X-100 (pH 7.5). Triton X-100 is diluted to 3% (v/v) in distilled water.
13. Detach the cells using rubber back of syringe plunger and add 100 µl into black 96-well plates. Cell scraper can also be used.
14. To quantify the binding of fungal particles to macrophages, measure the fluorescence at the excitation of 490 nm and the emission at 514 nm on a Titer-Tek Fluoroskan II. Expected results are as shown in Figure 4b in Marakalala *et al.* (2013).
15. For pro-inflammatory responses, use ELISA (OptEIA TNF kit) to measure the TNF concentrations of the supernatant aliquots which had been stored at -80 °C.
    Note: Pro-inflammatory responses to hyphae can be measured similarly, except that macrophages are added directly to the wells that already contain live or heat-killed hyphae. See below for detailed description of hyphal induction.

C. Pro-inflammatory responses to hyphae

1. To induce hyphae formation, grow and quantify *C. albicans* as stated in steps A1-3.
2. Incubate 2.5 x 10^6 yeast/well (cell density of about 3.2 x 10^6 yeast/ml or as per required amount) with RPMI medium for 3 h at 37 °C. View the cells under the microscope to confirm hyphae formation.
3. Heat-kill the hyphae by incubating at 65 °C for 2 h, or use live hyphae if desired.
4. Add 2.5 x 10^5 of thioglycollate-elicited macrophages (densisty is 5.0 x 10^5 cells/ml directly the hyphae-containing wells).
5. Incubate the plates overnight at 37 °C, 5% CO_2.
6. Take the supernatant samples and store at -80 °C until needed for cytokine analysis.

Acknowledgments

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