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Endothelial Cell Stimulation by *Candida albicans*

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Abstract

The opportunistic fungal pathogen, *Candida albicans*, enters the bloodstream and causes hematogenously disseminated infection in hospitalized patients. During the initiation of a hematogenously disseminated infection, endothelial cells are one of the first host cells to come in contact with *C. albicans*. Endothelial cells can significantly influence the local host response to *C. albicans* by expressing leukocyte adhesion molecules and pro-inflammatory cytokines. Thus, it is of interest to investigate the response of endothelial cells to *C. albicans* in vitro. We describe the use of real-time PCR and enzyme immunoassays to measure the effects of *C. albicans* on the endothelial cell production of E-selectin and tumor necrosis factor α in vitro.

Keywords

Endothelial cell; *Candida albicans*; E-selectin; IL-8; EIA; real-time PCR

1. Introduction

Endothelial cells form the lining of the blood vessels. In addition to regulating vascular permeability and vascular tone, endothelial cells are important for initiating and regulating the local host response to infection. Endothelial cells are capable of synthesizing a variety of factors that influence the activity of neutrophils and other leukocytes. These factors include proinflammatory cytokines such as tumor necrosis factor α (TNF- α), interleukin 1 (IL-1), IL-6, IL-8, and monocyte chemotactic protein 1 (MCP-1). Endothelial cells also express leukocyte adhesion molecules such as P-selectin, E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1). In general, the expression and secretion of these immunomodulators by endothelial cells help recruit leukocytes to foci of infection. The pro-inflammatory cytokines also activate the microbicidal activities of these recruited leukocytes.

During the initiation of hematogenously disseminated candidiasis, *C. albicans* cells in the bloodstream must traverse the endothelial cell lining of the vasculature to invade the deep tissues. Thus, endothelial cells are one of the first host cells that interact with this organism. Infection with *Candida albicans* in vitro stimulates endothelial cells to synthesize and release a variety of leukocyte adhesion molecules and pro-inflammatory cytokines (1, 2). For example, endothelial cells express E-selectin on their surface in response to *C. albicans* infection (1, 2). This leukocyte adhesion molecule mediates rolling adherence of neutrophils

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and monocytes to endothelial cells, and its presence likely contributes to the recruitment of these leukocytes at foci of *C. albicans* infection. Endothelial cells also secrete large amounts of IL-8 in response to *C. albicans* in vitro (1, 2). This cytokine is a potent neutrophil chemoattractant. Also, neutrophils that have been incubated in IL-8 have enhanced ability to kill *C. albicans* (3).

The effects of *C. albicans* on endothelial cell expression of E-selectin and IL-8 can be measured at the level of mRNA accumulation using real-time reverse transcriptase PCR. The surface expression of E-selectin and secretion of IL-8 into the medium can also be measured by enzyme immunoassays (EIAs).

2. Materials

2.1 *C. albicans* Culture

1. Yeast extract peptone dextrose (YPD) agar is made by dissolving 5 g of yeast extract (Becton Dickinson, Spark, MD), 10 g peptone (Becton Dickinson), 10 g glucose (Fisher Scientific, Pittsburgh, PA), and 10 g agar (Fisher Scientific) in 500 ml of water. Autoclave at 121°C for 15 min. Avoid overheating and remove medium from autoclave as soon as the sterilization cycle is completed to minimize caramelization).
2. Yeast nitrogen base (YNB) liquid medium is made by dissolving 6.7 g of YNB (Becton Dickinson) and 5 g of glucose (Fisher Scientific) in 100 ml sterile irrigation water and filter sterilize. This medium can be stored at 4°C for up to one month. For use, dilute 1:10 with endotoxin-free water (*See Note 1*).

2.2 Cell Culture

1. M199 tissue culture medium: M199 powder (Gibco/BRL, Bethesda, MD) is dissolved in 1 L endotoxin-free water. Next, add 100 ml of fetal bovine serum (FBS), 100 ml bovine calf serum (BCS) (both from Gemini Bio-products Woodland, CA; use serum lots with low endotoxin concentration), and 10 ml of a solution containing 1% penicillin, 1% streptomycin, and 2 mM L-glutamine (Gemini Bio-products). The FBS and BCS can be stored in 50 ml aliquots at -20°C. Store the penicillin-streptomycin-L-glutamine solution in 5 ml aliquots at -20°C. Filter sterilize the medium and store at 4°C. Warm medium up to 37°C in a water bath before adding it to the endothelial cells.
2. 96-well and 24-well flat bottom tissue culture plates, and Primaria 25cm² tissue culture flasks are from Becton Dickinson (Franklin Lakes, NJ).
3. Phosphate buffered saline without Ca⁺⁺ or Mg⁺⁺ (PBS) and Hanks balanced salt solution (HBSS) are from Mediatech, Inc (Herndon, VA) (*see Note 2*).
4. Solution of 10× trypsin (5g/L) and ethylenediamine tetraacetic acid (EDTA) 2g/L (Gemini Bioproducts) is stored in 4 ml aliquots at -20°C. Dilute 1:10 in PBS and warm to room temperature before use. The 1× solution can be stored at 4°C for 7 days.
5. Dilute 2% tissue culture quality gelatin stock solution (Sigma-Aldrich, St. Louis, MO) 1:10 in endotoxin-free water. Filter sterilize and store at 4°C for up to 1 mo.
6. RPMI 1640 medium is from Irvine Scientific (Santa Ana, CA)

2.2 RNA Extraction and Real-time PCR

1. RNA extraction (all reagents are molecular biology grade)

- a. TRI reagent from Ambion (Austin, TX)
 - b. Chloroform (Sigma-Aldrich)
 - c. Glycogen (Sigma-Aldrich)
 - d. RNase-free water (Ambion)
 - e. Isopropanol (Sigma-Aldrich)
 - f. 75% ethanol (Sigma-Aldrich)
2. DNase treatment and RNA clean up
 - a. TURBO DNase I and 10× TURBO DNase buffer (Ambion)
 - b. RNA clean up kit from Zymo Research (Orange, CA)
 3. Real-time RT-PCR
 - a. Retroscript (Ambion)
 - i. 10 unit/μl RNase inhibitor
 - ii. 10× reverse transcriptase buffer
 - iii. 25 mM dNTP mixture
 - iv. 100 units/μl M-MLV reverse transcriptase
 - v. 50 μM oligo(dT)
 - b. Taqman universal PCR master mix (Applied Biosystems, Foster City, CA)
 - c. IL-8 primers (Applied Biosystems)
 - d. E-selectin primer (Applied Biosystems)
 - e. GAPDH primer (Applied Biosystems)
 - f. 96-well microtiter reaction plate (Applied Biosystems)
 - g. Adhesive cover (Applied Biosystems)
 - h. Compressor pad (Applied Biosystems). The pad can be reused.

2.3 E-selectin EIA

1. 10× TBS-Glycine: Add 6.61 g Tris-HCl, 0.97 g Tris Base, 8.77 g NaCl, and 10.5 g glycine to ~99 ml water, adjust pH to 7.5 and volume to 100 ml, and filter sterilize. This solution can be stored at 4°C for up to 6 months. It should be diluted to 1× just before use.
2. The PBS used for rinsing fixed endothelial cells does not need to be endotoxin-free (*see Note 2*).
3. Blocking buffer: Add 1 ml of sheep serum (Sigma-Aldrich) and 3 g of bovine serum albumin (BSA; Sigma-Aldrich) to 99 ml of PBS, and filter sterilize. The solution can be stored at 4°C for up to 3 mo.
4. 0.1% BSA in PBS: Add 1 g of BSA to 1 L of PBS, and filter sterilize. The solution can be stored at 4°C for up to 3 mo.
5. 4% paraformaldehyde in PBS: Warm up 10 ml of PBS to about 90°C, and then add 0.4g of paraformaldehyde. Vortex to mix. Paraformaldehyde is virtually impossible

to dissolve in cold PBS. Do not inhale the vapors of this solution because they are toxic. Make this solution up the day of the experiment and always use when fresh.

6. M199 + 5% FBS should be made up right before.
7. Primary antibody: anti-E-Selectin antibody (Biodesign, Saco, MN). Antibody can be aliquoted in 100 μ l volumes and stored at -80°C . Thawed aliquots can be stored at 4°C for up to 2 wk.
8. Secondary antibody: sheep anti-mouse IgG conjugated with horse radish peroxidase is purchased from Sigma-Aldrich.
9. O-phenylenediamine dihydrochloride (OPD) tablets are from Sigma-Aldrich.

3. Methods

The endothelial cell response to *C. albicans* can be analyzed using real-time PCR to measure the accumulation of mRNA for E-selectin and IL-8. The advantages of this technique are that it is sensitive and requires a relatively small amount of endothelial cells. Also, primers for virtually all human leukocyte adhesion molecules and cytokines are commercially available, and the mRNA expression of multiple immunomodulators can be determined using cDNA prepared from a single sample.

It is frequently useful to verify that increased mRNA expression is associated with increased protein expression. To determine this association, changes in the expression of leukocyte adhesion molecules on the endothelial cell surface can be quantified by EIA. Also, the conditioned medium above the endothelial cells can be collected and analyzed for cytokine content using either commercially available EIAs or flow cytometric bead arrays.

3.1 Growth and preparation of *C. albicans*

1. *C. albicans* cells are maintained on YPD agar plates at room temperature. Approximately 16 hr before the experiment, a single colony is used to inoculate 10 ml of endotoxin-free YNB broth containing 0.5% glucose, which is incubated in rotary shaker at ~ 150 RPM at 30°C (see **Note 3**).
2. After incubation, vortex the culture tube vigorously to break up the clumps. Next, harvest the organisms by centrifuging them at $1,000 \times g$ for 5 min at room temperature.
3. Decant supernatant. Vortex pellet vigorously and then resuspend in 10 ml sterile, endotoxin-free PBS. Centrifuge at $1,000 \times g$ for 5 min at room temperature. Repeat this step a total of two times. After the last centrifugation, resuspend the pellet in 10 ml HBSS. Vortex vigorously again.
4. Dilute stock suspensions of *C. albicans* 1:100 in PBS and count the number of cells with a hemacytometer (yield should be about 10^8 organisms per ml).
5. Make up a suspension of organisms in prewarmed M-199 + 20% serum (see **Notes 4 and 5**).
6. Warm all solutions to 37°C immediately before adding them to the endothelial cells.

3.2 Endothelial Cell RNA Extraction

1. Human umbilical vein endothelial cells are maintained in 25 cm^2 tissue culture flasks coated with 0.2 % gelatin. When the cells reach confluency, they are detached from the bottom of the flask using trypsin-EDTA (see **Note 6**). When

cells are passaged for maintenance culture, they are split at a 1:3 ratio. For use in the real-time PCR experiments, the wells of a 24-well tissue culture plate are coated with 0.5 ml of 10 μ g/ml fibronectin in PBS for at least 30 min. Next, aspirate the fibronectin solution and add endothelial cells to the wells. One flask of confluent endothelial cells can be split into all wells of the 24-well tissue culture plate (see **Notes 7 and 8**). To obtain sufficient RNA for the experiments, each condition should be tested in duplicate.

2. The night before the experiment, place some M-199 +20% serum in the 5% CO₂ incubator for use in the experiment. Keep the cap on the container loose so that the tissue culture medium can equilibrate with the CO₂. The various stimuli (e.g., *C. albicans* and TNF- α) should be prepared in this medium on the day of the experiment.
3. On the day of the experiment, aspirate the medium from two wells at a time and add the desired stimulus in a total volume of 500 μ l (see **Note 9**). All experiments should include a set of wells that are incubated with medium alone. These wells are essential for using the $\Delta\Delta C_T$ method to calculate the extent of endothelial cell stimulation. It is also useful to include wells incubated with TNF- α (final concentration 400 pg/ml) as a positive control.
4. Incubate plate for 8 hr at 37°C in 5% CO₂. At the end of the incubation period, examine wells with the inverted microscope to look for loss of endothelial cells and determine the morphology of *C. albicans*. Wild-type strains should form extensive hyphae.
5. Gently aspirate medium from each well and wash each well once with 500 μ l of cold HBSS per well (See **Notes 10 and 11**).
6. Aspirate HBSS and add 250 μ l of Tri Reagent to each well. Homogenize the cells by repeatedly passing them up and down with a P1000 Pipetman. Change pipet tip between samples.
7. Incubate plate at room temperature for 5 min.
8. Transfer the lysates to 1.5 ml microfuge tubes, combining the lysates from each pair of wells that were exposed the same condition.
9. Samples can be either stored at -80°C or processed immediately.
10. Thaw lysates and incubate them at room temperature for 5 min to dissociate the nuclear proteins from the nucleic acids.
11. Add 100 μ l of chloroform and vortex for 20 sec.
12. Let sit at room temperature for 10 min.
13. Centrifuge samples in a microfuge at 14,000 rpm for 15 min at 4°C.
14. Carefully remove tubes from centrifuge and aspirate the upper aqueous layer to about 3 mm above the interface. Transfer this upper phase to a fresh tube.
15. To each tube add 1 μ l of glycogen (10 μ g/ μ l in RNase-free water), 250 μ l of RNase-free water and 500 μ l of isopropanol. Mix well using the vortex.
16. Incubate for 10 min at room temperature.
17. Centrifuge samples in a microfuge at 14,000 rpm for 15 min at 4°C.
18. Remove supernatant and wash pellet by adding 500 μ l of 75% ethanol and then vortexing.

19. Centrifuge samples in a microfuge at 14,000 rpm for 10 min at 4°C.
20. Aspirate supernatant and dry pellet by inverting the microfuge tubes on a paper towel for about 10 min. Do not let RNA dry out completely.
21. Resuspend pellets in 10 µl of RNase-free water.
22. Determine RNA concentration in the samples using a spectrophotometer.

3.3 Preparing cDNA

1. Remove DNA contamination by treating RNA samples with DNase I (2µ/µl) (*see Note 10*). Make up DNase I master mix by adding DNase I to DNase reaction buffer. Add 2 units of DNase I to 5 µg of each RNA sample and incubate at 37°C for 30 min.
2. Clean up the RNA samples with RNA clean-up Kit (such as kit R1015, from Zymo Research, Inc., Orange, CA). The kit is designed to treat up to 5 µg RNA with a recovery efficiency of 80 to 95%. The purified RNA can be used immediately or stored at -70°C for future use. Keep RNA samples on ice if they are not going to be stored at -70°C.
3. For reverse transcription of the RNA, label 2, 200 µl PCR tubes for each sample. One tube is for the reverse transcriptase reaction, and the other is for the no reverse transcriptase (RT) control.
 - a. Remove the secondary structure of the RNA and anneal the oligo(dT) primer to the RNA by adding oligo(dT) (final concentration of 5 µM) to 2 µg of RNA in RNase free water. Each sample should be added to two separate tubes and the final volume should be 12 µl per tube. Incubate the tubes 70°C for 3 min and then rapidly cool on ice.
 - b. The reverse transcription reaction is performed in a final volume of 20 µl per sample, which will contain 8 µl of the reverse transcriptase master mix and 12 µl of RNA + oligo(dT). Make up the reverse transcriptase master mix so that it contains 12.5µM oligo(dT), 12.5mM dNTP mixture, 1.25 units/µl of RNase inhibitor, and 12.5 units/µl of reverse transcriptase in reverse transcriptase buffer. You will need 8 µl of master mix for each sample, so plan on making slightly more (about 9 µl per sample).
 - c. Add 8 µl of the reverse transcriptase master mix to one tube from each sample (which contains the RNA and oligo(dT)) for a total volume of 20 µl. The other tube for each sample is for the no RT control. Add 8 µl of RNase free water to this tube; do not add the reverse transcriptase master mix to this tube.
 - d. Incubate all tubes at 42 °C for 1 hr for reverse transcription to take place. Next, incubate the tubes at 92°C for 10 min to inactivate the reverse transcriptase.
 - e. Place tubes on ice for immediate use or store at -20°C for later use.

3.4 Real-time PCR

1. These instructions assume the use of a real-time PCR thermocycler that can accept a 96-well microtiter plate. Also, the primers for E-selectin, IL-8, and GAPDH are proprietary Taqman primers obtained from Applied Biosystems. Proprietary primers for virtually all human and mouse genes are available from this and other companies. Alternatively, one can design one's own primers and monitor

amplification with Syber Green (Molecular Probes, Eugene, OR). GAPDH, which has fairly consistent expression, is used as the reference gene to control for differences in cDNA content among the various samples. Other reference genes, such as ACT1 can also be used (*see* **Note 12**).

2. Dilute all samples of cDNA, including the no RT control, 1:30 using RNase free water. Prepare a master mix of each primer set (E-selectin, IL-8, and GAPDH) in three clean microcentrifuge tubes. Add 10 μ l of 2 $\times$ Taqman PCR master mix, 1 μ l of 20 $\times$ primer mixture, and 9 μ l of diluted cDNA to the wells of the microtiter plate. Each sample should be tested for each primer set in duplicate. Add only the GAPDH master mix to the no RT controls as only a single set of primers is necessary for testing these controls.
3. Store any leftover diluted cDNA at -20°C .
4. Seal the plate with the adhesive pad, using the applicator to firmly seal the wells.
5. Place the sealed plate into the real-time thermocycler and determine the C_T for each well. The C_T of the no RT control wells should be either undetectable or more than 30 cycles.
6. Use the C_T results from the duplicate wells to calculate the average the C_T for each condition.
7. To calculate the relative change in gene expression, use the $\Delta\Delta C_T$ method: For each condition, first calculate the ΔC_T .

$$\Delta C_T = C_T (\text{target}) - C_T (\text{normalizer})$$

In this experiment, the target is either IL-8 or E-selectin, and the normalizer is GAPDH.

Next, calculate the $\Delta\Delta C_T$

$$\Delta\Delta C_T = \Delta C_T (\text{experimental}) - \Delta C_T (\text{control})$$

The experimental condition is endothelial cells exposed to either *C. albicans* or TNF- α , and the control condition is endothelial cells exposed to medium alone.

Finally, calculate the comparative expression level, which is $2^{-\Delta\Delta C_T}$.

Representative results of the real-time PCR experiment are shown in Figure 1.

3.5 Whole Cell EIA to Detect E-selectin on the Endothelial Cell Surface

1. This protocol is designed to determine relative levels of E-selectin expressed on the surface of endothelial cells exposed to various stimuli. As an alternative to this protocol, one can purchase an EIA from companies such as R&D Systems for measuring the amount of soluble E-selectin that is shed from the endothelial cells into the medium.
2. This assay is performed using endothelial cell grown on the bottom of the wells of a 96-well tissue culture plate. Coat the wells by adding 50 μ l of fibronectin (10 μ g/ml in HBSS) for at least 30 min. Next aspirate the fibronectin and add endothelial cells. One 25 cm^2 flask of confluent endothelial cells should be sufficient to seed 18 wells of the 96-well plate (See **Notes 1 and 5–8**). Endothelial cells grow very slowly when in 96-well plates. Therefore, a relatively large number of cells should

be added to each well so that the cells are at least 95% confluent within 3 to 4 hr after they have been added. As in the real-time PCR experiment, the endothelial cells should be exposed to medium alone (negative control), *C. albicans* (experimental condition), and TNF- α (400 pg/ml; positive control). Six well should be used for each condition; 3 wells will be incubated with both the primary and secondary antibodies, and 3 wells will be incubated with the secondary antibody alone.

3. The day before the experiment, change medium above the endothelial cells, adding 200 μ l of medium per well. Place sufficient M-199 +20% serum for the experiment in the 5% CO₂ incubator to the night before the experiment. Keep the cap on the container loose so that the tissue culture medium can equilibrate with the CO₂. Set up the liquid culture of *C. albicans* as described in section 3.1.
4. The next morning, aspirate the medium from the wells and add the various stimuli in a total volume of 85 μ l per well (*see Note 9*). Process 6 wells at a time to avoid letting the endothelial cells dry up.
5. Incubate the 96-well plate in 5% CO₂ at 37°C for 8 hr.
6. At the end of the incubation period, aspirate the medium from each well (*see Note 11*).
7. Gently add 200 μ l of M199 + 5% FBS to each well using a multi-channel pipettor at the slowest speed setting.
8. Remove the medium by gentle aspiration, and carefully add 200 μ l of 4% paraformaldehyde to each well. Incubate plate at room temperature for 20 min to fix the cells.
9. Aspirate paraformaldehyde and add 200 μ l of 1 $\times$ TBS-glycine. Incubate for 5 min at room temperature.
10. Aspirate the TBS-glycine. From this point onward, aspiration can be done by inverting the plate and shake out the medium into a large beaker. Wash each well 3 times with 200 μ l of PBS. Prepare anti-E-selectin primary antibody by diluting the E-Selectin monoclonal antibody 1:100 in PBS+0.1% BSA. Add 80 μ l of the diluted antibody to 3 of the 6 wells exposed to each condition.
11. Add 80 μ l of PBS + 0.1% BSA to the 3 wells of each condition that did not receive the anti-E-selectin primary antibody.
12. Place cover on plate and incubate at 4°C overnight (*see Note 13*).
13. Rinse each well 3 times with 200 μ l PBS+0.1% BSA and then 2 times with 200 μ l of ddH₂O.
14. Block each well by adding 50 μ l of PBS containing 3% BSA and 1% sheep serum. Incubate for 20 min at room temperature.
15. During this time, prepare the horse radish peroxidase labeled sheep anti-mouse secondary antibody by diluting it 1:2500 in PBS containing 3% BSA and 1% sheep serum.
16. Remove the blocking buffer from the well and add 50 μ l of the secondary antibody to all wells.
17. Incubate plate for 1 hour at room temperature.

18. Rinse each well 3 times with 200 μ l PBS + 0.1% BSA and the 3 times with 200 μ l ddH₂O.
19. Prepare OPD peroxidase substrate solution by adding one OPD tablet and one urea hydrogen peroxide phosphate-citrate buffer tablet into 20 ml water (*see Note 14*)
20. Add 150 μ l of OPD solution to each well, and allow the color to develop at room temperature. Keep plate in the dark and check it every few minutes. The negative control well should turn faintly yellow and positive control wells should be significantly more yellow. The usual incubation time is approximately 20 min.
21. Stop the reaction by adding 50 μ l of 3N HCl.
22. Transfer 150 μ l from each well to the corresponding well of an empty 96-well flat bottom microtiter plate. This plated does not need to be sterile.
23. Determine the optical density (OD) of each with 490 nm using a spectrophotometer designed for 96-well plates.
24. Calculate the corrected OD for each condition by subtracting the OD of each no primary antibody well from the OD of the corresponding well that received primary antibody. Because no standard curve is included in this experiment, the amount of E-selectin expression induced by the various is usually just expressed as the corrected optical density. Alternatively, it can be expressed as the fold-stimulation relative to the endothelial cells exposed to medium alone. Representative results are shown in Figure 2.

4 Notes

1. All containers, pipets, reagents, and media that will be in contact with the endothelial cells should have as little endotoxin as possible. Most sterile, disposable plastic items are free of endotoxin. Glass and metal items can be made endotoxin-free by baking them at 180°C for 4 h. Reconstitute dry media using endotoxin-free water, such as pyrogen free, sterile irrigation water.
2. PBS solution purchased commercially is endotoxin-free. Alternatively, PBS can be made from endotoxin-free ingredients as follows. Add 100 mg KCl, 100 mg KH₂PO₄, 4.0 g NaCl, and 1.08 g Na₂HPO₄·7H₂O, to 450 ml water, and adjust the pH to 7.4 and the volume to 500 ml. Filter sterilize.
3. The organisms can be grown in a disposable sterile 50 ml polypropylene centrifuge tube with the cap screwed on loosely. To avoid having the caps fall off in the rotary shaker, apply a piece of tape running across the top of the tube and down one side.
4. Add the organisms to the M-199 just before use. If the organisms are allowed to sit in this medium at 37°C for very long, they will germinate and form aggregates of hyphae, which will result in variable stimulation of the endothelial cells.
5. When human umbilical vein endothelial cells are confluent, there are approximately 5×10^4 cells per cm². For the real-time PCR experiments using 24-well tissue culture plates, a multiplicity of infection (MOI) of 1 works well. For the whole cell EIAs performed using endothelial cells grown in 96-well plates, use an MOI of 2.
6. Trypsinizing and splitting endothelial cells: aspirate media from the C²⁵ flask, and rinse flask with 1 ml of trypsin. Aspirate and add another 2ml of trypsin. Wait for 10–15 second, check the flask under the inverted microscope for the cell rounding. Once all endothelial cells are round up, slightly tap to lift cells off from the bottom of the flask. Neutralize the trypsin by adding 4 ml of M199+20% serum, and then

transfer the cells to a 50 ml centrifuge tube. Centrifuge at $500 \times g$ for 5 min without braking. Gently aspirate supernatant without disturbing the pellet. Suspend pellet with M199+20% serum and resuspend the cells by gently aspirating and then expelling the mixture with a 10 ml pipet.

7. Most trypsin contains endotoxin. Therefore, to minimize stimulation caused by exposure to the trypsin, the endothelial cells should be split into the 24-well plate at least 48 hr before use in the experiment.
8. The magnitude of endothelial cell stimulation by *C. albicans* is usually the greatest when endothelial cells are used at the first or second passage. Later passage cells usually have higher basal expression of leukocyte adhesion molecules and cytokines, and lower induced expressions of these pro-inflammatory molecules.
9. To aspirate media without removing or damaging the endothelial cells, use a sterile Pasteur pipette and connected with rubber tubing to a vacuum flask. Use as low vacuum as possible to aspirate the medium slowly. Avoid touching the bottom of the wells with the pipet tip. Also, process only two wells at a time so that the endothelial cells will not dry out.
10. Use RNase-free pipet tips and centrifuge tubes, wear gloves and keep samples on ice, except when specifically indicated.
11. Sometimes it is desirable to determine the cytokine concentration in the conditioned medium above the endothelial cells. In this case, collect the conditioned medium from each well and place it in a pre-labeled, sterile microfuge tube on ice. Centrifuge the microfuge tube at $500 \times g$ for 5 min at 4°C . Collect the supernatants and store them in 50 to 100 μl aliquots at -80°C . The cytokine concentration in the supernatants can be determined by commercially available EIA kits, such as from R&D Systems (Minneapolis, MN). Alternatively, the concentrations of multiple cytokines can be determined simultaneously the flow cytometric Cytokine Bead Array from R&D Systems.
12. When using the $\Delta\Delta\text{C}_T$ method of determining relative gene expression, it is important that the amplification efficiency of the primers for the reference gene is similar to that of the primers for the experimental genes. In our experience, some primers for ribosomal RNA have different efficiency compared to primers for non-ribosomal RNA genes, and we therefore avoid using ribosomal RNA as the reference.
13. The wells can be incubated with the primary antibody at 4°C for up to 72 hr.
14. OPD solution should be made within 5 minutes of use. The solution is carcinogenic, be careful when handling. The solution will eventually turn color if not used within 20 minutes. This solution should be disposed as a biohazard substance.

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Fig. 1. Real-time PCR measurement of endothelial cell stimulation by *C. albicans*. Endothelial cells were incubated with tissue culture medium (control), *C. albicans*, or TNF- α for 8 hr, after which the relative abundance of mRNA for E-selectin (A) and IL-8 (B) was measured using real-time PCR. Results are the mean $\pm$ SD of three independent experiments.

Fig. 2.

E-selectin expression and IL-8 secretion by endothelial cells infected with *C. albicans*.

Endothelial cells were incubated with tissue culture medium (control), *C. albicans*, or TNF- α for 8 hr. (A) Surface expression of E-selectin, as measured by whole cell EIA. Results are the mean $\pm$ SD of a single experiment performed in triplicate. (B) Concentration of IL-8 in the medium above endothelial cells grown in a 24-well tissue culture plate as measured using a commercial EIA. Results are the mean $\pm$ SD of a three experiments.