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## Activation of MAPK/c-Fos induced responses in oral epithelial cells is specific to *Candida albicans* and *Candida dubliniensis* hyphae

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### Abstract

Oral epithelial cells detect the human pathogenic fungus *Candida albicans* via NF- $\kappa$ B and a biphasic mitogen-activated protein kinase (MAPK) signaling response. However, discrimination between *C. albicans* yeast and hyphal forms is mediated only by the MAPK pathway, which constitutes activation of the MAPK phosphatase MKP1 and the c-Fos transcription factor and is targeted against the hyphal form. Given that *C. albicans* is not the only *Candida* species capable of filamentation or causing mucosal infections, we sought to determine whether this MAPK/MKP1/c-Fos mediated response mechanism was activated by other pathogenic *Candida* species, including *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata* and *C. krusei*. Although all *Candida* species activated the NF- $\kappa$ B signaling pathway, only *C. albicans* and *C. dubliniensis* were capable of inducing MKP1 and c-Fos activation, which directly correlated with hypha formation. However, only *C. albicans* strongly induced cytokine production (G-CSF, GM-CSF, IL-6 and IL-1 $\alpha$ ) and cell damage. *Candida dubliniensis*, *C. tropicalis* and *C. parapsilosis* were also capable of inducing IL-1 $\alpha$  and this correlated with mild cell damage and was dependent upon fungal burdens. Our data demonstrate that activation of the MAPK/MKP1/c-Fos pathway in oral epithelial cells is specific to *C. dubliniensis* and *C. albicans* hyphae.

### Keywords

*Candida albicans*; *Candida dubliniensis*; Hypha formation; MAPK; MKP1; c-Fos; NF- $\kappa$ B; Oral epithelium; Innate immunity

### Introduction

The most significant fungal pathogens of humans are *Candida* species, which are normal constituents of the oral and gut flora in approximately 50% of the population. However, under suitable predisposing conditions, *Candida* species cause a variety of mucosal diseases with significant morbidity [1]. Many *Candida* species are “polymorphic”, capable of growing either as yeasts, pseudohyphae or hyphae; however, only *C. albicans* and *C. dubliniensis* are thought to be capable of true hyphal growth. A number of studies have addressed the ability of *Candida* species to “infect” mucosal tissues and induce effector

cytokine responses by using monolayer culture systems or reconstituted human epithelial models. These studies demonstrate that *C. albicans* is the most “pathogenic” *Candida* species, as determined by hyphal penetration and induction of epithelial cell damage [2–7] and is the most immunostimulatory [4, 5, 7–10]. In all cases, fungal viability was required to induce epithelial cell damage and effector responses [4, 7, 9, 11]. *Candida albicans* superior ability to induce damage and effector responses appears to be due to hypha formation, as all non-*C. albicans* species including *C. dubliniensis* fail to form hyphae in epithelial cell culture systems [2, 5].

Given the apparent importance of hypha formation in pathogenicity and epithelial activation [12–14], we recently investigated how oral epithelial cells discriminate between the yeast and hypha form of *C. albicans* [7]. We reported that oral epithelial cells orchestrate innate immunity to *C. albicans* via NF- $\kappa$ B and a bi-phasic MAPK signaling response. Activation of NF- $\kappa$ B and the first MAPK phase, constituting c-Jun transcription factor activation, is independent of the morphological form and is likely due to the recognition of fungal cell wall polysaccharides (chitin, mannan,  $\beta$ -glucan). Activation of the second MAPK phase, constituting stabilization of the MAPK phosphatase MKP1 and activation of the c-Fos transcription factor, is specifically associated with hypha formation and correlates with proinflammatory responses in a dose-dependent manner. We concluded that this MAPK/MKP1/c-Fos activation system may be part of a “danger response” mechanism that permits epithelial tissues to remain quiescent in the presence of low fungal burdens while responding specifically and strongly to damage-inducing hyphae when burdens increase, and may be critical in identifying when this normally opportunistic fungus has become pathogenic.

However, *C. albicans* is not the only *Candida* species capable of filamentation or causing mucosal infections. Therefore, in this study, we sought to determine whether the MAPK/MKP1/c-Fos response mechanism was activated by other *Candida* species, including *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei* and the non-pathogenic brewer’s yeast *Saccharomyces cerevisiae*. Of particular interest was *C. dubliniensis*, which is regarded as the only other *Candida* species (apart from *C. albicans*) that forms true hyphae. Thus, a specific aim was to determine whether the MAPK/MKP1/c-Fos pathway was a common pathway activated by both these *Candida* species or a unique mechanism activated only by *C. albicans*.

## Materials and methods

### Epithelial cell lines and fungal strains

Experiments were carried out using the TR146 buccal epithelial carcinoma cell line. Monolayer TR146 cultures were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and experiments carried out in serum-free DMEM. Reconstituted human oral epithelium (oral RHE: 5 days) were purchased from SkinEthic Laboratories (Nice, France) and used as previously described [7, 12]. This model is created from the same cell line (TR146) and constitutes several layers of stratified squamous epithelium permitting the direct analysis of pathogen–epithelial interactions that are not complicated by non-epithelial factors. Rabbit monoclonal antibodies to human phospho-MKP1, phospho-c-Jun, phospho-I $\kappa$ B $\alpha$ , I $\kappa$ B $\alpha$  and c-Fos were purchased from Cell Signaling Technologies (New England BioLabs). Mouse monoclonal antibody to human  $\alpha$ -actin was purchased from Chemicon (Millipore) and goat anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies were purchased from Jackson Immunologicals Ltd (Strattech Scientific). The fungal strains used included *C. albicans* SC5314 [15], *C. dubliniensis* (CD36), *C. tropicalis* (ATCC 750), *C. parapsilosis* (NCPF 3191), *C. glabrata* (ATCC 2001), *C. krusei* (clinical isolate 2,325) and *Saccharomyces cerevisiae* (NCPF 3139). All strains were grown in YPD medium (1% yeast extract, 2%

peptone, 2% dextrose) overnight at 30°C to stationary phase prior to experimentation. *Candida dubliniensis* was pre-induced to form germ tubes/hyphae in 10% serum/sterile water for 3 h at 37°C [16].

### Fungal infection of epithelium and morphological analysis

*Candida* species and *S. cerevisiae* were inoculated at  $2 \times 10^6$  cells (in 50  $\mu$ l PBS;  $4 \times 10^7$  cells/ml stock) onto oral RHE, or at  $10^7$  cells/ml for signaling assays or between  $10^4$ – $10^8$  cells/ml for cytokine and PCR assays on monolayer epithelial cultures. The multiplicity of infection (MOI) ranged from 0.01 to 100 (fungal cells per epithelial cell) depending on the experiment. Oral RHE and monolayers were incubated at 37°C in 5% CO<sub>2</sub> for 30 min, 2 h, 6 h or 24 h as previously described [7, 12, 17]. Noninfected controls contained PBS alone. For morphological analysis, monolayers were fixed in 10% buffered formalin and examined by differential interference contrast (DIC) microscopy at  $\times 400$ .

### Western blotting

Epithelial cells were lysed using a modified RIPA lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton x-100, 1% Sodium deoxycholate, 0.1% SDS) containing protease (Sigma–Aldrich) and phosphatase inhibitors (Perbio), left on ice for 30 min, and then centrifuged for 10 min in a refrigerated microfuge. Supernatants were assayed for total protein using the BCA protein quantitation kit (Perbio). Twenty micrograms of protein were separated on 12% NuPAGE Bis/Tris minigels (Invitrogen) before transfer to PVDF membranes (GE Healthcare). After probing with primary (1:1,000 dilution) and secondary (1:10,000 dilution) antibodies, membranes were developed using Immobilon chemiluminescent substrate (Millipore) and exposed to ECL film (GE Health-care).  $\alpha$ -actin was used as a loading control.

### Transcription factor DNA binding assay

c-Fos DNA binding activity was assessed using the TransAM transcription factor ELISA system (Active Motif). Briefly, nuclear proteins were isolated from epithelial cells after 3 h infection with *Candida* using a nuclear protein extraction kit as per manufacturer's instructions (Active Motif). Protein concentration was determined as above and 5  $\mu$ g of nuclear extract was assayed in the TransAM system according to the manufacturer's protocol.

### Cytokine determination

Cytokine levels in cell culture supernatants was determined at 24 h using the Fluorokine MAP cytokine multiplex kits (R&D Systems), coupled with the Luminex 100™ machine according to the manufacturer's protocol. The trimmed median value was used to derive the standard curve and calculate sample concentrations.

### Epithelial cell damage assay

Epithelial cell damage was determined at 24 h by measuring lactate dehydrogenase (LDH) activity in the culture supernatant as described previously [7, 12, 17]. This was performed using the Cytox 96 NonRadioactive Cytotoxicity Assay kit (Promega) according to the manufacturer's protocol and using a recombinant porcine LDH (Sigma–Aldrich) to generate a standard curve. Sample values were then extrapolated from this curve.

### RNA isolation and analysis

Total RNA was isolated from TR146 oral epithelial monolayers using the Nucleospin II kit (Macherey–Nagel, ThermoFisher) and treated with Turbo DNase-(Ambion) to remove genomic DNA. cDNA was transcribed from total RNA using 1 U HIV reverse transcriptase

(Ambion) and real-time PCR was performed on cDNA using Jump-start SYBR green mastermix (Sigma–Aldrich) on a Rotorgene 6000 (Corbett Research). IL-6 primer sequences were: Forward 5'-AAC CTG AAC CTT CCA AAG ATG G-3'; Reverse 5'-TCT GGC TTG TTC CTC ACT ACT-3'; and cycling conditions were 95°C 5 min; 95°C 3 s, 58°C 10 s, 72°C 10 s for 40 cycles.

## Statistics

TransAM and cytokine data were analyzed using a paired *t* tests. In all cases,  $P < 0.05$  was taken to be significant.

## Results

### Activation of the NF- $\kappa$ B and MAPK signaling pathways

For comparative studies of signal protein activation, 30 min and 2 h were taken as representative time points to delineate the bi-phasic MAPK response against different *Candida* species and *S. cerevisiae*. Previously, we demonstrated that I $\kappa$ B- $\alpha$  phosphorylation (a key event in NF- $\kappa$ B pathway activation) and c-Jun transcription factor phosphorylation was activated by *C. albicans* yeasts and was not associated with the hyphal-mediated response [7]. Western blot analysis of TR146 oral epithelial cells demonstrated that all *Candida* species and *S. cerevisiae* induced I $\kappa$ B- $\alpha$  and c-Jun phosphorylation at both 30 min and 2 h postinfection, with little quantitative difference between time points or fungal species (Fig. 1a). The second stage of the bi-phasic MAPK response constitutes MKP1 phosphorylation and c-Fos activation at 2 h and is induced specifically by the hyphal form of *C. albicans* [7]. Figure 1 shows that *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei* and *S. cerevisiae* were all unable to stimulate production of phosphorylated (stabilized) MKP1 or c-Fos at 2 h. Nor were they able to increase c-Fos DNA binding activity at 3 h (Fig. 1b). These findings correlated with the lack of hypha formation under standard assay conditions (Fig. 2).

### *C. dubliniensis* germ tubes/hyphae can activate MKP1 and c-Fos

Under standard cell culture conditions only *C. albicans* was able to form hyphae in the presence of epithelial cells and this directly correlated with MKP1 and c-Fos activation (Figs. 1, 2). However, given that *C. dubliniensis* is able to form hyphae under nutrient poor conditions (and not nutrient rich conditions e.g., culture medium), this raised the possibility that *C. dubliniensis* may also be able to activate the MKP1/c-Fos response if first pre-induced to form hyphae. Therefore, we incubated *C. dubliniensis* in sterile water:10% serum for 3 h at 37°C to induce germ tube formation [16] prior to incubation with oral epithelial cells. Under these conditions, *C. dubliniensis* germ tubes were able to activate both MKP1 and c-Fos to a similar degree as *C. albicans* after 2 h (Fig. 3; Note: at 30 min, *C. albicans* is negative as it only starts forming germ tubes between 45 and 60 min). To confirm the transcription factor data, we additionally assessed c-Fos for DNA binding by TransAM ELISA and again demonstrated that *C. dubliniensis* germ tubes were equally efficient at activating c-Fos as *C. albicans* germ tubes. As expected, c-Jun phosphorylation was equal between the species and was morphology independent. Interestingly, after 4–6 h incubation, *C. dubliniensis* germ tubes reverted back to pseudohyphae and yeast cells as a result of nutrient-rich culture conditions (Fig. 4). Given this, we hypothesized that reversion to pseudohyphae/yeast cells may result in dephosphorylation or partial deactivation of MKP1 and c-Fos. Therefore, we analyzed MKP1 and c-Fos activation at 2, 6 and 24 h post-addition of *C. albicans* and *C. dubliniensis*, with and without pre-induction of germ tubes. For c-Fos, this was performed by Western blotting in order to directly compare data visually with MKP1. After 2 h, only *C. albicans* and *C. dubliniensis* pre-induced hyphal cells activated MKP1 and c-Fos (Fig. 4). By 6 h, *C. albicans* either markedly increased or sustained MKP1

phosphorylation and c-Fos, respectively, presumably as a result of increased hypha formation. In contrast, *C. dubliniensis*, which had reverted back to pseudohyphae/yeast cells by 6 h, demonstrated a marked or moderate reduction in MKP1 phosphorylation and c-Fos, respectively. By 24 h, MKP1 phosphorylation and c-Fos was still evident with *C. albicans* but absent with *C. dubliniensis*. *Candida dubliniensis*, when applied as yeast, did not significantly activate MKP1 and c-Fos at any stage.

### Cytokine activation and cell damage in organotypic epithelial models

Previously, using oral epithelial TR146 cell monolayers, we found that MKP1 and c-Fos activation correlated with cytokine induction by *C. albicans* [7]. As only *C. albicans* formed hyphae under standard assay conditions, we hypothesized that none of the other fungal species would significantly activate cytokine production. Using TR146 organotypic oral RHE models, we confirmed this as only *C. albicans* was capable of inducing high amounts of G-CSF, GM-CSF or IL-6 ( $P < 0.05$  or  $< 0.01$ ), although *C. dubliniensis* was capable of inducing low amounts of IL-6 (Fig. 5). Interestingly, in addition to *C. albicans*, *C. dubliniensis*, *C. tropicalis* and *C. parapsilosis* were all capable of inducing epithelial damage (Fig. 5) despite not forming hyphae (Fig. 2), and this correlated with an increase in secretion of the damage-associated cytokine IL-1 $\alpha$  ( $P < 0.01$ ; Fig. 5).

### Cytokine induction and cell damage by *C. dubliniensis* germ tubes is dose-dependent

Given that *C. dubliniensis* germ tubes were able to induce MKP1/c-Fos at 2 h but were unable to induce cytokines by 24 h due to a reversion back to pseudohyphae/yeasts, we explored whether an epithelial cytokine response could be forced by exposing epithelial cells to long hyphae of *C. dubliniensis* or by increasing the fungal burden. Unfortunately, we found that long hyphae could not be induced in *C. dubliniensis*, with maximal germ tube length peaking about 3–6 h post-induction in water:10% serum (data not shown). Also, it made no difference whether germ tubes were induced for 3 h or overnight (similar or shorter in length than at 3 h) because all the germ tubes reverted back to pseudohyphae/yeast soon after addition to the epithelial culture system. Thus, the transcriptional program from germ tubes back to pseudohyphae/yeast appears to be initiated very early in *C. dubliniensis* and so this hypothesis could not be fully tested.

We next hypothesized that since *C. dubliniensis* germ tube formation is maintained for the first 4–6 h, early induction of cytokine mRNA might be observed even though this does not ultimately result in secretion of a significant amount of cytokine protein at 24 h. This would indicate that the epithelial cells were activated in the early time points when *C. dubliniensis* germ tubes were present. To test this, we stimulated TR146 monolayer epithelial cells for 6 h with a high dose ( $10^8$  cells/ml) of pre-induced (3 h) *C. dubliniensis* germ tubes and looked for IL-6 mRNA transcripts. As hypothesized, pre-induced *C. dubliniensis* germ tubes strongly increased production of IL-6 mRNA (~30-fold) as compared with *C. dubliniensis* yeasts (Fig. 6), confirming that *C. dubliniensis* germ tubes were capable of activating epithelial cells. However, *C. albicans* increased IL-6 mRNA transcripts to a much higher extent (~500-fold) than *C. dubliniensis* germ tubes. The difference in IL-6 mRNA induction between *C. albicans* and *C. dubliniensis*, together with the reversion of *C. dubliniensis* germ tubes back to pseudohyphae/yeasts by 4–6 h, probably explain why *C. dubliniensis* only induced minimal amounts of IL-6 protein at 24 h (Fig. 5).

Our previous work indicated that fungal burdens and threshold levels of activation were crucial for the induction of cytokine responses and damage from epithelial cells [7]. With *C. albicans*, cytokine and damage assays are normally performed with a start dose of  $10^4$  cells/ml (MOI of 0.01) as this gives the fungus an opportunity to grow, produce hyphae and induce cytokine responses before the epithelial cells are killed. *Candida dubliniensis* was

unable to induce cytokine production at this MOI due to the reversion of the germ tubes back to pseudohyphae/yeast at 4–6 h post-addition. Therefore, TR146 cells were stimulated with higher doses of  $10^6$  or  $10^7$  *C. dubliniensis* germ tubes/ml (MOI of 1 or 10) and cytokine and damage induction was assessed at 24 h. We found that *C. dubliniensis* germ tubes at  $10^6$  cells/ml induced a significant increase in IL-6 protein release, but minimal induction of LDH and the damage-associated cytokine IL-1 $\alpha$  (Fig. 6). However, at a dose of  $10^7$  germ tubes/ml *C. dubliniensis* also significantly induced LDH and IL-1 $\alpha$  to similar levels as *C. albicans*. It should be noted that IL-6 release by *C. albicans* and *C. dubliniensis* germ tubes at  $10^7$  cells/ml was markedly reduced by 24 h because at these high doses significant damage to the epithelial cells occurs early in the assay, thereby preventing de novo synthesis of IL-6 protein as a result of cell death. This does not necessarily apply to IL-1 $\alpha$  as significant stores of IL-1 $\alpha$  protein are constitutively present in epithelial cells and are released rapidly upon stimulation.

## Discussion

*C. albicans* is regarded as the most pathogenic *Candida* species and one of its key virulence attributes is hypha formation. Previously, we reported that a bi-phasic innate immune MAPK response constituting activation of MKP1 and c-Fos discriminates between the yeast and hyphal forms of *C. albicans* in oral epithelial cells [7], which leads to the induction of a proinflammatory cytokine response and ultimately protection against infection [7, 11]. Since *C. albicans* is not the only *Candida* species capable of filamentation or causing mucosal infections, we sought to determine whether this MAPK/MKP1/c-Fos mediated mechanism was also activated by other pathogenic *Candida* species, particularly *C. dubliniensis*, which is also a hypha-forming species.

Under standard cell culture conditions (DMEM, serum-free) all *Candida* species and *S. cerevisiae* were able to induce the NF- $\kappa$ B pathway and the first MAPK phase constituting c-Jun activation, demonstrating that the epithelial cells detect and respond to the presence of fungus. Only *C. albicans* was able to activate the second MAPK phase constituting MKP1/c-Fos activation and proinflammatory cytokines, and given that *C. albicans* was the only fungal species able to form hyphae under these culture conditions this supports the conclusion that the MAPK/MKP1/c-Fos response mechanism is hypha associated. However, when pre-induced to form germ tubes prior to addition to epithelial cells, *C. dubliniensis* was also able to induce MKP1/c-Fos activation, but since *C. dubliniensis* germ tubes reverted back to the pseudohyphal/yeast form after 4–6 h post-addition to epithelial cells this provided insufficient time for the production and secretion of cytokine proteins. Notably, the reversion of *C. dubliniensis* germ tubes back to pseudohyphae/yeast correlated with the subsequent deactivation of the MKP1/c-Fos response. The data indicate that sustained epithelial activation and cytokine induction requires the maintenance of *Candida* hypha formation together with continual activation of the MAPK/c-Fos pathway.

In addition to hypha formation, fungal burdens and threshold levels of activation are also crucial for the induction of cytokine responses and epithelial damage by *C. albicans* [7]. Given this, we hypothesized that stimulating epithelial cells with higher doses of *C. dubliniensis* germ tubes may force a cytokine response since the exposure to the “hyphal” form would be greater. Indeed this is what we found, as stimulating epithelial cells with between  $10^6$  and  $10^8$  *C. dubliniensis* germ tubes/ml induced transcription of cytokine (IL-6) mRNA and protein, albeit lower than that induced by *C. albicans*. At the high doses ( $10^7$  cells/ml), *C. dubliniensis* germ tubes were also capable of causing epithelial damage and inducing secretion of the damage-associated cytokine IL-1 $\alpha$  (see below). The data indicates that *C. dubliniensis* germ tubes/hyphae have the ability to activate epithelial cells and induce cytokine secretion if present in sufficient quantity and confirms the importance of fungal

burdens in mediating mucosal innate responses. It also suggests that *C. dubliniensis* possesses an ortholog of the *C. albicans* hyphal factor that activates epithelial cells, but that it may be present in lower quantities (or is modified) and is thus less immunostimulatory. However, we would like to stress that such high burdens of *C. dubliniensis* are unlikely to be present during infection in vivo. Therefore, since hyphal burdens are key for activation of the MAPK/c-Fos pathway and that *C. dubliniensis* cannot maintain hyphal formation, this may in part explain why *C. dubliniensis* is a relatively poor activator of epithelial cells and a weak pathogen of humans.

Apart from *C. albicans* and *C. dubliniensis*, *C. tropicalis* and *C. parapsilosis* were also able to induce IL-1 $\alpha$  and cell damage despite not forming hyphae. Given the established link between IL-1 $\alpha$  and cell damage in myeloid cells [18, 19], it is likely that IL-1 $\alpha$  production in oral epithelial cells also correlates with cell damage. This is probably induced independently of hypha formation or MKP1/c-Fos activation, with IL-1 $\alpha$  production most likely mediated via the NF- $\kappa$ B pathway as previously demonstrated [7]. However, precisely how IL-1 $\alpha$  production, cell damage and NF- $\kappa$ B activation are linked in epithelial cells is unclear and requires more detailed investigation. The data also indicate that the mechanisms associated with cell damage are, in part, separate from the mechanisms inducing cytokines since *C. tropicalis* and *C. parapsilosis* were unable to induce G-CSF, GM-CSF or IL-6 despite causing mild damage.

Our data are in line with some studies demonstrating minimal or no invasion of oral epithelial cells [2, 3] and lack of cytokine production [5] with non-*C. albicans* species, but in contrast to others that have demonstrated invasion and/or cytokine production by *C. tropicalis*, *C. parapsilosis* or *C. glabrata* [4, 20–23]. An important and common feature of these latter studies, though, is that invasion and cytokine production by the non-*C. albicans* species was significantly lower than that induced by *C. albicans*. Although these non-*C. albicans* species are capable of causing human mucosal infections, it is unclear whether they induce epithelial responses in vivo. One possibility is that *C. tropicalis*, *C. parapsilosis* and *C. krusei* may form “hyphal-like” structures in vivo to activate epithelial cells. However, it is unlikely that these structures will parallel the true hyphae produced by *C. albicans* and *C. dubliniensis* and are thus unlikely to possess the same hyphal moiety that seems to be shared by *C. albicans* and *C. dubliniensis* to activate epithelial cells via the MAPK/MKP1/c-Fos pathway. Unfortunately, the morphology of these non-*C. albicans* species cannot be efficiently manipulated to test this hypothesis using in vitro systems. Another possibility is that other *Candida* species might not be as metabolically active as *C. albicans* hyphae, which may lead to minimal epithelial responses. However, this is also unlikely given that heat-killed *C. albicans* hyphae, which are also metabolically inactive, are able to induce MKP1 and c-Fos activation, albeit to a lesser degree than viable *C. albicans* hyphae [7]. These arguments, together with the reduction in MKP1 and c-Fos activation that accompanied the loss of hyphal growth of *C. dubliniensis* and the lack of MKP1 and c-Fos activation by *C. albicans* strains that are unable to form true hyphae (such as 529L) [7], strongly indicate that epithelial immune activation via the MAPK/MKP1/c-Fos pathway is specifically associated with a hyphal moiety that is shared by *C. albicans* and *C. dubliniensis* hyphae. Conceptually, this is important as it means that both the epithelial mechanism and the hyphal moiety could be essential components in *Candida* pathogenesis and induction of host immunity.

The identity of the shared *C. albicans* and *C. dubliniensis* hyphal moiety responsible for epithelial activation via the MAPK/MKP1/c-Fos pathway is unknown, but cell wall associated chitin, mannan or  $\beta$ -glucan are not the activatory factors [7]. The *C. dubliniensis* genome has recently been sequenced, and comparative analysis demonstrates that it possesses a near identical gene repertoire to *C. albicans*, with *C. dubliniensis* possessing

only 29 species-specific genes and *C. albicans* 168 species-specific genes [24]. Notably, *C. dubliniensis* lacks certain hypha-specific genes of interest, including *SAP4/5* and *HYR1*; therefore, these proteins are unlikely to be the epithelial activating factors. This leaves a host of potential hyphal genes/proteins shared between the two species that could activate the MAPK-based MKP1/c-Fos mediated discriminatory response. Future studies will endeavor to identify this common hyphal component, which appears to be a crucial target of oral epithelial cells and a key activator of mucosal innate immunity.

## Acknowledgments

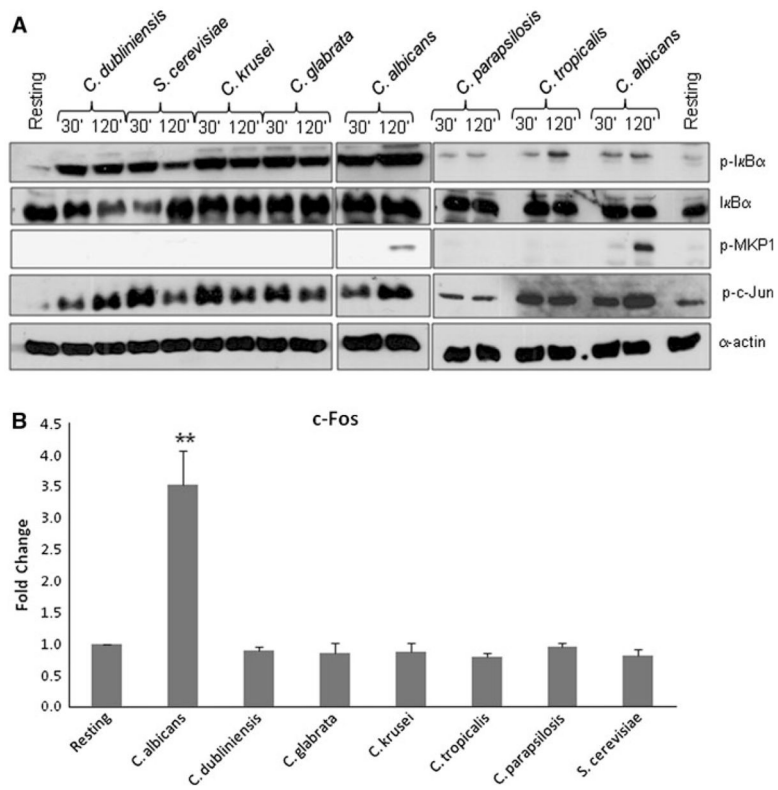
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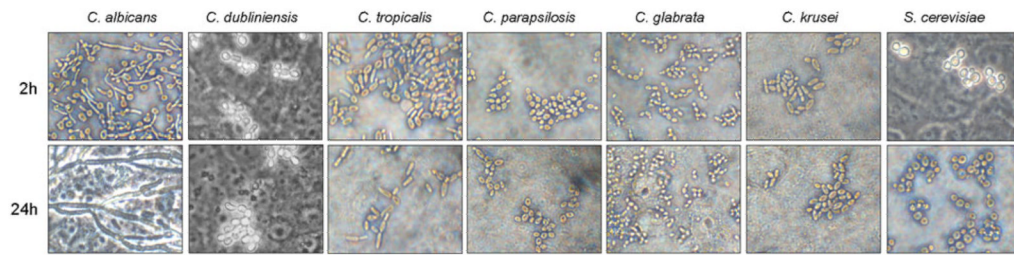
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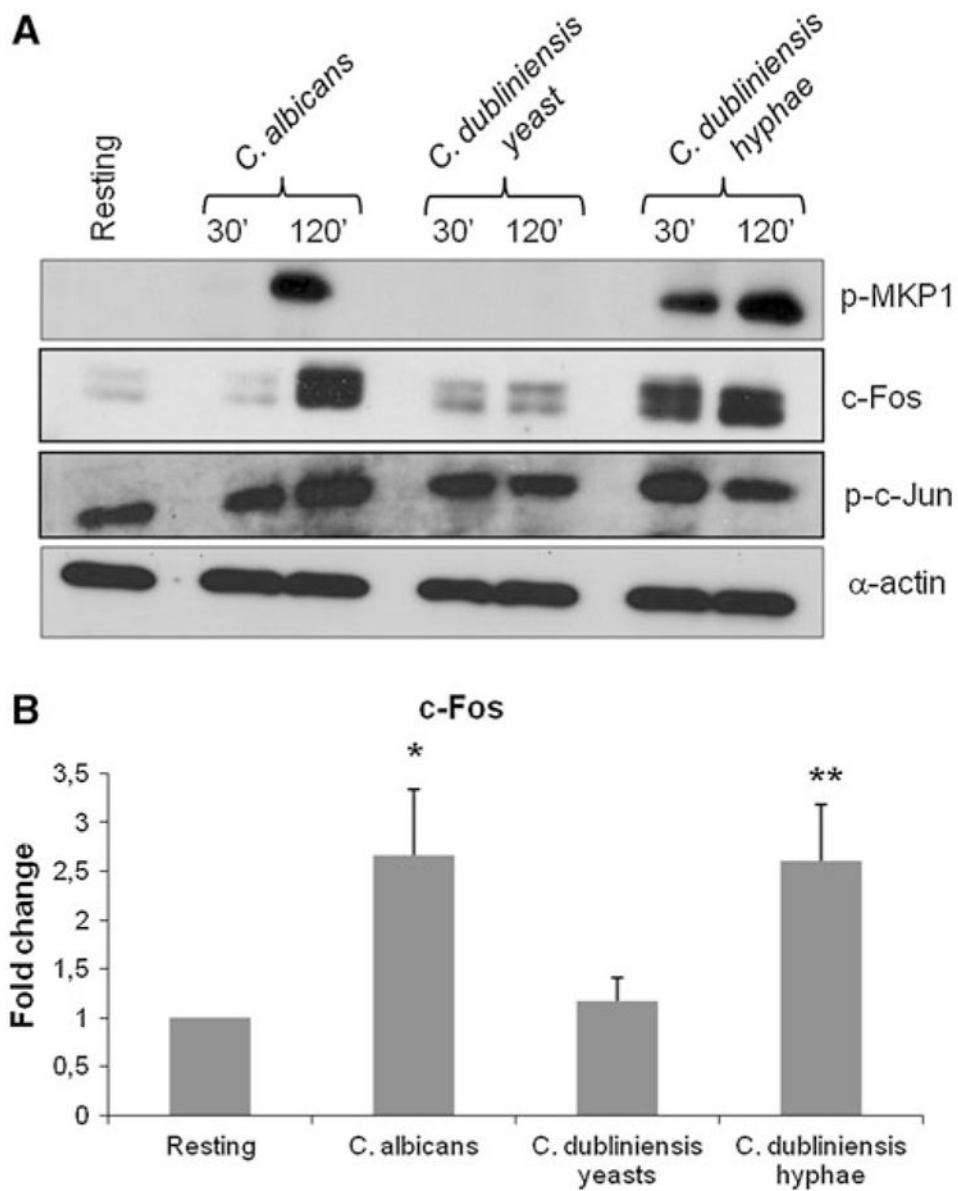
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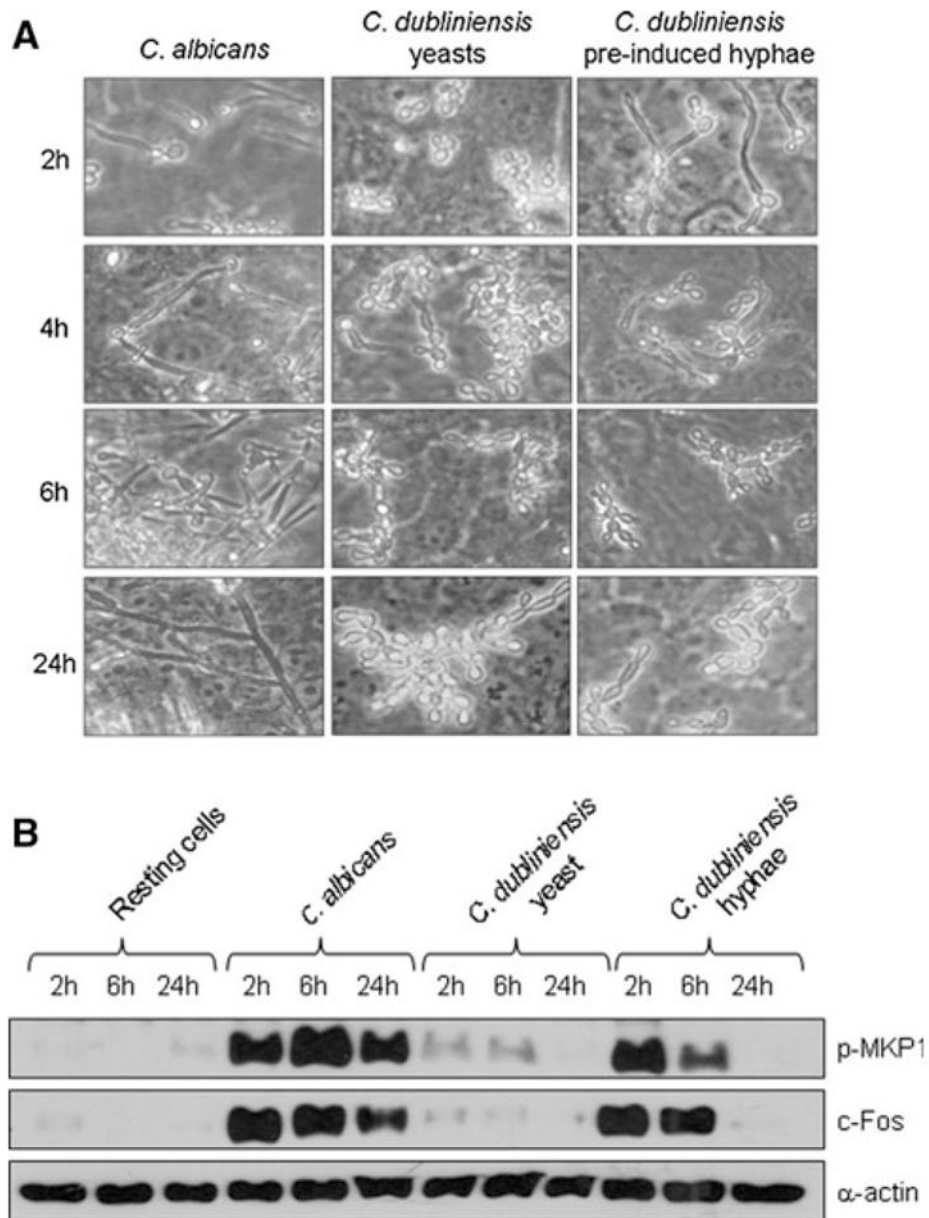
**Fig. 1.** Activation of NF- $\kappa$ B and MAPK signaling by different *Candida* species and *S. cerevisiae*. Different *Candida* species and *S. cerevisiae* were added to TR146 oral epithelial cells under standard culture conditions for 2 h. **a** Total protein was isolated and phosphorylation of I $\kappa$ B- $\alpha$  and MKP1 and c-Jun assessed as well as production of c-Fos. **b** Nuclear extracts were isolated and c-Fos DNA binding activity measured by TransAm ELISA. Bands are shown relative to  $\alpha$ -actin loading control. A fungal/epithelial cell MOI of 10:1 was used. Data are representative (assembled from different experiments) (**a**) or mean (**b**) of three independent experiments  $\pm$ SEM. \*\* $P < 0.01$



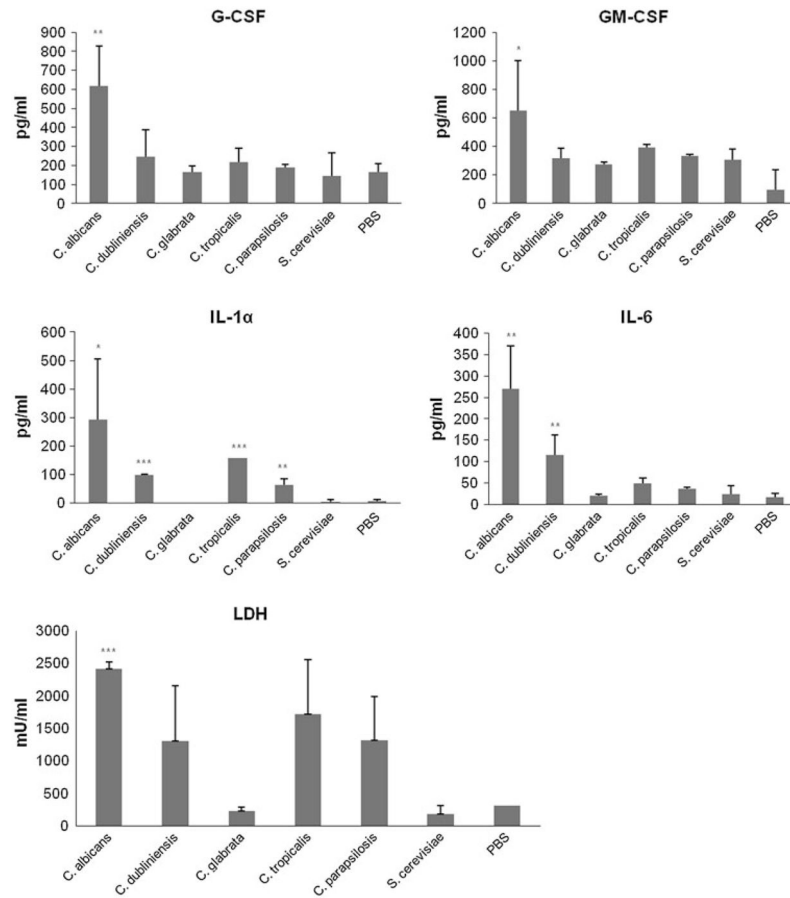
**Fig. 2.** Morphology of different *Candida* species and *S. cerevisiae*. Different *Candida* species and *S. cerevisiae* were added to TR146 oral epithelial cells for 2 or 24 h and formalin fixed. Morphology was assessed by DIC microscopy at  $\times 400$



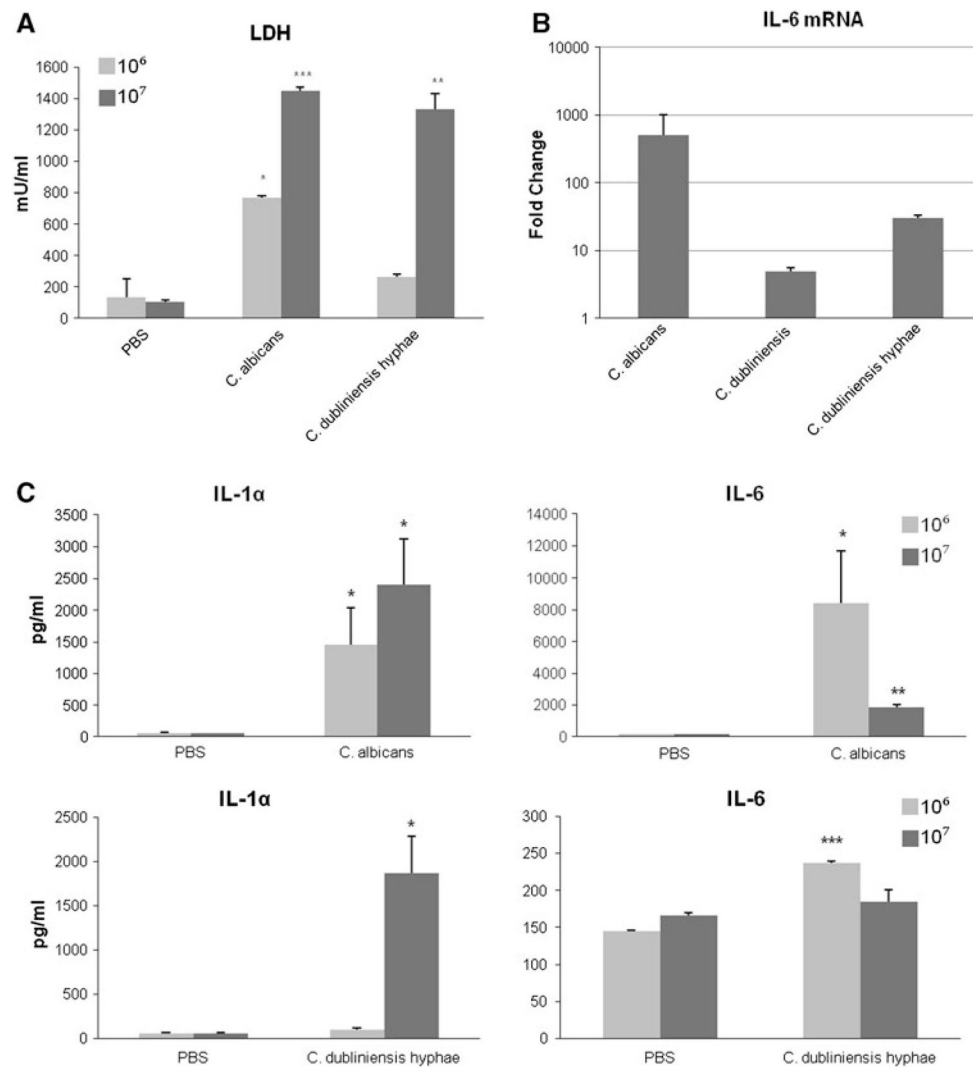
**Fig. 3.** Activation of MKP1 and c-Fos by *C. dubliniensis* hyphae. *Candida dubliniensis* was pre-induced to form hyphae in water/10% serum for 3 h at 37°C and added to TR146 cells for 2 h together with control strains (*C. dubliniensis* and *C. albicans* in the yeast phase). Total protein and nuclear extracts were isolated and assessed for **a** MKP1 and c-Jun phosphorylation as well as c-Fos production by Western blot (relative to  $\alpha$ -actin loading control) or **b** c-Fos DNA binding activity by TransAm ELISA. A fungal/epithelial cell MOI of 10:1 was used. Data are representative of three (assembled from different experiments) (a) or the mean of 5 (b) independent experiments ( $\pm$ SEM). \* $P < 0.05$ ; \*\* $P < 0.01$



**Fig. 4.** Reduction of MKP1 and c-Fos activation upon reversion of *C. dubliniensis* hyphae to pseudohyphal/yeast cells. *Candida dubliniensis* was pre-induced to form hyphae in water/10% serum for 3 h at 37°C and added to TR146 cells. At 2, 6 and 24 h postinfection **a** the morphology of *C. dubliniensis* or **b** MKP1 phosphorylation and c-Fos production was assessed by microscopy or Western blot, respectively. Bands are shown relative to  $\alpha$ -actin loading control. A fungal/epithelial cell MOI of 10:1 was used for the 2 and 6 h time points and 0.01 for the 24 h time point. Data are representative of three independent experiments



**Fig. 5.** Cytokine activation and cell damage by different *Candida* species and *S. cerevisiae*. Different *Candida* species and *S. cerevisiae* were added ( $2 \times 10^6$  cfu/ml) to organotypic oral RHE models for 24 h and the cell culture medium collected and assessed for cytokine proteins by multiplex microbead assay (luminex) or cell damage by LDH (lactate dehydrogenase) release. Data are representative of three independent experiments. \* $P < 0.05$ ; \*\*\* $P < 0.001$  (compared with PBS control)



**Fig. 6.** Cytokine activation and cell damage is dependent upon fungal burdens. Increasing concentrations of *C. albicans* and *C. dubliniensis* were added to TR146 monolayers and incubated at 37°C. **a** Cell damage was assessed 24 h postinfection with either *C. albicans* or pre-induced *C. dubliniensis* germ tubes (3 h in water:10% serum at 37°C) at 10<sup>6</sup> or 10<sup>7</sup> cfu/ml (MOI of 1 and 10, respectively). **b** IL-6 mRNA production was assessed 6 h postinfection with *C. albicans*, *C. dubliniensis* and pre-induced *C. dubliniensis* germ tubes. Results are expressed as fold change in mRNA transcripts relative to the PBS control. **c** IL-1α and IL-6 protein production in culture supernatants 24 h postinfection with *C. albicans* or pre-induced *C. dubliniensis* germ tubes at 10<sup>6</sup> or 10<sup>7</sup> cfu/ml (MOI of 1 and 10, respectively). Data are the mean of two experiments. \**P* < 0.05; \*\*\**P* < 0.001 (compared with PBS control)