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Invasive Phenotype of *Candida albicans* Affects the Host Proinflammatory Response to Infection

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***Candida albicans* is a major opportunistic pathogen in immunocompromised patients. Production of proinflammatory cytokines by host cells in response to *C. albicans* plays a critical role in the activation of immune cells and final clearance of the organism. Invasion of host cells and tissues is considered one of the virulence attributes of this organism. The purpose of this study was to investigate whether the ability of *C. albicans* to invade host cells and tissues affects the proinflammatory cytokine responses by epithelial and endothelial cells. In this study we used the invasion-deficient *RIM101* gene knockout strain DAY25, the highly invasive strain SC5314, and highly invasive *RIM101*-complemented strain DAY44 to compare the proinflammatory cytokine responses by oral epithelial or endothelial cells. Using a high-throughput approach, we found both qualitative and quantitative differences in the overall inflammatory responses to *C. albicans* strains with different invasive potentials. Overall, the highly invasive strains triggered higher levels of proinflammatory cytokines in host cells than the invasion-deficient mutant triggered. Significant differences compared to the attenuated mutant were noted in interleukin-1α (IL-1α), IL-6, IL-8, and tumor necrosis factor alpha in epithelial cells and in IL-6, growth-related oncogene, IL-8, monocyte chemoattractant protein 1 (MCP-1), MCP-2, and granulocyte colony-stimulating factor in endothelial cells. Our results indicate that invasion of host cells and tissues by *C. albicans* enhances the host proinflammatory response to infection.**

Candida albicans is present in most humans as a commensal organism; however, for immunocompromised individuals *C. albicans* is an opportunistic pathogen which causes localized invasive mucosal infections or life-threatening disseminated and deep-seated organ infections (33). Several virulence attributes have been shown to be positively associated with invasive infection during the infectious process. For example, the ability of *C. albicans* to switch among yeast, pseudohyphal, and true hyphal morphologies is thought to be critical for invasion, since yeast cells are thought to disseminate hematogenously more efficiently, while filamentous organisms have a greater potential to locally invade host tissues by promoting cell damage (for a review see, reference 1). Consequently, mutants with defects in filamentation have reduced virulence in animal models of disseminated candidiasis (10) and are unable to establish mucosal infections in animal models of oropharyngeal and vaginal candidiasis (34, 40). Adhesins, secreted aspartyl proteinases, and phospholipases expressed at high levels by hyphal morphotypes, which enable *C. albicans* to interact with, damage, and invade host cells, may play significant roles in establishing invasive infections locally or systemically (20, 35).

We and others have shown that only live, filamentous organisms growing in contact with host cells are capable of stimulating proinflammatory cytokine responses in nonimmune cells (13, 14, 19, 37, 47). In this study we hypothesized that the ability of *C. albicans* to invade host tissues may affect the intensity and composition of the proinflammatory cytokine response of host cells to infection. To investigate this hypothesis, we compared *C. albicans* strains that differ in the capacity to establish an invasive infection with respect to the ability to trigger an array of proinflammatory cytokine responses by epithelial and endothelial cells in vitro.

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MATERIALS AND METHODS

Organisms. Homozygous deletion of the *RIM101* gene from *C. albicans* has been shown to severely curtail the ability to hematogenously invade organs and tissues in a mouse model of candidiasis (11). Therefore, in the present study we used a *RIM101* gene knockout strain (DAY25) to represent the strains having a reduced invasive phenotype. The control strains, which displayed a strong invasive phenotype in vivo, included the *RIM101*-complemented strain DAY44 and the clinical strain SC5314, which was originally isolated from a patient with invasive disseminated candidiasis (21). The prototrophic mutant strain DAY25 (*rim101*$^-$/*rim101*$^-$) and complemented strain DAY44 have been described elsewhere (11). The complete genotype of DAY25 is *ura3*Δ::λ*imm434*/*ura3*Δ::λ*imm434 HIS1*::*his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG rim101*::*ARG4*/*rim101*::*URA3*. The complete genotype of DAY44 is *ura3*Δ::λ*imm434*/*ura3*Δ::λ*imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG rim101*::*ARG4*::pRIM101::*HIS1*/*rim101*::*URA3*. All of the strains used in this study had similar growth rates in keratinocyte or endothelial cell media, as determined by direct cell counting of yeast cells or by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazoluim-hydroxide (XTT) assay when germinated organisms were tested. XTT is converted into a colored formazan product in the presence of metabolically active *C. albicans*, and the assay is based on the linear relationship between *C. albicans* cell number and the formazan colorimetric signal within a certain range of *C. albicans* concentrations (26). The XTT assay showed that there was no statistically significant difference between the growth rates of SC5314 and DAY25, between the growth rates of SC5314 and DAY44, and between the growth rates of DAY25 and DAY44 ($P = 0.94$, $P = 0.39$, and $P = 0.28$, respectively). The organisms were routinely propagated in YPD agar (Difco Laboratories, Detroit, MI) at 25°C.

Cell cultures. Because epithelial and endothelial cells represent the first line of defense against invasive mucosal and hematogenously disseminated infections, respectively, proinflammatory responses were characterized in these two cell types. The epithelial cells included a well-differentiated squamous cell carcinoma

of the ventral tongue (cell line SCC15) (30) obtained from D. Wong (University of California at Los Angeles). SCC15 cells were maintained in keratinocyte serum-free media (KSFM) (Invitrogen, Carlsbad, CA) supplemented with 0.4 mM $CaCl_2$, 0.1 ng/ml epidermal growth factor, 50 μg/ml bovine pituitary extract (Invitrogen, Carlsbad, CA), and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin).

The endothelial cells used (HMEC-1 cells) originated from transformation of primary human dermal endothelial cells with the simian virus 40 large T antigen and were graciously provided by F. Candal (Centers for Disease Control and Prevention, Atlanta, GA). These cells retain the endothelial cell morphology and array of endothelial cell surface antigens (45, 46). HMEC-1 cells were maintained in MCDB 131 medium (GibcoBRL, Rockville, MD) enriched with 10% fetal bovine serum (FBS), 2 mM L-glutamine (Sigma, St. Louis, MO), 1 μg/ml hydrocortisone (Sigma, St. Louis, MO), and 0.01 μg/ml epidermal growth factor (Roche, Indianapolis, IN).

Coculture of *C. albicans* with epithelial and endothelial cell monolayers. Stationary-phase yeast cells were prepared by growth for 18 h at room temperature in YPD agar broth (Difco Laboratories, Detroit, MI) supplemented with 2% (wt/vol) dextrose. The fungal cells were harvested by centrifugation and washed in phosphate-buffered saline (PBS). Subsequently, yeast cells were counted with a hemacytometer, and the final concentration was adjusted in complete KSFM and MCDB 131 medium before the cells were added to epithelial and endothelial cells, respectively.

SCC15 and HMEC-1 cells were seeded at or near confluence (4×10^5 cells/well and 8×10^5 cells/well, respectively) in six-well polystyrene plates (Corning Incorporated, Corning, NY) and were incubated overnight in complete KSFM or MCDB 131 medium at 37°C in a 5% CO_2 atmosphere. The following day the media were discarded, and the cells were challenged with suspensions of stationary-phase viable organisms at various fungal cell-to-host cell ratios for up to 20 h. The negative controls for these experiments included uninfected cultures and *C. albicans* alone. At the end of the experimental period supernatants or cell lysates were collected and stored at −70°C until they were assayed.

Cell lysates were prepared after removal of supernatants using PBS containing 1% IGEPAL (NP-40) (Sigma, St. Louis, MO), 1 mM EDTA, 0.02% NaN_3, and inhibitors of proteases (0.5 mM phenylmethylsulfonyl fluoride [Sigma, St. Louis, MO], 2 μg/ml of pepstatin [Sigma, St. Louis, MO], and 1 μg/ml of antipain [Sigma, St. Louis, MO]) for 15 min on ice. The adherent cells were scraped from the plates with a rubber policeman, and the cell lysates were sedimented by centrifugation at 4°C in a microcentrifuge at the maximum speed for 10 min. The protein concentration of each cell lysate was determined by using the Bio-Rad protein determination assay (Bio-Rad, Hercules, CA).

Cytokine detection. (i) Cytokine protein array. Multiple proinflammatory cytokines were simultaneously detected in cell supernatants and lysates using a commercially available enzyme-linked immunosorbent assay (ELISA)-based cytokine protein array (Ray Bio cytokine array; RayBiotech, Norcross, GA). The cytokine protein array system used contained 23 pairs of capture monoclonal antibodies spotted onto a nitrocellulose membrane, and the procedure was performed according to the manufacturer's instructions. Briefly, after membrane blocking, 1-ml portions of supernatants or lysates (at a protein concentration of 350 μg/ml) were added and incubated for 2 h, and this was followed by addition of a cocktail of biotinylated detection antibodies at a dilution of 4:1,000. The membranes were developed by addition of horseradish peroxidase-conjugated streptavidin at a dilution of 1:1,000 for 2 h, followed by addition of an enhanced chemiluminescence-type solution. The membranes were exposed to X-ray film (Kodak BioMax film; Kodak, Rochester, NY) for 10 min and processed by autoradiography. Positive control signals (provided by the manufacturer) on each membrane were used to normalize cytokine signal intensities. Signals in films were then scanned and stored as TIF images, and their intensities were determined by densitometry analysis using the Chemilmager software (Alpha Innotech Corporation, San Leandro, CA).

(ii) ELISA. To confirm the findings obtained with cytokine arrays and to quantify certain cytokine responses, culture supernatants obtained from duplicate or triplicate experiments were analyzed by ELISA. In each experiment supernatants from two replicate wells were pooled and assayed by duplicate sandwich ELISAs using commercially available antibody pairs (Endogen Mini-Kit; Pierce, Rockford, IL). Briefly, flat-bottom 96-well microtiter plates (Fisher Scientific, Springfield, NJ) were coated with 1 μg/ml of anti-human interleukin-1α (IL-1α), IL-6, IL-8, or tumor necrosis factor alpha (TNF-α) monoclonal antibodies in carbonate buffer and blocked with 1% bovine serum albumin in PBS for 4 h. For IL-1α and IL-6 analyses, 50 μl of supernatant and 50 μl of rabbit anti-human cytokine antibody (Endogen, Woburn, MA) at a dilution of 1:5,000 were added at the same time in each well and incubated for 2 h. For IL-8 and TNF-α analyses, 100 μl of supernatant was added to each well and incubated for 1 h, followed by addition of 100 μl of rabbit anti-human cytokine antibody at a 1:5,000 dilution. The reaction color was developed by addition of horseradish peroxidase-conjugated streptavidin, followed by addition of a tetramethylbenzidine substrate solution (1 Step TM Ultra tetramethylbenzidine ELISA; Pierce, Rockford, IL). The reaction was stopped with 0.2 M H_2SO_4 (100 μl per well). Absorbance values and corresponding cytokine concentrations were determined with an Opsys MR microplate reader (Dynex Technologies Inc., Chantilly, VA) using the Revelation QuickLink software (Thermo Labsystems, Chantilly, VA). The sensitivities of the assays ranged from 8 to 16 pg/ml.

Microscopic examination of *C. albicans* strains. In order to examine *C. albicans* morphogenesis during coculture with oral epithelial and dermal microvascular endothelial cells, we used the calcofluor white fluorescent dye. Calcofluor white is a chitin-binding fluorescent agent that stains the cell wall of *C. albicans* (9). Epithelial and endothelial cells were seeded on glass slides (4×10^5 cells/slide and 8×10^5 cells/slide, respectively) contained in six-well polystyrene plates (Corning Incorporated, Corning, NY) and were incubated overnight in complete KSFM or MCDB 131 medium at 37°C in 5% CO_2. The following day the media were discarded, and the cells were challenged with 1×10^5 stationary-phase viable organisms in complete KSFM or MCDB 131 medium. After 5 h of incubation at 37°C in 5% CO_2, the cover slides were carefully removed from each well and were placed on frosted microscope slides. Each cover glass was stained with 100 μl of 0.05 mM calcofluor white (calcofluor white fluorescent brightener 28; Sigma, St. Louis, MO), and the morphology of each strain was examined by fluorescence microscopy.

Assessment of the invasive phenotypes of *C. albicans* strains in vitro. (i) Monolayer cultures. One mechanism by which *C. albicans* can penetrate through mucosal and vascular barriers is by damaging and eventually killing epithelial and endothelial cells, respectively. Therefore, in this study we first measured the invasive capacities of different *C. albicans* strains in vitro by determining their abilities to damage a cell monolayer. The ability of *C. albicans* to injure oral epithelial and dermal microvascular endothelial cells was assessed by the Cyto-Tox-96 assay (Promega, Madison, WI), which measures the release of lactate dehydrogenase (LDH) from dying cells. In these experiments SCC15 or HMEC-1 cells were cocultured with increasing doses of strain SC5314, mutant strain DAY25, and complemented strain DAY44 for 8 h, and the LDH released from the coculture system was quantified by spectrophotometry performed according to the manufacturer's instructions. Uninfected cultures (control 1) and *C. albicans* alone (control 2) incubated under identical conditions were included as negative controls. The total amount of LDH released was estimated by treating control uninfected SCC15 or HMEC-1 cells with 9% Triton X-100 for 1 h. The LDH released in the presence of *C. albicans* was quantified by using the following formula: [(experimental − control 1 − control 2)/(total − control 1)] × 100. The values were expressed as percentages of the total amount of LDH released.

The abilities of the microorganisms to invade a cell monolayer were also directly assessed by determining their abilities to traverse through a confluent cell monolayer in vitro. To set up this assay, 24-transwell polystyrene plates (Corning Incorporated, Corning, NY) were used. The upper compartment of a transwell plate was seeded at a concentration slightly above the confluence concentration (5×10^4 cells/well), and epithelial cells were allowed to adhere overnight at 37°C in 5% CO_2. To confirm the monolayer integrity and test its permeability to macromolecules in vitro, the dextran permeability assay was used, as previously described (3). The negative and positive controls included membranes without cells, membranes with subconfluent SCC15 cells, and membranes with confluent MDCK cell (ATCC CCL-34) monolayers. MDCK cells are known to form tight junctions which are relatively impermeable to dextran under these in vitro conditions.

Stationary-phase viable *C. albicans* (1×10^4 yeast cells suspended in 0.1 ml of KSFM) were added to the upper compartment. After 4 h of incubation, the transwell insert was removed. *C. albicans* cells that moved through the epithelial cell monolayer into the lower compartment were quantified using the XTT assay. To set up this assay, 0.2 ml of PBS containing 0.25 mg/ml of XTT (Sigma Chemical Co., St. Louis, MO) and 40 μl/ml of coenzyme Q (Sigma Chemical Co., St. Louis, MO) were added to each well, and the plates were incubated at 37°C for 2.5 h (26). One hundred microliters of supernatant was transferred to a 96-well plate, and the optical density at 492 nm was measured by using an Opsys MR microplate reader (Dynex Technologies Inc., Chantilly, VA) and the Revelation QuickLink software (Thermo Labsystems, Chantilly, VA). Data obtained with the XTT assay correlated well ($r^2 = 0.73$) with direct CFU counts obtained from the upper and lower compartments after incubation of their contents for 48 h in YPD agar plates (not shown).

(ii) Three-dimensional model of the human oral mucosa. To further characterize the tissue-invasive properties of the strains used in these studies, we

developed a three-dimensional model system of the oral mucosa. This system is composed of gingival fibroblasts embedded in a biomatrix of collagen type I, overlaid by a multilayer of oral epithelial cells. In order to develop the oral mucosal model, gingival fibroblasts from primary cultures were harvested and diluted with a precooled gel medium (4°C; 2× Dulbecco's modified Eagle's medium [DMEM]) containing 100 mM HEPES to obtain a final concentration of 3×10^5 cells/ml. The cell suspension was mixed with an equal volume of an acidic collagen solution (4 mg/ml of collagen type I from rat tails[Sigma] in 0.1% acetic acid), and 200 μl of this solution was placed in the upper compartment of a Millicell culture plate insert (Millicell-MA; diameter, 13 mm; Millipore, Bedford, MA). The gel was allowed to solidify for 15 min at 37°C in 5% CO_2. Each insert was transferred to a well of a 24-well plate containing 0.5 ml of DMEM–10% FBS (Mediatech, Herndon, VA). Two days later 200 μl of KSFM (Invitrogen, Carlsbad, CA) containing 1×10^5 keratinocytes was added to each insert. The culture medium was changed every other day using KSFM inside the inserts and DMEM–10% FBS basolaterally. After 6 days under submerged conditions, the inserts were transferred into six-well plates, and the cells were exposed to air. Airlift medium (KSFM containing 5% FBS, 1.88 mM $CaCl_2$, and 0.025 mM glucose) was added only basolaterally. The cultures were grown for 14 additional days with daily changes of the medium until a multilayer consisting of 10 to 15 keratinocyte cell layers was formed.

To study invasion, 100 μl of airlift medium containing 1×10^5 *C. albicans* yeast cells was added inside the inserts. Two days later all cultures were fixed with 10% formaldehyde–PBS and embedded in paraffin. Sections (thickness, 5 μm) were stained with periodic acid-Schiff stain and were evaluated under a light microscope.

Statistical analyses. The statistical significance of the differences in cytokine levels (detected by ELISA), invasion, and cytotoxicity between pairs of *C. albicans* strains was determined by a two-tailed *t* test, assuming equal variances. Cytokine array data from the three different strains were compared by analysis of variance. Differences were considered statistically significant at a *P* value of <0.05.

RESULTS

***C. albicans* SC5314, the *rim101*$^-$/*rim101*$^-$ mutant, and the RIM101-complemented strain exhibited similar patterns of filamentation when they were cocultured with epithelial and endothelial cells.** Because the proinflammatory response of epithelial (47) or endothelial (38) cells to *C. albicans* in vitro depends on the capability of the organism for true hyphal transformation, we first confirmed that all strains formed true hyphae when they infected oral epithelial and endothelial cells in our coculture systems. Oral epithelial cells and dermal microvascular endothelial cells nonspecifically absorbed some of the calcofluor white stain and can be seen in the background in Fig. 1. Clinical strain SC5314 germinated within 2 h over the epithelial cell monolayer and produced long hyphae with parallel walls along the entire length, as shown by calcofluor white staining (Fig. 1A). Similarly, within 2 h strains DAY25 (Fig. 1B) and DAY44 (Fig. 1C) germinated over the epithelial cell monolayer and showed a morphological pattern that is consistent with true hyphal transformation. The germination patterns of the three strains when they were cocultured with endothelial cells were similar (not shown).

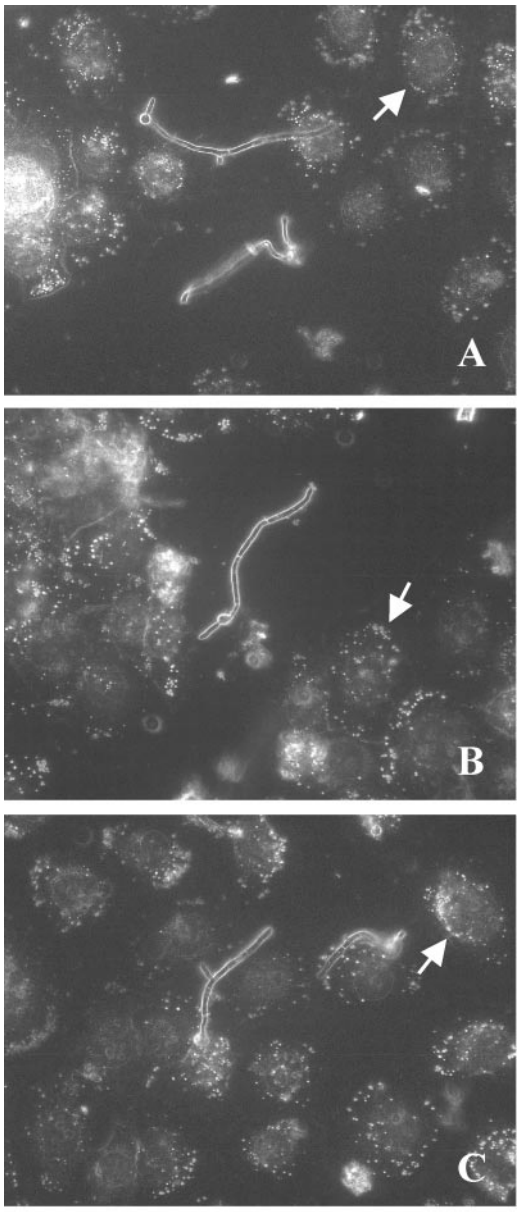

FIG. 1. Cellular morphology of strain SC5314 (A), DAY25 (*rim101*$^-$/*rim101*$^-$ mutant) (B), and complemented strain DAY44 (*rim101*$^-$/*rim101*$^-$ + RIM101) (C) cocultured with epithelial cells for 5 h. The infectivity ratios were the same, and the numbers of cells germinating in the strains were roughly equivalent. Epithelial cells are indicated by the arrows. Cultures were stained with calcofluor white, and fluorescence micrographs are shown. Bar = 10 μm.

***rim101*$^-$/*rim101*$^-$ mutant had a reduced invasive phenotype in vitro.** Although the *rim101*$^-$/*rim101*$^-$ mutant (DAY25) has been described as a strain that displays a defect in hematogenous tissue invasion in vivo (11, 42), we had to confirm that the reduced invasive phenotype was expressed in vitro as well. It is widely accepted that the ability of *C. albicans* to invade through cells correlates well with its ability to inflict injury to these cells (38, 18); therefore, we first tested the ability of each strain to damage an epithelial or endothelial cell monolayer. To quantify cell injury, we compared the levels of LDH released when mutant strain DAY25, complemented strain DAY44, and clinical strain SC5314 were cocultured with SCC15 or HMEC-1 cells at different infectivity ratios for up to 20 h. In general, cell damage was time and infectious dose dependent (not shown). Clinical strain SC5314 and complemented strain DAY44 were the most powerful strains in promoting both oral keratinocyte and dermal microvascular endothelial cell lysis at all the infectious doses tested. After 8 h of coincubation, strain SC5314 induced the lysis of 13.8% ± 1.3% of the oral epithelial cells and 48.8% ± 4.7% of the microvascular endothelial cells when

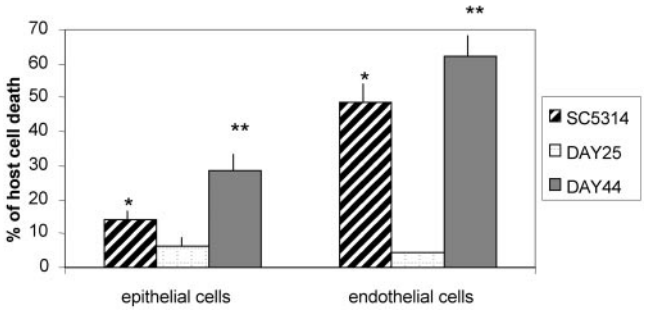

FIG. 2. Abilities of the different *C. albicans* strains to injure oral epithelial cells or microvascular endothelial cells. SCC15 or HMEC-1 cells were cocultured with clinical strain SC5314, mutant DAY25, or complemented strain DAY44 for 8 h, and the LDH release in culture supernatants was quantified. Mean values were obtained by analysis of at least three separate experiments, and the error bars indicate one standard deviation of the mean. One asterisk indicates that the *P* value is ≤0.005 for a comparison with cells infected with DAY25. Two asterisks indicate that the *P* value is ≤0.001 for a comparison with cells infected with SC5314.

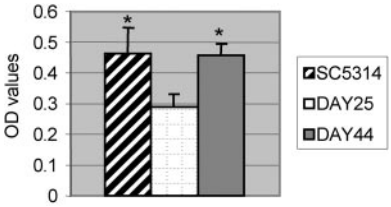

FIG. 3. Abilities of the different *C. albicans* strains to invade through an oral epithelial cell monolayer. SCC15 cells seeded in an upper compartment of a transwell plate were challenged with clinical strain SC5314, mutant DAY25, or complemented strain DAY44 for 4 h. The abilities of the strains to invade through the SCC15 monolayer are expressed as the XTT metabolic activity in the lower compartment of the transwell plate. Mean values were obtained by analysis of triplicate wells in two separate experiments, and the error bars indicate one standard deviation of the mean. An asterisk indicates that the *P* value is ≤0.0005 for a comparison with strain DAY25. OD, optical density.

it was added to host cells at a 10:1 yeast-to-host cell ratio (Fig. 2). At the same infectious dose, the complemented strain also promoted a high level of cell damage, promoting the lysis of 28.3% ± 3.7% of the oral epithelial cells and 62.2% ± 6.5% of the microvascular endothelial cells (Fig. 2). Under these conditions, mutant strain DAY25 exhibited a reduced capacity to injure host cells, damaging only 6.3% ± 1.9% of the epithelial cells ($P = 0.005$ for a comparison with SC5314; $P = 0.0008$ for a comparison with DAY44) and 4.1% ± 0.9% of the endothelial cells ($P = 0.0002$ for a comparison with SC5314; $P = 0.0003$ for a comparison with DAY44) (Fig. 2). Strain DAY44 was able to inflict greater cellular injury than SC5314 on epithelial and endothelial cells ($P = 0.003$ and $P = 0.01$, respectively).

Since the ability of the *rim101*$^-$/*rim101*$^-$ mutant to damage an epithelial or endothelial cell monolayer was compromised, we hypothesized that the ability of this mutant to traverse through a cell monolayer is also impaired. To test this hypothesis, we used a transwell invasion assay as described above. Prior to infection we verified that the epithelial cell monolayer was relatively impermeable to dextran, as determined spectrophotometrically by comparison to a confluent MDCK cell monolayer (not shown). Strain SC5314 and complemented strain DAY44 had the greatest ability to traverse through an oral epithelial cell monolayer, as determined by the XTT assay (Fig. 3) or colony counting (not shown). Although mutant strain DAY25 was able to cross the epithelial cell monolayer, it showed a significantly attenuated invasive phenotype compared to the SC5314 and complemented strains ($P = 0.00028$ and $P = 0.00018$, respectively) (Fig. 3).

Finally, the ability of the *rim101*$^-$/*rim101*$^-$ mutant to invade through an in vitro mucosal model system was evaluated and was compared to the ability of highly invasive clinical strain SC5314 (Fig. 4). In this model, both strains germinated into hyphae and formed a biofilm over the oral epithelium. Consistent with prior reports (12), strain SC5314 invaded past the basal epithelial cell layer, through the entire length of the collagen embedded fibroblasts, and through the porous membrane of the culture insert (Fig. 4A). In contrast, strain DAY25 (*rim101*$^-$/*rim101*$^-$) was observed only within the superficial layers of the oral epithelium and did not cross the basal cell layer into the submucosa (Fig. 4B).

Highly invasive strains stimulate a stronger proinflammatory cytokine response to infection. We next hypothesized that the invasion-deficient strain DAY25 may trigger a host cell proinflammatory response that differs in intensity and/or composition from the response triggered by the more invasive strains SC5314 and DAY44. Using antibody-based arrays, we found both qualitative and quantitative differences in the overall inflammatory responses to *C. albicans* strains with different invasive potentials. The highly invasive strains (SC5314 and DAY44) induced significantly higher levels of all the proinflammatory cytokines detected by the cytokine array in both endothelial and epithelial cells compared to the levels in uninfected cells ($P < 0.05$) (Table 1). The attenuated mutant triggered significant induction of only a small subset of the cytokines (IL-6 [$P < 0.05$] and IL-1α [$P < 0.01$]) above the basal levels in the epithelial cell system, but it was unable to trigger significant upregulation of any other cytokine. Similarly, this mutant was unable to trigger significant upregulation of any cytokine detected in uninfected endothelial cells, but it caused statistically significant downregulation of the constitutive monocyte chemoattractant protein 1 (MCP-1) ($P < 0.005$) and MCP-2 ($P < 0.05$) levels. The cytokines induced at significantly higher levels by both invasive strains than by mutant strain DAY25 included IL-6, IL-1α, IL-8, and TNF-α in epithelial cells and IL-6, GRO, IL-8, MCP-1, granulocyte colony-stimulating factor (G-CSF), and MCP-2 in endothelial cells (Table 1).

To confirm the differences in cytokine responses among the strains used in this study, culture supernatants were analyzed by ELISA for IL-1α and TNF-α in epithelial cells and for IL-8, IL-6, and TNF-α in endothelial cells. In general, the cytokine responses (upregulation or no change from the basal results) to the different strains as determined by the array methodology were confirmed by ELISA for all the cytokines tested (Fig. 5). SCC15 cells responded to strains SC5314 and DAY44 with greater IL-1α release than the release observed with DAY25 ($P = 0.0002$ and $P = 0.0006$, respectively). Strain DAY44 triggered significantly greater release of IL-1α than strain SC5314 triggered ($P < 0.05$). Furthermore, only strains SC5314 and DAY44 stimulated TNF-α secretion by SCC15

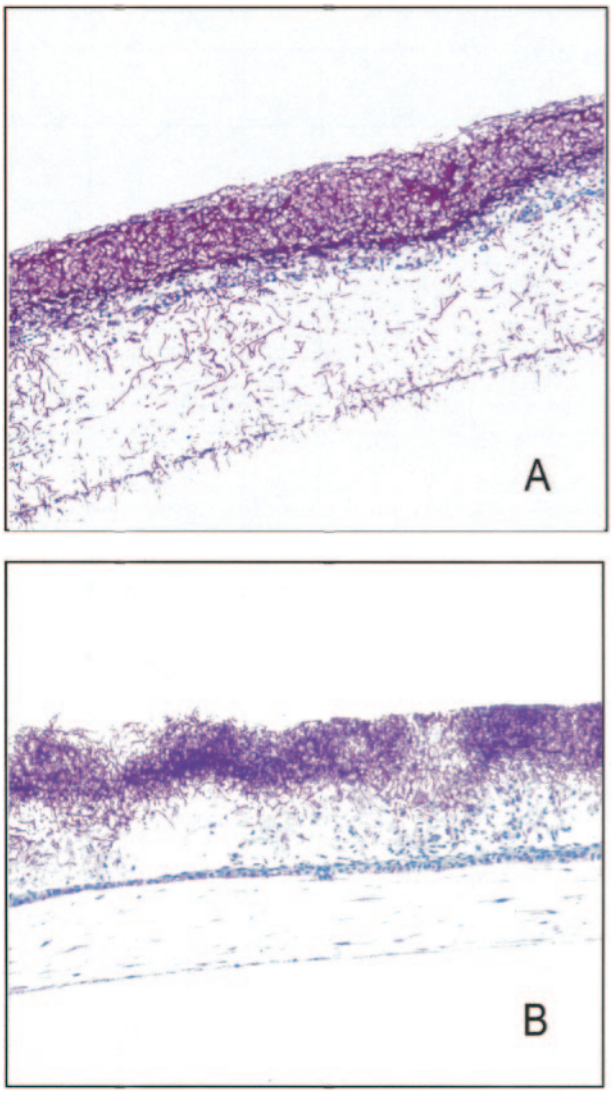

FIG. 4. Infection of a three-dimensional model of the oral mucosa with strain SC5314 (A) or invasion-deficient mutant DAY25 (B) for 48 h. Periodic acid-Schiff-stained paraffin sections (thickness, 5 μm) are shown at a magnification of ×10. The results are the results of one of three representative experiments.

cells (32.7 ± 6.1 and 46.1 ± 11.6 pg/ml, respectively). Similarly, only strains SC5314 and DAY44 stimulated significant IL-6 secretion above uninfected levels by endothelial cells ($P = 0.001$ and $P = 0.016$, respectively). Finally, consistent with the array results, strains SC5314 and DAY44 were able to induce a higher level of IL-8 secretion by microvascular endothelial cells than DAY25 was able to induce ($P = 0.0005$ and $P = 0.008$, respectively). TNF-α was not detected in any of the infected endothelial cell supernatants tested by ELISA (not shown), confirming the array findings.

DISCUSSION

In previous studies we showed that *C. albicans* transformation into true hyphae enhances the interaction of *C. albicans* with oral epithelial cells by increasing the ability of the organism to adhere to the cells and to trigger a potent proinflammatory response by the cells (13, 15, 47). In this study we investigated the role of *C. albicans* invasion in the host proinflammatory response to infection. As the first cells to interact with *C. albicans*, epithelial and endothelial cells represent the first line of defense against invasive mucosal infection and hematogenously disseminated infection, respectively. We found that highly invasive strains triggered a greater proinflammatory cytokine response by epithelial and endothelial cells than an invasion-deficient mutant triggered, suggesting that a stronger invasive phenotype contributes to a more potent inflammatory response to infection.

Consistent with our results, it has been reported previously that only internalized live germinated *C. albicans* organisms are capable of stimulating the synthesis of IL-6, IL-8, and MCP-1 and the expression of E-selectin, ICAM-1, and VCAM-1 by human umbilical vein endothelial cells (HUVEC) (19). More importantly, the amount of cytokines synthesized by HUVEC in response to *C. albicans* is closely associated with the level of host cell invasion and injury promoted by this organism (19). Our study is the first study to report on the coordinated production of additional proinflammatory cytokines, including GRO, G-CSF, and MCP-2, by endothelial cells in response to invasive fungal infection. While we significantly extended the array of cytokines produced by endothelial cells using a high-throughput approach, the presence of serum in the endothelial cell samples may have caused certain low-level cytokines to be diluted below the sensitivity level of the cytokine array. Therefore, it is possible that the complete proinflammatory profile of the endothelial cell response to invasive fungal infection includes additional, as-yet-unidentified cytokines.

We were unable to demonstrate the synthesis of TNF-α and IL-1α by HMEC-1 cells in response to *C. albicans*, as has been previously reported for HUVEC (37). There are several possible explanations for this discrepancy. First, in the previous study HUVEC produced a very low level of TNF-α (<10 pg/ml), which in fact is very close to the detection limit of most ELISAs. Second, IL-1α synthesized by HUVEC in response to *C. albicans* was cell associated and detected mainly in whole-cell lysates (37). In our study cell lysates were centrifuged at the maximal speed for 10 min, and supernatants were analyzed for cytokine content, which would have excluded most membrane-associated proteins, such as IL-1α. Third, there are significant differences between the phenotype of HUVEC and the phenotype of microvascular endothelial cells, including differential constitutive expression of von Willebrand factor and CD34 (46) and induction of VCAM-1 and E-selectin in response to TNF-α (28), supporting the notion that these cells may respond differently to infection. Finally, different strains were used to challenge endothelial cells in the two studies (37).

Cytokines were quantified in cell lysates in addition to supernatants in order to capture cytokines that are not actively secreted but are stored intracellularly upon synthesis in response to fungal challenge. For example, we have previously reported that the IL-1α induced in oral keratinocytes in response to the invasive strain SC5314 accumulates intracellularly during the first 12 h of infection, before it is released into culture supernatants (15). Consistent with our prior report, using a cytokine protein array, we detected both intracellular IL-1α in 8-h cell lysates and extracellular IL-1α in 20-h super-

TABLE 1. Cytokine array analysis of SCC15 and HMEC-1 cells following infection with viable *C. albicans* strain SC5314, mutant DAY25, and complemented strain DAY44 at a fungal cell-to-host cell ratio of 1:1[a]

Conditions	Cytokine	Fold induction compared with uninfected cells		
		SC5314	DAY25	DAY44
SCC15 cell lysate	IL-6	28.0 ± 5.4[b]	4.4 ± 1.7	27.8 ± 7.3[b]
	GRO	2.3 ± 0.4	1.3 ± 0.2	4.0 ± 2.0
	IL-1α	2.7 ± 0.6[c]	1.2 ± 0.3	2.4 ± 0.3[c]
	IL-8	3.0 ± 0.9[c]	1.0 ± 0.1	2.7 ± 0.7[c]
SCC15 supernatant	IL-6	4.0 ± 0.3	2.9 ± 0.8	4.4 ± 0.6
	GRO	4.2 ± 1.6	3.0 ± 1.4	5.0 ± 1.9
	IL-1 α	11.8 ± 2.5[c]	5.7 ± 2.1	9.6 ± 0.1[c]
	IL-8	1.8 ± 0.1[d]	1.2 ± 0.1	1.6 ± 0.2[d]
	TNF-α	12.7 ± 9.8[c]	1.0 ± 0.0	13.1 ± 5.3[c]
	TNF-β1	6.0 ± 4.2	0.9 ± 0.1	3.1 ± 0.2
HMEC-1 cell lysate	IL-6	2.0 ± 0.2[b]	1.2 ± 0.3	3.9 ± 0.3[b]
	GRO	2.8 ± 0.8[c]	1.4 ± 0.3	2.4 ± 0.2[c]
	IL-8	1.8 ± 0.2[d]	1.1 ± 0.2	1.8 ± 0.0[d]
	IL-15	2.5 ± 0.7	1.4 ± 0.2	2.0 ± 0.0
	MCP-1	4.3 ± 0.0[d]	0.6 ± 0.0	3.0 ± 0.7[d]
	G-CSF	2.3 ± 0.0[c]	1.0 ± .00	1.8 ± 0.5[c]
	RANTES	2.2 ± 0.1	1.1 ± 0.1	2.4 ± 0.3
HMEC-1 supernatant	IL-6	5.5 ± 1.4[c]	1.8 ± 1.0	2.8 ± 1.8[c]
	GRO	1.6 ± 0.0[c]	1.0 ± 0.2	1.3 ± 0.0[c]
	IL-8	1.5 ± 0.1[d]	0.9 ± 0.1	1.3 ± 0.2[d]
	MCP-2	1.6 ± 0.2[c]	0.7 ± 0.1	1.5 ± 0.4[c]

[a] Twenty-hour supernatants and 8-h cell lysates were analyzed by using a cytokine array. Cytokine signals were quantified by densitometry. The results are expressed as the fold induction above basal (uninfected) levels (means ± standard deviations of the means). Each cytokine was analyzed in duplicate on each membrane. Samples from three independent experiments were analyzed, and each experiment was analyzed separately on a single array membrane.
[b] $P < 0.001$ compared to strain DAY25.
[c] $P < 0.05$ compared to strain DAY25.
[d] $P < 0.01$ compared to strain DAY25.

natants. Most other keratinocyte cytokines detected by the antibody array in cell lysates (i.e., IL-6, GRO, and IL-8) also appeared in cell supernatants, suggesting that, similar to IL-1α, these cytokines accumulate intracellularly in infected cells in sufficient amounts to be detected by this assay prior to active secretion or release. Newly synthesized cytokines can be stored intracellularly or immediately secreted, and their early or delayed release depends largely on the cell type and the type of

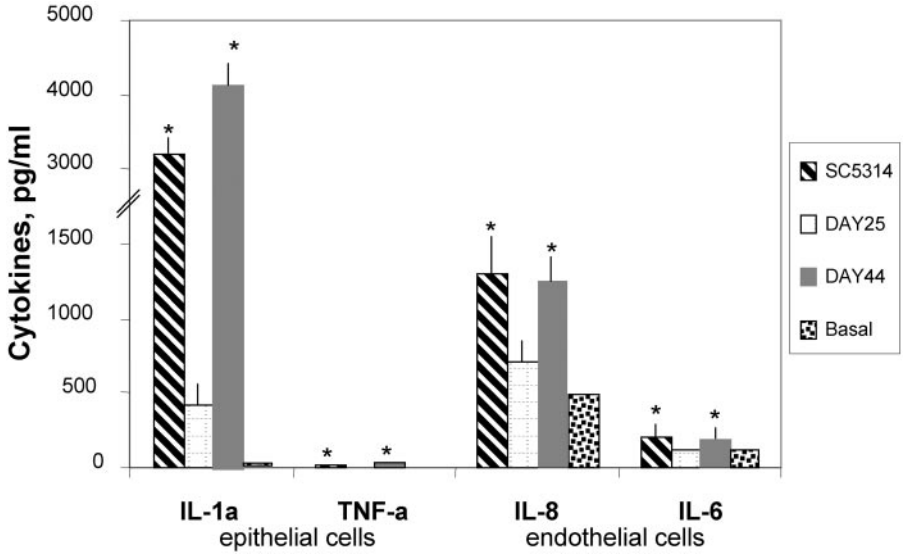

FIG. 5. Analysis of cytokine responses of SCC15 and HMEC-1 cells to different *C. albicans* strains by ELISA. Aliquots of supernatants used in array experiments were analyzed for the presence of cytokines by sandwich ELISA. The error bars indicate one standard deviation of the mean of triplicate assays. An asterisk indicates that the *P* value is ≤0.05 for a comparison with cells infected by DAY25.

stimulus (36, 41). In our study TNF-α and TNF-β1 in oral keratinocytes and MCP-2 in endothelial cells were detected only in culture supernatants and not in cell lysates, suggesting that there is a rapid processing and secretion mechanism after synthesis in response to infection. This is consistent with previous studies of macrophages, in which TNF-α was found almost exclusively in the extracellular compartment (24), but it partially contradicts findings obtained with endothelial cells, in which the TNF-α formed in response to *Candida* infection was found to be both cell associated and secreted (37). These discrepant findings obtained with different cell systems further underscore the importance of the specific cell type and/or stimulus in the processing and the kinetics of release of newly formed cytokines. In the endothelial cell system, we also observed that a group of cytokines (IL-15, MCP-1, G-CSF, RANTES) which were detected in 8-h cell lysates were not present in cell supernatants after 20 h of infection. This finding suggests that although these cytokines accumulate intracellularly upon synthesis, they are not secreted in sufficient amounts after 20 h of culture to be detected in culture supernatants. This is consistent with findings of other investigators for endothelial cells which showed that most secretion of RANTES, G-CSF, and MCP-1 in culture supernatants in response to a variety of stimuli takes place within 48 to 72 h after stimulation (4, 6, 8).

The finding that mucosal epithelial cells respond to invasive fungal organisms with an exaggerated proinflammatory cytokine profile compared to the profile observed with less invasive organisms has not been reported previously. In this study we used the oral epithelial cell line SCC15, which was shown previously to have a cytokine response to invasive strain SC5314 similar to that of primary oral epithelial cells (13, 15). Our findings are in agreement with previous studies showing that live hyphal organisms that are capable of inflicting damage to oral epithelial cells are able to trigger elevated IL-8 and IL-1α responses (13, 15). Our findings are also in agreement with reports from other investigators who found that infection of human colon epithelial cells with invasive strains of bacteria induces significantly larger amounts of proinflammtory cytokine secretion than infection with noninvasive gram-negative or gram-positive bacteria induces (25). Our results are also supported by the finding that human intestinal epithelial cells respond to invasive infection by *Cryptosporidium parvum* with a threefold increase in IL-8 and GRO secretion, showing a time course overlap between cell invasion, cell lysis, and cytokine release (27). The process of invasion by aggressive pathogens is likely to be important for generating signals that activate proinflammatory pathways within mucosal epithelial cells in the gastrointestinal tract, which is normally colonized by over 400 microbial species, including commensals and opportunistic pathogens. One of the signal transduction molecules that might be responsible for the coordinated regulation of such cytokines and chemokines during invasion of epithelial and endothelial cells is the transcription factor NF-κB, which is activated in response to infection with invasive bacteria and intracellular parasites (16, 22, 23, 43).

Our results are consistent with the increased expression of IL-1α, IL-6, IL-15, MCP-1, and RANTES in the tissues of patients with oropharyngeal candidiasis compared to the expression in healthy subjects (29). In fact, the release of these proinflammatory cytokines and chemokines by epithelial and/or endothelial cells might contribute to the characteristic histological feature of oral *C. albicans* infection of intraepithelial abscesses surrounded by a large number of neutrophils (7, 17). Moreover, the reduced ability of the invasion-deficient mutant to trigger a strong proinflammatory cytokine response in our study may at least partially explain the previous findings obtained in a mouse model of infection in which the *rim101*$^-$/*rim101*$^-$ mutant failed to stimulate migration of neutrophils and formation of microabscesses in kidneys recovered from animals with disseminated candidiasis (11).

We studied the role of *C. albicans* invasion during its interaction with host cells by using mutant strain DAY25 (*rim101*$^-$/*rim101*$^-$). We chose this mutant strain because it satisfied two criteria: (i) it had previously been shown to have a reduced invasive potential in an animal model of hematogenously disseminated candidiasis (11); and (ii) the mutation did not affect its ability to germinate into true hyphae or its XTT-converting metabolic activity when it was cocultured with epithelial and endothelial cells in vitro. The reduced ability of the mutant strain to interact with host cells can be explained by the disruption of the *rim101* gene, since gene complementation restored its ability to damage and invade host cells and its ability to stimulate a proinflammatory response by host cells. In fact, the complemented strain exhibited a greater ability to lyse host cells than its parental clinical strain, strain SC5314, exhibited. Although we do not completely understand the basis for this difference, the two strains differ by nine successive transformations, as well as intervening subculturing, so there has been ample opportunity for genetic divergence between them.

The *RIM101* pathway regulates changes in the expression of several pH-conditioned genes (11). Most of the genes regulated by the *RIM101* pathway encode secreted and cell surface-associated proteins (39), including *HWP1* and *ALS1* (31, 39). Hw1p and Als1p are cell surface proteins responsible for adherence of *C. albicans* to host cells (32, 44); therefore, downregulation of these proteins in the *rim101*$^-$/*rim101*$^-$ mutant might contribute to its reduced ability to invade and damage host cells and tissues, as demonstrated in this study and by other workers (11, 42). Although it has been hypothesized that the capacity of *C. albicans* to invade host tissues depends mainly on its ability to form hyphae (2), our study indicates that formation of true hyphae is not sufficient for expression of a highly invasive phenotype in vitro. Indeed, our study further supports the fact that a combination of several virulence factors, including germination into true hyphae, adhesion to host cells, and expression of invasion-related proteolytic enzymes, is required to establish an invasive infection (5).

In summary, our data suggest that in addition to morphogenesis, invasion of host cells and tissues is important for a strong proinflammatory host response to infection by *C. albicans*. Future studies directed at identifying *C. albicans* and host cell recognition systems and signaling pathways responsible for the proinflammatory cytokine response are needed to fully elucidate the mechanism of the host response to invasive fungal infection.

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