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Epithelial GM-CSF Induction by *Candida glabrata*

APPENDIX

MATERIALS & METHODS

Cytokine Detection

ELISA

To quantify cytokine protein levels, we used ELISA to analyze culture supernatants obtained from duplicate experiments. In each experiment, supernatants from 2 replicate wells were pooled and assayed by duplicate sandwich enzyme-linked immunosorbent assays (ELISA), with commercially available antibody pairs (Endogen MiniKit, Pierce, Rockford, IL, USA), as previously described (Dongari-Bagtzoglou and Kashleva, 2003; Dongari-Bagtzoglou *et al*., 2004). Absorbance values and corresponding cytokine concentrations were determined with an Opsys MR Microplate reader (Dynex Technologies Inc., Chantilly, VA, USA) equipped with Revelation QuickLink software (Thermo Labsystems, Chantilly, VA, USA).

Real-time RT-PCR

Expression of GM-CSF mRNA by OKF6/TERT-2 cells was assessed quantitatively by real-time RT-PCR, and data were analyzed with iCycle iQ system software (Bio-Rad, Hercules, CA, USA). Briefly, a 3-μg quantity of total RNA of each sample was reverse-transcribed into cDNA with random primer oligonucleotides (Invitrogen, Carlsbad, CA, USA) and BD sprint™ PowerScript™ (BD Biosciences, Palo Alto, CA, USA). Real-time PCR was performed on 96-well optical reaction plates (BioRad, Hercules, CA, USA). All PCR reaction mixtures contained 7.5 μL of iQ™ SYBR® Green Supermix (BioRad, Hercules, CA, USA), 0.5 μL of forward primer (10 μM), 0.5 μL of reverse primer (10 μM), 4 μL of each diluted RT product, and 2.5 μL of distilled water *per* well. The sequences of the primers used for real-time PCR were as follows: human GM-CSF (forward) 5′-ATGTGAATGCCATCCAG-GAG-3′ and (reverse) 5′-AATCTGGGTTGCACAGGAAG-3′, and human cyclophilin (forward) 5′-CGGGTCCTGGCATCTTGT-3′ and reverse (reverse) 5′-GCAGATGA-AAAACTGGG-AACCA-3′. Negative controls included water and RNA extracted from *C. glabrata* incubated under the same conditions in the absence of oral epithelial cells. The specificity of each primer pair was confirmed by agarose gel electrophoresis. The PCR reaction was performed at 95°C for 3 min, followed by 50 cycles of 30 sec each at 95 °C, 60 °C, and 72 °C. The cyclophilin-normalized data were expressed as fold-induction of GM-CSF gene expression in infected compared with uninfected cultures.

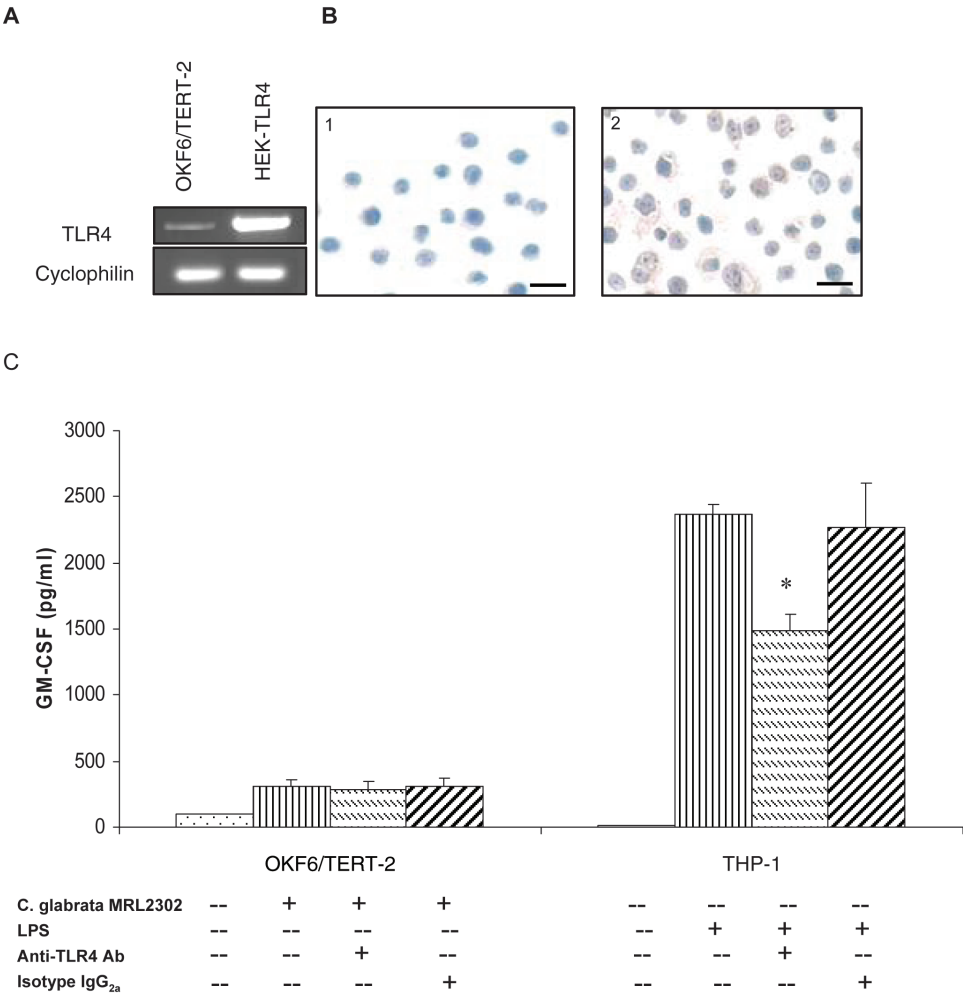
Adhesion Assay

To compare the adherence potential of various *C. glabrata* strains with that of oral epithelial cells, we seeded OKF6/TERT-2 cells at confluence (2 x 10^5 cells/well) in 96-well plates (Corning Inc., Corning, NY, USA) and allowed them to adhere overnight. The next day, media were discarded, and 1 x 10^6 *C. glabrata* suspended in 200 μL KSFM were added. Oral epithelial cell cultures incubated under identical conditions were included as a negative control. After 4 hrs of incubation, wells were washed by PBS twice to remove the unattached yeast cells. The attached *C. glabrata* cells were quantified by the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium-hydroxide (XTT) assay, which was based on the linear relationship between *C. glabrata* cell number and the colorimetric signal, within a certain range of *C. glabrata* concentrations. The colorimetric signal developed by attached yeast cells was quantified according to the formula: $OD_{attached\ yeast} = OD_{washed\ culture} — OD_{control}$. The colorimetric signal developed by total yeast cells was quantified according to the formula: $OD_{total\ yeast} = OD_{unwashed\ culture} — OD_{control}$. The percentage of attached yeast cells was calculated as: % attached yeast cell = (attached yeast cells/total yeast cells) x 100. Briefly, 0.2 mL of PBS containing 0.25 mg/mL of XTT (Sigma Chemical Co., St. Louis, MO, USA) and 40 μL/mL of co-enzyme Q (Sigma Chemical Co.) were added to each well, and the plates were incubated at 37°C for 2.5 hrs. A 100-μL quantity of supernatant was transferred to a 96-well plate, and the optical density at 492 nm was measured by means of an Opsys MR microplate reader (Dynex Technologies Inc., Chantilly, VA, USA) and Revelation QuickLink software (Thermo Labsystems, Chantilly, VA, USA).

TLR4 Detection

Immunocytochemistry

OKF6/TERT-2 cells were seeded on eight-chamber glass slides, fixed with 4% paraformaldehyde for 15 min, and treated with 0.5% Triton X-100 for 15 min for detection of



Appendix Figure 1. Role of TLR4 in *C. glabrata*-mediated GM-CSF production in OECs. **(A)** Expression of TLR4 mRNA in OKF6/TERT-2 cells was determined by RT-PCR. HEK/TLR4 cells were used as positive control. **(B)** OKF6/TERT-2 monolayers were stained with anti-TLR4 monoclonal antibody and $IgG2_a$ as Ab specificity control. Images were taken at a magnification of X20. Bar = 40 μm. **(C)** OKF6/TERT-2 cells were challenged with *C. glabrata* MRL2302 (0.1:1 yeast-to-epithelial-cell ratio) for 36 hrs in the presence or absence of anti-TLR4 Ab. As positive control, THP-1 cells were pre-incubated with anti-TLR4 Ab and stimulated with LPS (10 μg/mL) for 24 hrs. Supernatants were collected for ELISA analysis. Error bars indicate one standard deviation of the mean. * $P < 0.05$ for a comparison with LPS-only-treated THP-1 cells.

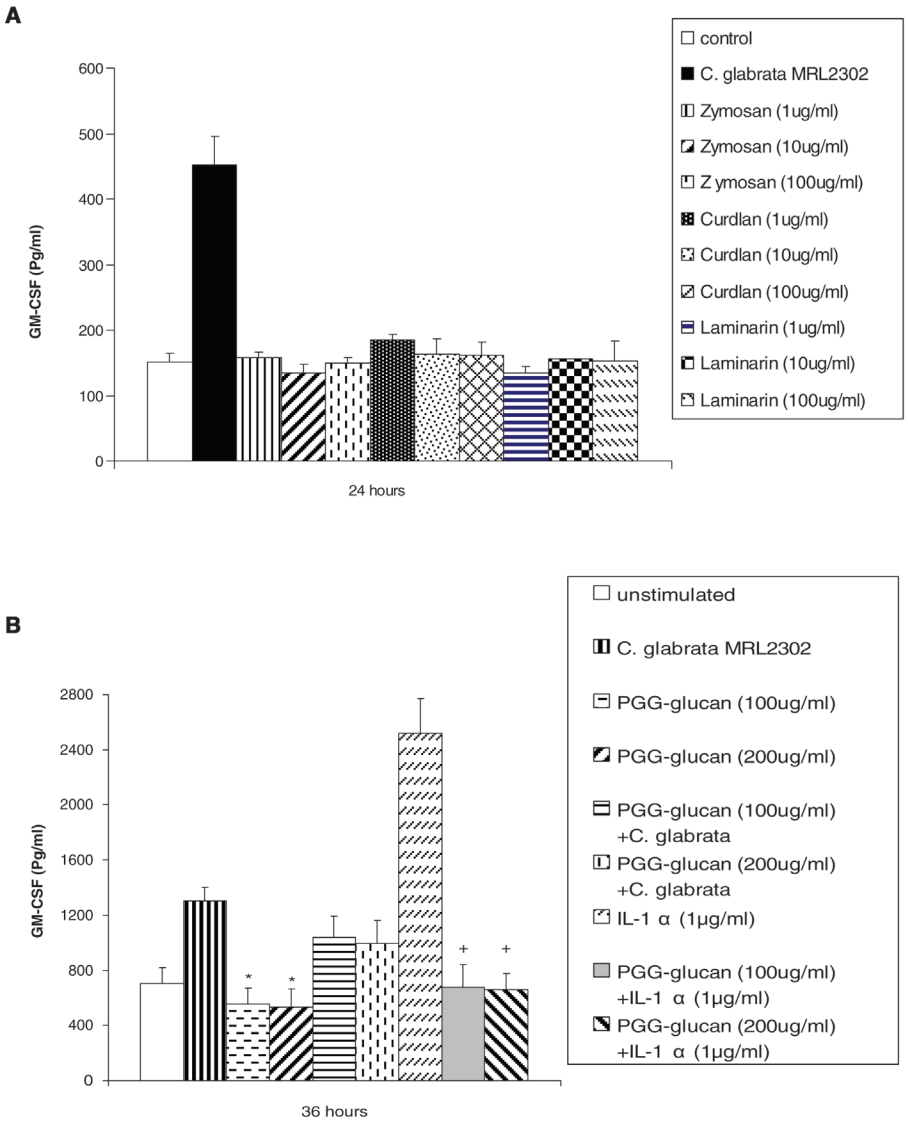
intracellular expression (Sugawara *et al*., 2006). Cells were then incubated with 20 μg/mL anti-human TLR4 monoclonal antibody (clone HTA125, eBioscience, San Diego, CA, USA) or mouse IgG2a as a negative control (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 hrs at room temperature. HEK293 cells stably transfected with TLR4 were used as positive controls for TLR4 staining. After being washed with PBS, cells were incubated with biotinylated secondary IgG antibody followed by avidin-biotin-peroxidase complex, according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). Sites of peroxidase binding were revealed with a combination of 300 μL 3% H_2O_2 and 0.023% 3,3′-diaminobenzidine tetrahydrochloride solution. All samples were counterstained with Mayer's hematoxylin solution for 30 sec, dehydrated in graded ethanol, cleared in xylene, and mounted in Diatex (Becker Industrifärg AB, Marsta, Sweden).

RT-PCR

Total RNA was extracted from 1 x 10^6 OKF6/TERT-2 or HEK-TLR4 cells by means of the Trizol RNA isolation system (Invitrogen Life Tech, Carlsbad, CA, USA), according to the manufacturer's instructions. A 3-μg quantity of total RNA was reverse-transcribed into cDNA with random primer oligonucleotides (Invitrogen) and BD sprint™ PowerScript™ (BD Biosciences). The following primers were used to amplify a specific fragment of the following genes: TLR4 (forward) 5′-AGTCAAGGAACCCATGACAA-3′, (reverse) 5′-GAGAATGACCAGGATCC TTG-3′; and cyclophilin (forward) 5′-CGGGTCCTGGCAT-CTTGT-3′ and (reverse) 5′-GCAGATGA-AAAACTGGG-AACCA-3′. Reaction conditions were as follows: 95°C for 30 sec followed by 35 cycles of 30 sec each at 95 °C, 60 °C, and 72 °C. Reactions were also amplified in the absence of reverse transcriptase as negative controls. Following PCR, a 10-μL quantity of the total amplified product was subjected to electrophoresis on ethidium-bromide-stained 1% agarose gels and visualized under UV fluorescence.

RESULTS

TLR4 was expressed by OKF6/TERT-2 cells, but was not involved in the GM-CSF response to *C. glabrata.* Although the expression of TLR4 has been identified in primary oral epithelial cells and certain oral epithelial cell lines (Uehara *et al*., 2001; Sugawara *et al*., 2006; Uehara and Takada, 2007), the involvement of this receptor in the recognition of *Candida* pathogens remains controversial (Van de Graaf *et al*., 2005; Gil and Gozalbo, 2006). In this study, we demonstrated, using RT-PCR, that the oral epithelial cell line OKF6/TERT-2 constitutively expressed mRNA for TLR4 (Appendix Fig. 1A). Consistent with the RT-PCR findings, constitutive expression of TLR4 protein in OKF6/TERT-2 cells was observed by immunocytochemistry (Appendix Fig. 1B). To investigate the role of TLR4 in *C. glabrata*-induced GM-CSF induction, we pre-incubated OKF6/TERT-2 cells with neutralizing monoclonal anti-TLR4 antibody for 30 min and subsequently exposed them to *C. glabrata* MRL2302 for up to 36 hrs. The blocking efficiency of the anti-TLR4 antibody was verified in LPS-stimulated THP-1 cells. IL-6 production by LPS-stimulated



Appendix Figure 2. Role of β-glucan in the GM-CSF responses of OECs. **(A)** OKF6/TERT-2 cells were stimulated with zymosan, curdlan, or laminarin with concentrations ranging from 1 to 100 µg/mL for 24 hrs. **(B)** OKF6/TERT-2 cells were challenged with IL-1α (1 µg/mL) or *C. glabrata* MRL2302 (0.1:1 yeast-to-epithelial-cell ratio) in the presence or absence of PGG-glucan (100 µg/mL or 200 µg/mL), for 36 hrs. Supernatants were analyzed by ELISA. Mean values were obtained by analysis of triplicate wells in two separate experiments, and error bars indicate one standard deviation of the mean. * $P < 0.05$ for a comparison with the *C. glabrata*-alone group. + $P < 0.05$ for a comparison with the IL-1α-alone group.

THP-1 cells was significantly inhibited in the presence of this antibody (Appendix Fig. 1C). In contrast, this antibody had no inhibitory effect in *C. glabrata*-challenged OECs, suggesting that GM-CSF responses to *C. glabrata* were not mediated by TLR4.

APPENDIX REFERENCES

Dongari-Bagtzoglou A, Kashleva H (2003). *Candida albicans* triggers interleukin-8 secretion by oral epithelial cells. *Microb Pathog* 34: 169-177.

Dongari-Bagtzoglou A, Kashleva H, Villar CC (2004). Bioactive interleukin-1alpha is cytolytically released from *Candida albicans*-infected oral epithelial cells. *Med Mycol* 42:531-541.

Gil ML, Gozalbo D (2006). TLR2, but not TLR4, triggers cytokine production by murine cells in response to *Candida albicans* yeasts and hyphae. *Microbes Infect* 8:2299-2304.

Sugawara Y, Uehara A, Fujijmoto Y, Kusumoto S, Fukase K, Shibata K, *et al.* (2006). Toll-like receptors, NOD1, and NOD2 in oral epithelial cells. *J Dent Res* 85:524-529.

Uehara A, Takada H (2007). Functional TLRs and NODs in human gingival fibroblasts. *J Dent Res* 86:249-254.

Uehara A, Sugawara S, Tamai R, Takada H (2001). Contrasting responses of human gingival and colonic epithelial cells to lipopolysaccharides, lipoteichoic acids and peptidoglycans in the presence of soluble CD14. *Med Microbiol Immunol* 189:185-192.

Van der Graaf CA, Netea MG, Verschueren I, Van der Meer JW, Kullberg BJ (2005). Differential cytokine production and Toll-like receptor signaling pathways by *Candida albicans* blastoconidia and hyphae. *Infect Immun* 73:7458-7464.