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Epithelial GM-CSF Induction by *Candida glabrata*

ABSTRACT

The main cytokine induced by the interaction of oral epithelial cells with *C. glabrata* is granulocyte monocyte colony-stimulating factor (GM-CSF); however, the mechanisms regulating this response are unknown. Based on previously published information on the interactions of *C. albicans* with oral epithelial cells, we hypothesized that interaction with viable *C. glabrata* triggers GM-CSF synthesis *via* NF-κB activation. We found that *C. glabrata*-induced GM-CSF synthesis was adhesion-dependent, enhanced by endocytosis, and required fungal viability. NF-κB activation was noted during interaction of epithelial cells with *C. glabrata,* and pre-treatment with an NF-κB inhibitor partly inhibited GM-CSF synthesis. Blocking TLR4 with anti-TLR4 antibody did not inhibit GM-CSF production. In contrast, an anti-CDw17 antibody triggered significant inhibition of NF-κB activation and GM-CSF synthesis. β-glucans did not stimulate GM-CSF synthesis, suggesting that the CDw17/NF-κB/GM-CSF pathway may be β-glucan-independent. This study provides new insights into the mechanism of GM-CSF induction by *C. glabrata*.

KEY WORDS: *C. glabrata*, oral epithelial cell, GM-CSF, TLR4, CDw17.

INTRODUCTION

Candidiasis is the most common oral fungal infection in humans (Muzyka, 2005). *C. glabrata* has emerged as the second or third most frequently isolated *Candida* species in the oral flora of persons with diabetes mellitus, advanced cancer, and HIV infection (Masia Canuto *et al*., 2000; Davies *et al*., 2002; Kadir *et al.*, 2002). In addition to maintaining an intact invasion barrier, oral epithelial cells (OECs) can regulate the inflammatory host response to *Candida* by releasing a wide array of chemotactic and priming molecules for innate immune effector cells (Dongari-Bagtzoglou and Kashleva, 2003; Dongari-Bagtzoglou *et al*., 2005).

We have shown that *C. glabrata* is a potent inducer of granulocyte monocyte colony-stimulating factor (GM-CSF) in human oral keratinocytes (Li and Dongari-Bagtzoglou, 2007; Li *et al*., 2007), and that strains differ in eliciting GM-CSF responses, with certain oroesophageal candidiasis isolates possessing higher GM-CSF induction capacity than oral commensal isolates (Li and Dongari-Bagtzoglou, 2007). By enhancing the proliferation, activation, as well as fungicidal activity of immuno-effector cells such as neutrophils and monocytes, GM-CSF may play a major role in local control of *Candida* and the prevention of invasive infection, as suggested in clinical reports (Nicolatou-Galitis *et al*., 2001). Given the potential importance of this cytokine in the host defense against *Candida* infection, a better understanding of the specific mechanisms involved in GM-CSF induction in response to *C. glabrata* is needed. The current study was therefore undertaken to investigate the mechanisms of *C. glabrata*-induced GM-CSF expression in OECs.

MATERIALS & METHODS

Organisms

C. glabrata strains GDH2269 and 94-11 were obtained from ATCC. Two pathogenic esophageal candidiasis isolates, MRL2302 and MRL7525, were kindly provided by Dr. M. Ghannoum (Case Western Reserve University, Cleveland, OH, USA). Stationary-phase *C. glabrata* yeast cells were prepared by growth for 18 hrs at room temperature in yeast extract, peptone, and dextrose (Difco Laboratories, Detroit, MI, USA), supplemented with 2% glucose. All of the strains used in this study had similar growth rates in Keratinocyte Serum-free culture media (KSFM, Invitrogen, Carlsbad, CA, USA), as determined by direct cell counting of yeast cells**.** Non-viable *C. glabrata* were prepared by fixation in 4% formaldehyde-PBS for 30 min and overnight washing in PBS.

Cell Cultures

OKF6/TERT-2 cells represent normal oral mucosal epithelium immortalized by forced expression of telomerase *via* retroviral transduction (Dickson *et al.*,

2000). The SCC15 oral carcinoma line was obtained from ATCC. OECs were maintained in KSFM, supplemented with 0.4 mM $CaCl_2$, 0.1 ng/mL bovine pituitary extract, and antibiotics. Leukocytic cell lines HL-60 (ATCC) and THP-1 (ATCC) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. HEK293 cells stably transfected with TLR4 were a generous gift from Dr. Douglas Golenbock (University of Massachusetts) and were cultured in DMEM containing 10% FBS.

Experimental System

OKF6/TERT-2 cells were seeded at or near confluence in 6-, 12-, or 24-well polystyrene plates (Corning Incorporated, Corning, NY, USA) and incubated overnight in complete KSFM at 37ºC in 5% CO_2 atmosphere. The following day, media were discarded, and cells were challenged with suspensions of stationary-phase organisms at various fungal cell-to-host cell ratios. Supernatants and cell or nuclear extracts were collected and stored at –80ºC until they were assayed. Cytokines were quantified by ELISA and/or Q-RT-PCR (see Appendix).

In adhesion experiments, OKF6/TERT-2 cells were co-cultured with *C. glabrata* for 4 hrs, and the attached yeast cells were quantified by a modification of the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazoliumhydroxide (XTT) assay (see Appendix).

In receptor blocking experiments, cells were pre-incubated for 30 min with 20 μg/mL anti-TLR4 mAb (IgG2a, eBioscience, San Diego, CA, USA), or 20 μg/mL anti-CDw17 mAb (IgMκ, Ancell, Bayport, MN, USA) and then challenged with *C. glabrata* for up to 36 hrs. To block endocytosis, we pre-treated OKF6/TERT-2 cells with cytochalasin D (100 ng/mL) for 30 min (Neal *et al*., 2006), followed by challenge with *C. glabrata* in the presence of cytochalasin D for up to 20 hrs.

To examine the effect of β-glucan on GM-CSF production, we stimulated OKF6/TERT-2 cells with curdlan, zymosan, or laminarin (all from Sigma, St. Louis, MO, USA) at concentrations ranging from 1 to 100 μg/mL for 24 hrs, or with PGG-glucan (Biothera, Eagan, MN, USA), at 100-200 μg/mL, for 36 hrs. The effect of PGG-glucan was also tested in cells stimulated by *C. glabrata* or IL-1α.

Transmission Electron Microscopy

OKF6/TERT-2 and SCC15 cells (5 x 10^4/well) were co-cultured with viable or non-viable *C. glabrata* cells (1.5 x 10^5/well) for up to 12 hrs and processed for TEM as described elsewhere (Villar *et al*., 2007). Sections were collected on grids, stained with 2% uranyl acetate and lead citrate, and examined and photographed by transmission electron microscopy (Philips CM10; Philips, Eindhoven, the Netherlands).

TLR4 and CDw17 Detection

Formalin-fixed OKF6/TERT-2 cells were stained with 20 μg/mL anti-TLR4 mAb (IgG2a, eBioscience HTA125) with the use of an avidin-biotin-peroxidase kit (Vector Laboratories, Burlingame, CA, USA). Cells stained with isotype control antibody (IgG2a)

were used as a negative control. Expression of TLR4 was confirmed by RT-PCR (Appendix).

CDw17 was detected by flow cytometry with HL-60 cells as positive control (Iwabuchi and Nagaoka, 2002). Briefly, cells were dislodged with a Hanks' salt-based, enzyme-free cell dissociation buffer (Sigma-Aldrich). After Fc receptor was blocked with IgG from human serum (Sigma-Aldrich), cells were stained with anti-CDw17 mAb or control mouse IgMκ on ice for 30 min, followed by Cy3-conjugated affiniPure F(ab′)2 fragment goat anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for an additional 30 min. Cells were analyzed with a FACSCalibur cytometer (BD Biosciences, Palo Alto, CA, USA).

NF-κB Activation Assays

To detect NF-κB activation, we co-cultured OKF6/TERT-2 cells with *C. glabrata* MRL2302 at 0.1:1, 1:1, 10:1, and 100:1 yeast-to-epithelial-cell ratios, for 5 min-18 hrs. In certain experiments, we also added IL-1 receptor antagonist (IL-1ra, 1 μg/mL; R&D Systems, Minneapolis, MN, USA) to rule out a secondary role of IL-1 in NF-κB activation. To inhibit NF-κB activation, we pre-treated cells with 15 μM pyrrolidine dithiocarbamate (PDTC, Sigma) for 1 hr (Evans *et al*., 2005), prior to the addition of *C. glabrata*. At this concentration, PDTC did not affect the growth or binding of *C. glabrata* to epithelial cells (not shown). Nuclear protein extracts were obtained from cell cultures by means of a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA). P65 and P50 DNA binding activity was assessed by the TransAM™ NF-κB Kit (Active Motif).

Statistical Analysis

The statistical significance of the differences between experimental and control groups was determined by two-tailed *t* test, assuming equal variances.

RESULTS

C. glabrata-induced GM-CSF Responses in Oral Epithelial Cells Were Dependent on Host Cell-*Candida* Contact and Fungal Viability

Previous dose response studies showed that a maximal GM-CSF response is detected after prolonged incubation at a 0.1:1 *C. glabrata*-to-epithelial-cell ratio (Li and Dongari-Bagtzoglou, 2007; Li *et al*., 2007), and we obtained similar data with strain MRL2302 in this study (Fig. 1A). When fungal and epithelial cells were separated by well inserts, GM-CSF secretion was completely inhibited (Fig. 1A). Increasing the fungal challenge (1000:1 yeast-to-epithelial-cell ratio) in the presence of well inserts did not result in GM-CSF induction (Fig. 1A). These results showed a requirement of fungal-to-epithelial-cell contact in GM-CSF induction.

Variable GM-CSF-inducing potentials among *C. glabrata* strains have been noted in our previous studies (Li and Dongari-Bagtzoglou, 2007). Because the types and quantities of surface adhesins differ in genetically distinct *C. glabrata* strains
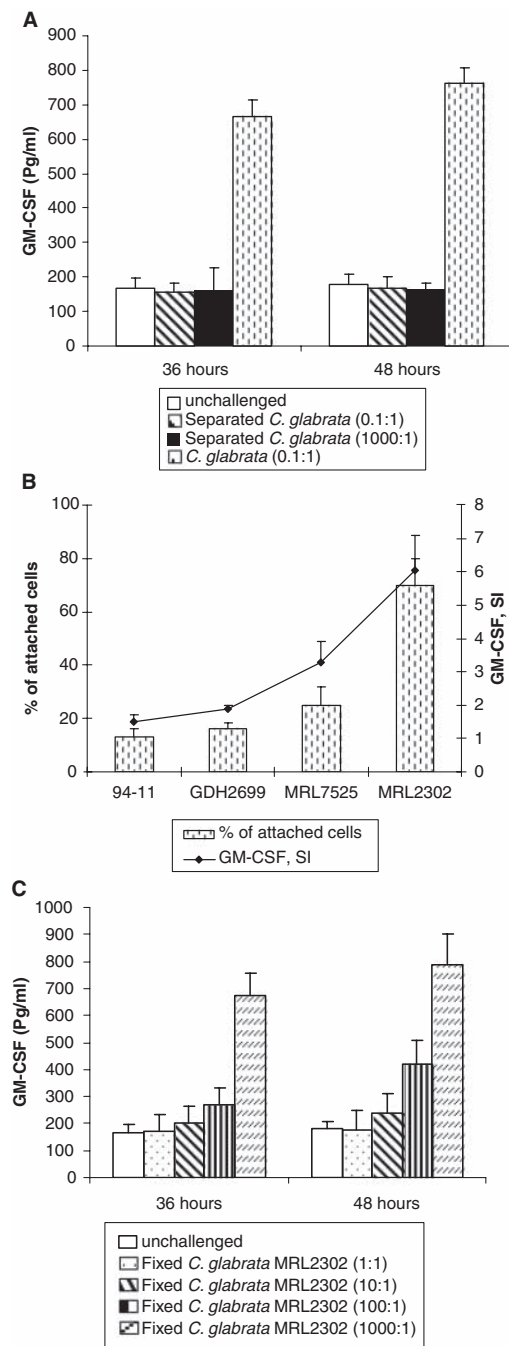
Figure 1. Effects of fungal-epithelial cell contact, yeast viability, and adhesion on GM-CSF responses by OKF6/TERT-2 cells. **(A)** Epithelial cells were challenged with *C. glabrata* MRL2302 in the presence (no contact) or absence (contact) of a separating well insert for up to 48 hrs, and GM-CSF release was quantified in culture medium. **(B)** OKF6/TERT-2 cells were co-cultured with *C. glabrata* (strains 94-11, GDH2269, MRL2302, and MRL7525) at a 1:5 epithelial-to-yeast-cell ratio for 4 hrs, and adhesion was quantified by XTT assay. Culture supernatants were tested for the presence of GM-CSF. **(C)** Epithelial cells were challenged with fixed *C. glabrata* MRL2302 at various epithelial-to-yeast-cell ratios for up to 48 hrs, and GM-CSF release was quantified in culture medium. Replicate samples from at least two independent experiments were analyzed. Error bars indicate one standard deviation of the mean.

(deGroot *et al.*, 2008), we next examined whether the GM-CSF induction potential was commensurate with the adhesion potential of these strains. Adhesion of *C. glabrata* strains to OECs was time-dependent, with maximum levels obtained after 4 hrs of co-culture (not shown). GM-CSF stimulation was commensurate with each strain's ability to adhere to OECs, with strain MRL2302 having the greatest adhesion potential and triggering the highest GM-CSF stimulation, followed by strains MRL7525, GDH2269, and 94-11 (Fig. 1B).

To determine whether fungal viability is required for GM-CSF induction, we used fixed *C. glabrata* MRL2302 yeast cells in cell interaction assays. Extremely high (1000:1) doses of fixed yeast cells were needed to induce GM-CSF levels comparable with those induced by low (0.1:1) doses of viable yeasts, indicating that *C. glabrata* viability is important in GM-CSF induction (Fig. 1C).

C. glabrata-induced GM-CSF Responses in Oral Epithelial Cells Were Enhanced by Endocytosis

It has been reported that *C. glabrata* can be detected within the cytoplasm of murine vaginal epithelial cells, raising the possibility that endocytosis of this organism by epithelial cells leads to internalization (Fidel *et al.*, 1999). Using two OEC lines, we observed the intracytoplasmic presence of *C. glabrata* yeast cells as early as 4 hrs after co-culture, with extension of membrane-bound pseudopods around yeast cells (Figs. 2A, 2B). Pre-treatment of OKF6/TERT-2 cells with endocytosis inhibitor cytochalasin D prevented internalization (not shown) and triggered a significant, but incomplete, inhibition of GM-CSF gene expression (Figs. 2C, 2D). Cytochalasin D treatment did not affect IL-1α-triggered GM-CSF induction, suggesting that this treatment did not result in a non-specific adverse effect on cell metabolism (Fig. 2C). Analysis of these data, taken together, suggested an active involvement of actin polymerization in the uptake of *C. glabrata* by OECs and confirmed a role for endocytosis in *C. glabrata*-induced GM-CSF release.

CDw17, but not TLR4, was Involved in the GM-CSF Response to *C. glabrata*

Although OKF6/TERT-2 cells constitutively express TLR4 (Appendix Figs. 1A, 1B), a neutralizing anti-TLR4 mAb did not inhibit GM-CSF production (Appendix Fig. 1C), suggesting that the GM-CSF response to *C. glabrata* was not mediated by TLR4.

CDw17 has been suggested to be an adhesion receptor for several fungal organisms, including *Candida* (Jimenez-Lucho *et al.*, 1990). Moreover, interaction of *Pneumocystis carinii* cell wall β-glucan with CDw17 has been reported to induce release of pro-inflammatory cytokines from alveolar epithelial cells (Hahn *et al.*, 2003). Therefore, we hypothesized that *C. glabrata*-induced GM-CSF response was mediated by β-glucan-CDw17 interaction. Expression of CDw17 was detected in both OKF6/TERT-2 and HL-60 cells, with slightly higher expression levels in the latter (Fig. 3A). Next, an anti-CDw17 mAb was
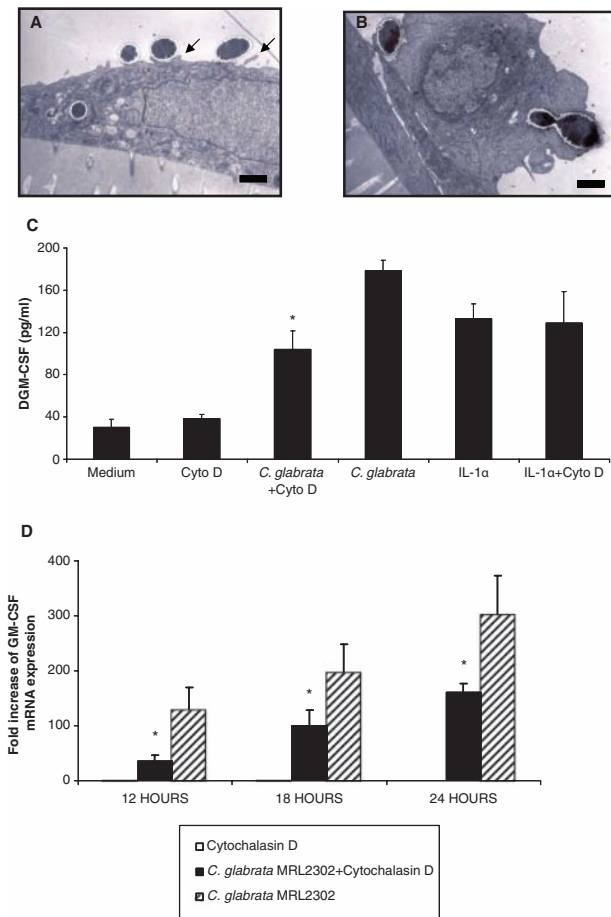
Figure 2. Endocytosis of *C. glabrata* by OECs and effect of cytochalasin D on GM-CSF responses. Epithelial cells OKF6/TERT-2 **(A)** and SCC15 **(B)** were co-cultured with *C. glabrata* MRL2302 at a 1:5 epithelial-to-yeast-cell ratio for 4 hrs. The intracellular presence of yeast cells was observed by TEM. Bar = 1 μm. **(C,D)** OKF6/TERT-2 cells were pre-treated with cytochalasin D and challenged with *C. glabrata* MRL2302 (0.1:1 fungal-to-epithelial-cell ratio) for up to 24 hrs. IL-1α-stimulated GM-CSF induction was used as a negative control for the effects of cytochalasin D. Supernatants were analyzed by ELISA (C), and cell lysates were analyzed by real-time RT-PCR (D). Mean values were obtained by analysis of three individual experiments, with each condition set up in duplicate, and error bars indicate one standard deviation of the mean. * $P < 0.05$ for a comparison with the *C. glabrata* MRL2302-stimulated group.

used to block surface interaction with OECs. Pre-treatment of OKF6/TERT-2 cells with anti-CDw17 significantly suppressed GM-CSF production (Fig. 3B), indicating that this molecule partly regulates secretion of GM-CSF from OECs. However, challenging OECs with increasing concentrations of zymosan, PGG-glucan, curdlan, or laminarin (Appendix Fig. 2A), or seeding OECs on curdlan- or laminarin-coated plates (not shown), did not induce a GM-CSF response. Furthermore, PGG-glucan inhibited both *C. glabrata*- and IL-1α-induced GM-CSF synthesis (Appendix Fig. 2B). Analysis of these data, taken together, suggests that β-glucan is not the moiety on the yeast cell triggering GM-CSF induction.
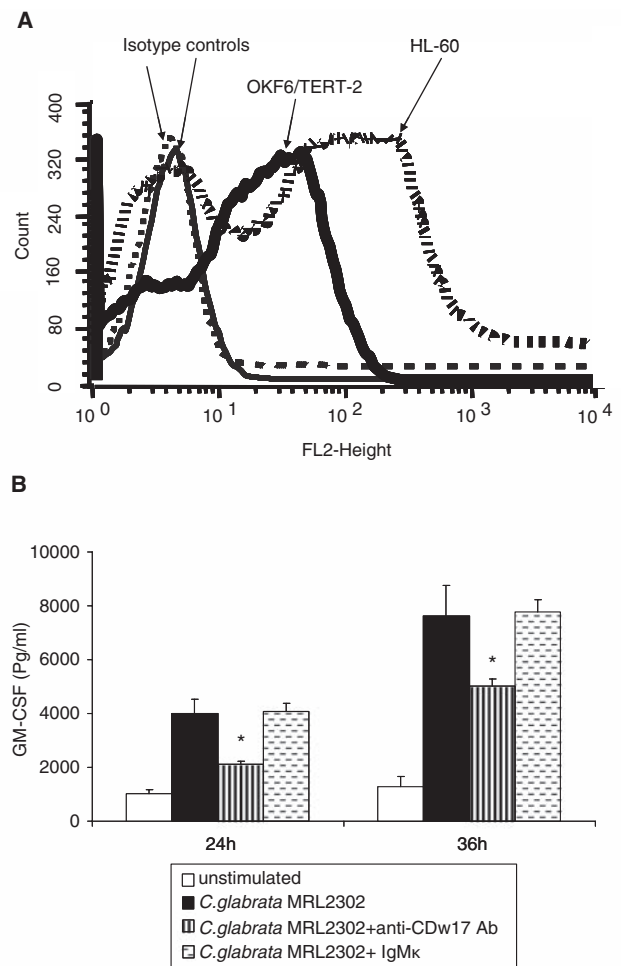

Figure 3. Involvement of CDw17 in the GM-CSF responses of OECs to *C. glabrata*. **(A)** Expression of CDw17 in OKF6/TERT-2 cells and HL-60 cells was assessed by flow cytometry. Results are representative of 3 experiments. **(B)** OKF6/TERT-2 cells were challenged with *C. glabrata* MRL2302 (0.1:1 yeast-to-epithelial-cell ratio) for up to 36 hrs, in the presence of anti-CDw17 or isotype control Ab IgMκ. * $P < 0.05$ for a comparison with *C. glabrata*-challenged cells in the absence of Ab. Error bars indicate one standard deviation of the mean.

Activation of NF-κB was Involved in *C. glabrata*-induced GM-CSF Production

Since NF-κB activation is involved in pro-inflammatory cytokine synthesis of keratinocytes in response to fungal pathogens (Pivarcsi *et al.*, 2003), we investigated its role in GM-CSF stimulation. P65 and p50 DNA binding activity in challenged OKF6/TERT-2 cells increased significantly after 2 hrs of co-culture (Fig. 4A) and continued to be significantly elevated even after 12 hrs of challenge (not shown). NF-κB activation was inhibited by PDTC, but not by IL-1ra (Fig. 4A), excluding a role of IL-1 in NF-κB activation. Pre-treatment of OKF6/TERT-2 with anti-CDw17 significantly inhibited activation of NF-κB in *C. glabrata*-challenged OECs (Fig. 4B). The NF-κB inhibitor PDTC also triggered a significant
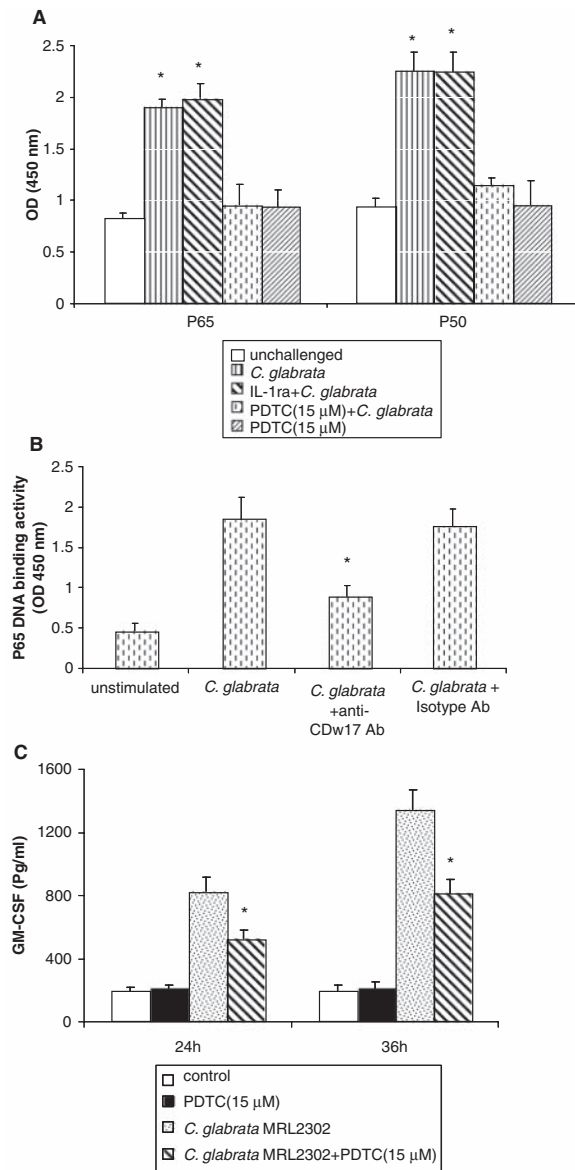
Figure 4. NF-κB activation in *C. glabrata*-challenged OECs. **(A)** OKF6/TERT-2 cells were pre-treated with PDTC (15 μM) or IL-1ra (1μg/mL) for 1 hr, followed by *C. glabrata* challenge (100:1 yeast-to-epithelial-cell ratio) for 2 hrs. * P < 0.05 for a comparison with unchallenged control. **(B)** Pre-treatment with anti-CDw17 Ab (20 μg/mL) partially inhibited NF-κB activation. *P < 0.05 for a comparison with *C. glabrata*-challenged cells in the absence of antibody. **(C)** OKF6/TERT-2 cells were pre-treated with PDTC (15 μM) for 1 hr prior to fungal challenge, and supernatants were analyzed by ELISA for GM-CSF. *P < 0.05 for a comparison with *C. glabrata*-challenged cells without PDTC treatment. Error bars indicate one standard deviation of the mean.

reduction in GM-CSF release (Fig. 4C). Analysis of these data, taken together, suggests that the GM-CSF response to *C. glabrata* is mediated, at least partly, by a CDw17-NF-κB signaling pathway.

DISCUSSION

Oral *Candida* infections are characterized by intense and persistent intra-epithelial inflammation (Eversole *et al.*, 1997). Previous work from our lab has shown that OECs respond to *C. glabrata* infection by secreting substantial amounts of the phagocyte priming and growth enhancing cytokine GM-CSF (Li and Dongari-Bagtzoglou, 2007; Li *et al.*, 2007). To our knowledge, this is the first study showing that the *C. glabrata*-induced GM-CSF response in OECs: (a) requires direct physical contact and fungal viability; (b) is enhanced by endocytosis; and (c) is at least partly regulated by a CDw17-NF-κB signaling pathway.

The involvement of TLR4 in *Candida*-induced inflammatory cytokine production has been controversial (Netea *et al.*, 2006). It has been reported that activation of TLR4 signaling pathways was required for *Candida*-induced inflammatory cytokine expression by human monocytes (Van der Graaf *et al.*, 2005). However, TLR4 was also reported as dispensable for the production of inflammatory cytokines in host cells in response to *Candida albicans in vivo* (Murciano *et al.*, 2006) and *in vitro* (Villamón *et al.*, 2004). In OECs, the expression of TLRs, especially TLR4, remains controversial (Asai *et al.*, 2001; Kusumoto *et al.*, 2004). Although this study demonstrated expression of TLR4 in the oral epithelial cell line OKF6/TERT-2, analysis of our data indicated that TLR4 was not involved in *C. glabrata*-induced GM-CSF production.

We next examined the role of CDw17 in *C. glabrata*-induced GM-CSF response in OECs. CDw17 was reported to act as receptor for bacteria (Krivan *et al.*, 1989), viruses (Markwell *et al.*, 1981), and fungi, including *C. albicans* (Jimenez-Lucho *et al.*, 1990). Expression of CDw17 was detected in human buccal and bronchial epithelial cells (Baker *et al.*, 1990). In this study, we detected surface expression of CDw17 in the oral epithelial cell line OKF6/TERT-2 for the first time. Blocking of CDw17 resulted in a significant, but partial, inhibition of NF-κB activity and GM-CSF response, suggesting the existence of additional surface receptor(s) on OECs which may interact with *C. glabrata* to stimulate GM-CSF expression. Although β-glucan-CDw17 interaction has been reported to mediate cytokine synthesis in epithelial cells (Hahn *et al.*, 2003; Evans *et al.*, 2005), β-glucans failed to activate GM-CSF in our system, and thus we conclude that the CDw17/NF-κB/GM-CSF pathway may be β-glucan-independent. It is possible that binding and signaling are mediated by an alternative *C. glabrata* lectin-type ligand (Fantini *et al.,* 2006) from the Epa family of adhesins, which has the potential to bind to a core structural motif shared by CDw17 and other membrane glycosphingolipids (Cormack *et al.*, 1999). However, given the relatively late activation of NF-κB in epithelial cells after contact with *C. glabrata*, there is also a distinct possibility that NF-κB activation is not the result of immediate recognition of the fungus, but rather a more indirect effect of downstream mediators other than IL-1. To our knowledge, this is the first report of CDw17 receptor involvement in generating a pro-inflammatory response to *C. glabrata* infection, suggesting its potential role in host innate immune defense against fungal infections.

In summary, we demonstrated that *C. glabrata*-induced GM-CSF responses in OECs were contact-dependent, and enhanced by fungal viability and endocytosis. We further demonstrated that the surface receptor CDw17, but not TLR4, participated in GM-CSF gene expression, *via* NF-κB activation. This is the first report describing an inflammatory signaling pathway triggered by *C. glabrata* in OECs.

ACKNOWLEDGMENT

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