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Interaction of *Candida albicans* with periodontal ligament fibroblasts limits biofilm formation over elastomer silicone disks

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ABSTRACT

Objective: Candida albicans is the most numerous commensal and potentially pathological yeast in the human oral cavity. The purpose herein is to investigate the ability of *C. albicans* to form a biofilm in the presence of periodontal ligament (PDL) fibroblasts.
Material and methods: Silicone elastomer disks (SE) were transferred to wells containing PDL cells. *C. albicans* suspension was added to each well. The whole mixed culture was then allowed to form a biofilm for 48 h. Biofilms were quantified by tetrazolium-salt-based (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl amino) carbonyl]- 2H-tetrazolium hydroxide (XTT). Furthermore, biofilm was visualized by confocal scanning laser and scanning electron microscopy. Migration of *C. albicans* and its ability to form biofilms in presence of PDL cells was determined by using a transwell system. Last, elutes obtained from co-culturing *C. albicans* and PDL cells were added to SE disks and covered with *C. albicans*. The culture plate was then incubated to allow biofilm formation. Biofilms formed over SE disks were quantified using XTT.
Results: PDL cells significantly limited the biofilm formation at incubation interval of 48 h. PDL cells induced less biofilm compared to mature and thick hyphae in the absence of PDL cells as seen in confocal scanning laser and scanning electron microscopy. The presence of PDL cells limited the migration and formation of biofilm by *C. albicans*. Elutes obtained from co-culturing PDL cells with C. *albicans* for one hour induced significantly less biofilm.
Conclusions: This is the first study to report that PDL cells exhibit antifungal activity. While the exact mechanism of how PDL cells limited biofilm formation is yet unknown, it was clear that competent PDL cells promote resistance to *C. albicans* biofilm formation.

1. Introduction

Candida albicans, being the most frequent commensal and periodically pathogenic yeast in the oral cavity (Conti & Gaffen, 2010). Like many oral microbes, *C. albicans* form and live within a biofilm matrix composed of exopolysaccharides, proteins, and nucleic acids that protect them from the environment and immune system (Siqueira & Sen, 2004; Gomes, Fidel, Fidel, & de Moura Sarquis, 2010). Biofilm formation, leading to immune-evasion and immune-modulation of the host defense, is considered a key virulence factor of *C. albicans*. Formation of a biofilm can provide the *C. albicans* community protection against antimicrobial agents as compared with those in a nomadic state (e.g. planktonic cells). *C. albicans* in biofilm can be 100-fold more resistant to antifungal fluconazole and 20- to 30-fold more resistant to antifungal amphotericin B than planktonic cells (Kumamoto, 2002). Several studies and *in vitro* models have been established to characterize *C. albicans* biofilm formation on common bio-prosthetic materials such as polymethylmethacrylate, which is used in the construction of dentures as well as silicone elastomer, a model material used for indwelling devices including catheters. Previous studies have indicated that biofilm development occurs in three distinct phases. The first phase begins with the adherence of *C. albicans; yeast forms,* to its substrate (≈0 to 11 h). Intermediate developmental phase features attached cells proliferation to form microcolonies and begin to deposit an extracellular matrix (≈12 to 14 h). Finally, the maturation phase (≈24 to 72 h) characterized by forming a dense network of filamentous forms (pseudohyphae and hyphae), and become encased in the exopolymeric matrix (Ramage, Mowat, Jones, Williams, & Lopez-Ribot, 2009; Chandra et al., 2001).

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Periodontitis is a chronic inflammatory disease affecting the integrity of tooth supporting tissues induced by variety of microorganisms, including yeast (Slots, Rams, & Listgarten, 1988). More recently, 30% of patients with chronic periodontitis compared to 15% with healthy subjects had *C. albicans* in periodontal pockets (Canabarro et al., 2013). *C. albicans* can evade the host defense and induce a complex immune response that ultimately determines the clinical outcome of the infection. Polymorphonuclear neutrophils and macrophages are known to be the most important inflammatory cells involved in the defense against *C. albicans* (Ashman & Papadimitriou, 1995). Periodontal ligament fibroblasts (PDL cells) are involved in the formation and maintenance of periodontal fibrous tissue connecting teeth to the alveolar bone (Beertsen, McCulloch, & Sodek, 1997). PDL cells play a crucial role in the early infection as well as resolution stage of infection at root surfaces (Jonsson, Nebel, Bratthall, & Nilsson, 2011). Thus, studying the interaction between *C. albicans* infection and periodontal cells should advance current understanding of the mechanism involved in the etiology of chronic periodontitis related to fungal infections.

The central hypothesis of this investigation was that PDL cells may influence the biofilm formation by *C. albicans*. There were no data published on the interaction of PDL cells with *C. albicans*. It is unknown whether PDL cells have any antifungal activity during *C. albicans* colonization. Using co-culture model system, the current study sought to determine whether PDL cells results in less biofilm formation by *C. albicans* on silicone disks (SE). The flat sheeting of SE disks was chosen to facilitate biofilm formation and produce undistorted images of biofilm formed by microscopic techniques as described previously (Kuhn, Chandra, Mukherjee, & Ghannoum, 2002). It also allows to design future studies aimed to understand the pathogenicity in other *ex/in vivo* oral models.

2. Material and methods

2.1. Cell culture, C. albicans, and growth conditions

Primary human PDL fibroblasts isolated from human periodontal tissues were obtained from ScienCell (Carlsbad, CA) and grown in complete culture medium; Dulbecco's modified Eagle's medium (Life Technologies, CA) supplemented with antibiotics and 10% fetal bovine serum (FBS), at 37 °C in a humidified 5% CO_2 atmosphere. Cells between the 4th and 6th passages were used in the present study.

The *C. albicans* wild strain CA42 (formerly known as SC5314 (Fonzi & Irwin, 1993) was used. A non-filamentous; unable to form biofilms, cph1Δ/efg1Δ double mutant strain CA80 (Lo et al., 1997), was used to validate model presented. Both strains were a courtesy of Dr. Audrey Atkin, University of Nebraska, Lincoln and were grown in yeast extract-peptone-dextrose (YPD) medium (Difco Laboratories, Detroit, MI.) from fresh Sabouraud dextrose agar plates (Difco) and incubated for 24 h at 37 °C in a shaker at 250 rpm. Cells were harvested and washed twice with phosphate buffered saline (PBS). Cells were then re-suspended in 10 mL of PBS, counted following serial dilution, standardized, and used immediately.

2.2. Silicone disks preparation

Silicone elastomer (SE) was purchased (Invotec International Incorporated, Jacksonville, FL). This material was shown to promote *C. albicans* biofilm and often used in indwelling devices (Hawser & Douglas, 1994). The SE is supplied as a flat sheet that facilitates quantifications and imaging the biofilm formed. They were cut with a carpenter's hole punch to produce standardized samples of 1.5 mm thick, and 3 mm diameter. SE disks were washed, autoclaved and incubated in fetal bovine serum (FBS) for 24 h at 37 °C. The pretreatment with FBS is known to promote hyphal formation (Chandra et al., 2001).

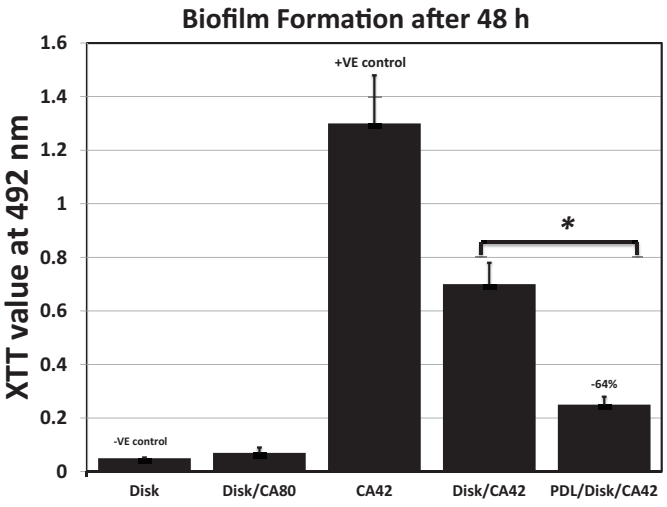

Fig. 1. Metabolic activity (XTT) of *C. albicans* biofilms formed for 48 h on SE disks in the presence (PDL/Disk/CA42) or absence (Disk/CA42) of PDL cells. Disks without inoculation of *C. albicans* was used as negative control. *C. albicans* (CA42) grown onto wells was used as positive control. Disks inoculated with *C. albicans* (CA80) was used to validate model used. All groups had $n = 6$. Metabolic activity is presented as an optical density at 492 nm. *P value of groups compared was significantly different at $\alpha = 0.05$.

2.3. Quantitative measurement of biofilms

PDL cells that reached confluence in culture medium were collected, washed, and counted with a haemocytometer. A total of 10^5 cells/well were plated in complete culture medium onto 24-well plates and grown overnight in 5% CO_2 at 37 °C to allow adherence to the surface. The SE disks were transferred to wells according to following groups ($n = 6$, repeated three times): (Group 1) no PDL cells or *C. albicans*, (Group 2) CA80 strain, (Group 3) CA42 strain, and (Group 4) CA42 strain and PDL cells. Group 1 and 2 served as negative controls. Additional wells pretreated with FBS with no SE disks received CA42 strain served as positive control. *C. albicans* suspension containing 10^5 cells was used. The whole mixed culture, in complete culture medium; was then allowed to form a biofilm at 37 °C in a humidified 5% CO_2 atmosphere. Following maturation for 48 h, SE disks were transferred to new culture plates and biofilms were quantified by tetrazolium-salt-based (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl amino) carbonyl]-2H-tetrazolium hydroxide (XTT) as described previously (Chandra et al., 2001). The reduction of XTT to formazan crystals can only take place in the presence of viable cells and the necessary reductase enzymes.

2.4. Confocal microscopy analysis of biofilms

Biofilm formation by *C. albicans* on SE disks in the presence of PDL cells was investigated using confocal scanning laser microscopy(CSLM) as described previously (Chandra, Mukherjee, & Ghannoum, 2008). New experiments were designed as described above. After co-culturing SE disks with *C. albicans* in the presence of PDL cells, SE disks were stained with FUN-1 (L7009, Molecular Probes, Eugene, Oreg.). Following biofilm formation, disks were removed and transferred to new 24-well plates. Wells containing biofilm disks were submerged in FUN-1 at a final concentration of 10 μM (from 10 mM stock). The plates were then incubated for 30 min at 37 °C. The disks were removed from the wells, placed in 35-mm glass-bottom microwell dishes (MatTek Corp., Ashland,

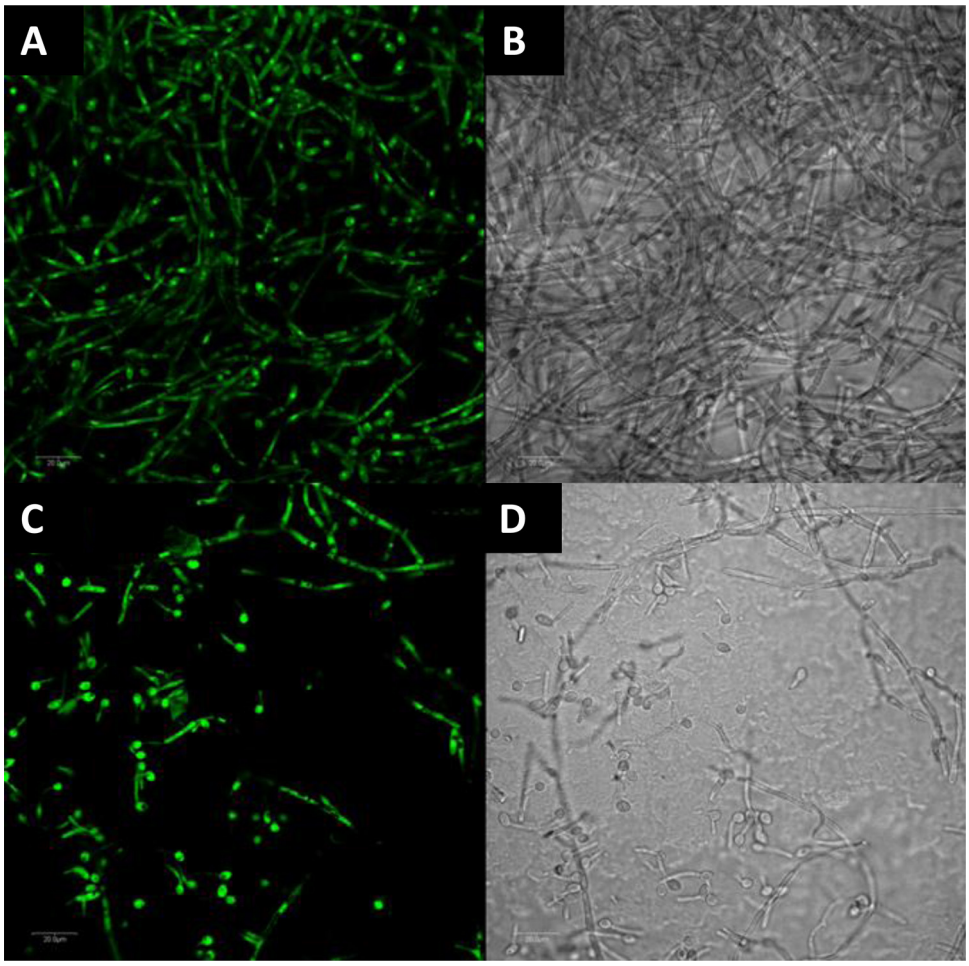

Fig. 2. Confocal laser scanning microscopy (CLSM) of *C. albicans* (CA42) biofilms grown on SE disks and stained with 10 μM FUN-1 in the absence (A) or presence (C) of PDL cells. (B) and (D) are differential interference contrast images of (A) and (C) respectively. Images taken with an Olympus FV500-IX81 inverted CSLM system. Fluorescence optical and DIC images were collected with a 20× lens using 488 nm excitation and 522 nm emission ($n = 3$).

Mass.), and observed by using an Olympus FV500-IX81 inverted CSLM system. Fluorescence optical and DIC images were collected with a 20x lens using 488 nm excitation and 522 nm emission.

2.5. Scanning electron microscopy

In a parallel experiment, SE disks were prepared for SEM using standardized method described previously (Chandra et al., 2008). They were fixed with 10% glutaraldehyde, followed by fixation with osmium tetroxide. This was followed by a series of ethanol dehydration steps, and the prepared samples were sputter coated with Au-Pd (60/40 ratio) and viewed with a model JEOL 6100 microscope.

2.6. Migration of C. albicans in the presence of PDL cells

Migration of *C. albicans* and its ability to form biofilms was inspired from previous studies (Wozniok et al., 2008), by using the same experimental design described above. First, migration of *C. albicans* was studied using transwell system without a substrate; SE disks. A total of 10^5 of PDL cells were plated in culture medium onto a transwell system (0.4 μm pore size, Corning Incorporated, Lowell, MA) for 24-well plates and grown overnight in 5% CO_2 at 37 °C to allow adherence to the surface. The SE disks were placed onto lower compartment of the system of wells had PDL cells. *C. albicans* suspension containing 10^5 cells was added to upper compartment of the system. An addition SE disks were placed onto empty well; with no PDL cells which served as positive controls. Biofilms formed over SE disks were quantified using XTT after 72 h as described above.

2.7. C. albicans growth in conditioned media

Supernatant obtained from co-culturing *C. albicans* (CA42) with PDL cells was used as previously described (Chandra, McCormick, Imamura, Mukherjee, & Ghannoum, 2007) to analyze biofilms formation. PDL cells were seeded at a concentration of 2×10^6 cells onto 75-cm^2 flasks and grown overnight in 5% CO_2 at 37 °C to allow adherence to the surface. A suspension of *C. albicans* wild strain CA42 was added to flasks containing PDL cells at ratio of 1:1, and was labeled solution A. An additional flask of PDL cells was left untreated, and was labeled solution B. After one hour of incubation, elutes were collected and centrifuged at low speed (1000 RPM) for 10 min. Each solution was filtered through a 0.2 μm syringe filter. SE disks treated with FBS overnight were placed onto flat-bottom 96-well plates. All SE disks received 2×10^4 cells of *C. albicans* (CA42) and covered with 200 μL of elute A or B ($n = 6$). The culture plate was then incubated for 48 h at 37 °C, 5% CO_2 to allow biofilm formation. Biofilms formed over SE disks were quantified using XTT described above.

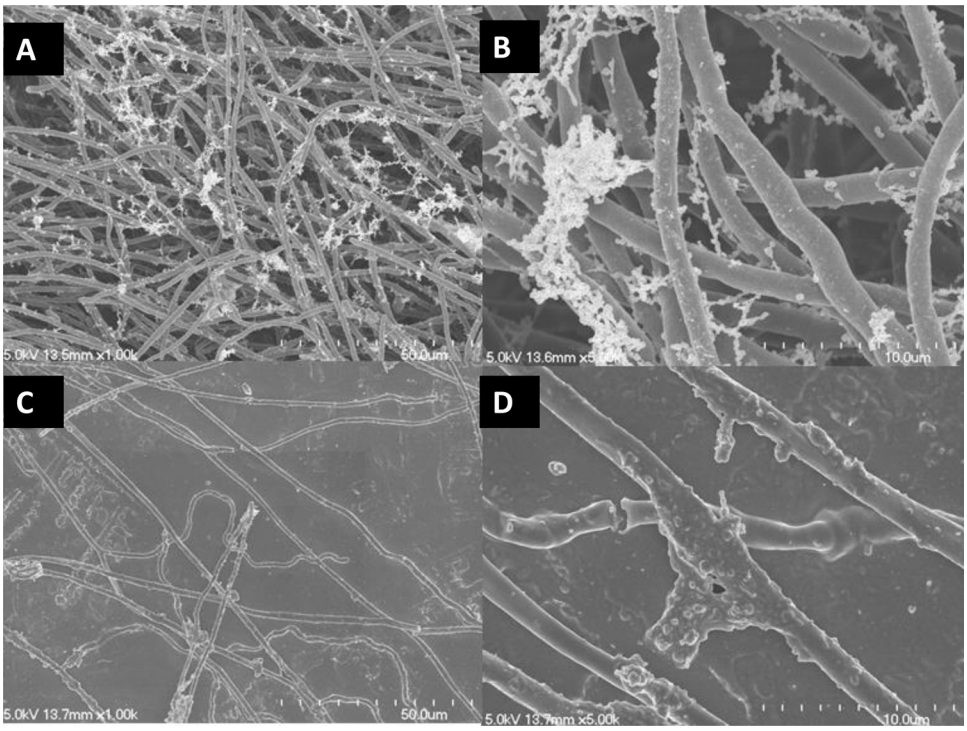

Fig. 3. SEM showing biofilms formed by *C. albicans* (CA42) alone on SE disks (A and B), or with PDL cells (C and D). SEM analyses at accelerating voltage of 10 kV and magnifications at 1.00 k and 5.0 k ($n = 3$).

2.8. Statistical analysis

Each experiment was independently performed at least three times on separate days; data shown in the figures are from one representative experiment. For quantification of biofilms by XTT (Sections 2.3,2.6 and 2.7), one-way analysis of variance was performed to compare means of multiple groups, and the one-tailed Student's *t*-test was used for analysis of two groups ($n = 6$). Results with a *P*-value less than 0.05 were considered statistically significant.

3. Results

3.1. Quantitative and microscopy analysis of C. albicans biofilms in the presence or absence of PDL cells

The results presented have shown that PDL cells significantly limited the biofilm formation at incubation interval of 48 h ($P < 0.001$) (Fig. 1). The reduction in biofilm formation was 64% (Fig. 1). The mutant form of *C. albicans strain* (CA80) was unable to form biofilm and was used to validate model presented. *C. albicans* that was allowed to grow on FBS-coated wells formed significant biofilm and was used as positive control (Fig. 1).

To visualize the biofilm formed over SE after 48 h, CLSM was utilized. In the absence of PDL cells, *C. albicans* (CA42) consistently produced mature and thick hyphae (Fig. 2A and B). The presence of PDL cells induced less mature and scattered hyphae formation (Fig. 2C and D). Furthermore, SE disks were analyzed by SEM. Biofilm formation was more mature and thick (Fig. 3A and B) compared to disks co-cultured with PDL cells (Fig. 3C and D).

3.2. PDL cells and conditioned media limit C. albicans migration and biofilms formation

It was noted that *C. albicans* were able to migrate and form mature biofilms on FBS-coated wells after 72 h compared to wells with PDL cells (data not shown). Therefore, studies were designed to analyze the effect of PDL cells on the migration of *C. albicans* to form biofilm on SE disks. The presence of PDL cells limited the migration and formation of biofilm by *C. albicans* (Fig. 4A). Furthermore, elutes obtained from co-culturing PDL cells with *C. albicans* for one hour induced significantly less robust biofilms compared to elutes obtained from *C. albicans* culture; solution B (Fig. 4B; $P < 0.05$).

4. Discussion

Oral *C. albicans* infections have to overcome the immune response mediated by supporting tissue and cells of roots. Therefore, the present study has utilized an *in vitro* biofilm model to investigate the ability of *C. albicans* to form a biofilm on SE disks in the presence of PDL cells. Results presented clearly demonstrated that PDL cells limit the formation of biofilms by *C. albicans* on its substrates. To our knowledge, this is the first report to study the interaction between PDL cells and *C. albicans* and its ability to form a biofilm.

The present study was motivated by Chandra et al. (2007) who demonstrated that co-cultured *C. albicans* with blood mononuclear cells (BMCs) enhanced biofilm formation. The current studies showed that the coculture of PDL cells with *C. albicans* limits the ability of this pathogen to form biofilms. Therefore, the dynamic interactions of *C. albicans* with different immune cells may result in different outcome. In their study, *C. albicans* were allowed to adhere to synthetic disks prior to co-culturing with BMCs. It is generally accepted that the phagocytosis-induced apoptosis of BMCs and subsequent clearance by macrophages is a prerequisite for the resolution of infection-associated inflammation, while the failure to undergo apoptosis creates a pathological situation (Zhang, Hirahashi, Cullere, & Mayadas, 2003; Kobayashi et al., 2003). One aspect of *C. albicans* pathogenicity is related to its ability to form biofilms (Kumamoto & Vinces, 2005). Utilizing a quantitative viability assay (XTT), it was shown that PDL cells

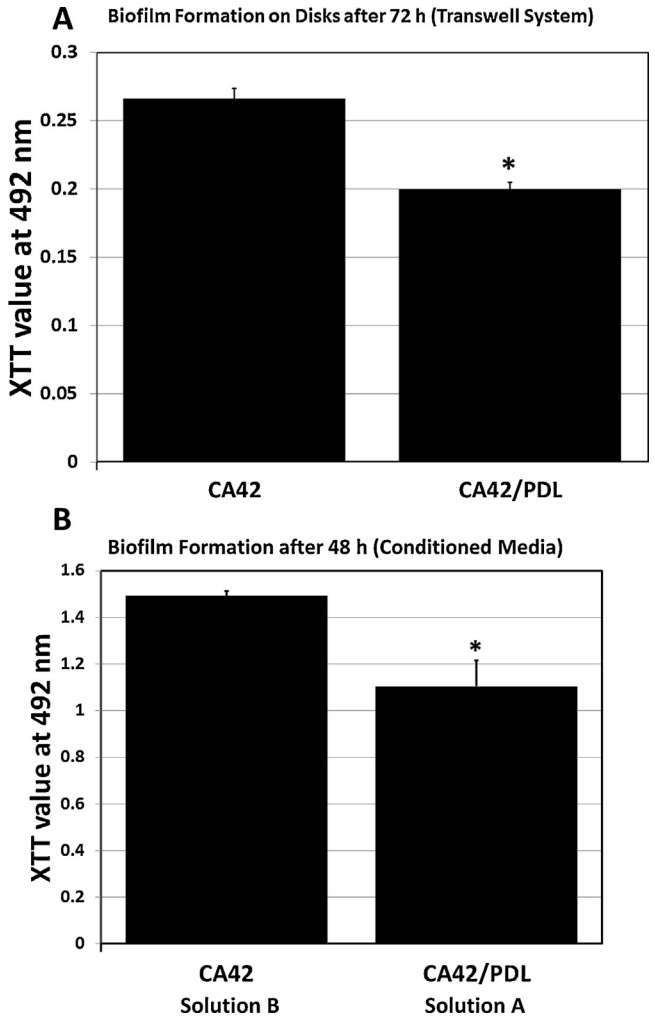

Fig. 4. (A) Metabolic activity (XTT) of *C. albicans* biofilms formed after migration through transwell system for 72 h on SE disks in the absence or presence of PDL cells ($n = 6$). (B) Metabolic activity (XTT) of *C. albicans* biofilms formed after 48 h on SE disks submerged in solution A (PDL cells stimulated by *C. albicans*, CA42); or solution B (*C. albicans* alone, CA42) for one hour ($n = 6$). Metabolic activity is presented as an optical density at 492 nm. $^{*}P$ value of groups compared was significantly different at $\alpha = 0.05$.

limited *C. albicans* biofilm formation. The CLSM and SEM analysis was conducted to visualize the morphology and architecture of biofilms. It was clear that the presence of PDL cells resulted in less mature biofilms over SE disks. While the mechanism of how PDL cells limited biofilm formation is yet unknown, it is clear that competent PDL cells promote resistance to *C. albicans* biofilm formation.

Although, *C. albicans* can cause infectious lesions in yeast and filamentous forms, studies have shown that its ability to form biofilm is a key factor to host invasion and tissue destruction (Kurzai, Schmitt, Brocker, Frosch, & Kolb-Maurer, 2005; Farrell, Hawkins, & Ryder, 1983; Hausauer, Gerami-Nejad, Kistler-Anderson, & Gale, 2005). Several studies have indicated that Efg1 and/or Cph1 transcription factors of *C. albicans* are involved in the biofilm formation (Kumamoto & Vinces, 2005). The strain CA80, used in herein, lacks both genes and was used to validate model presented. XTT assay has been used as a viability assay of different organisms including mammalian cells, bacteria and fungi (Chandra et al., 2001; Scudiero et al., 1988; McCluskey, Quinn, & McGrath, 2005). In the present studies, PDL cells alone also metabolized XTT (Fig. 1). However, signals produced by *C. albicans* biofilm in the absence of PDL cells were significantly higher than that of biofilm formed by *C. albicans* co-cultured with PDL cells. These findings confirm that PDL cells exert anti-biofilm activity and perhaps down regulated Efg1 and/or Cph1 genes. The current model presented is able to test such hypothesis.

The current study shed some light on antifungal activity of PDL cells. By studying the interaction of PDL cells and *C. albicans*, it was demonstrated that PDL cells exerted a slow-down of *C. albicans* migration (Fig. 4A). Furthermore, results presented clearly demonstrated that elutes from such interaction caused a reduction in biofilm formation (Fig. 4B). It is plausible that PDL cells are able to change the environment by secreting inflammatory cytokines. IL-17 is proinflammatory cytokine secreted by a subset of CD4+ T helper cells, named Th17 cells. It has been shown that Th17 cells regulate host defense through neutrophil trafficking (Yu et al., 2007) and are involved in several inflammatory diseases including chronic lesions of human periodontal disease (Cardoso et al., 2009). Interestingly, the IL-17 receptor (IL-17R) is expressed by human PDL cells (Zhu et al., 2011), suggesting that PDL cells respond to Th17 cells of adaptive immunity to promote inflammation. It should be noted that Th17 cells are known to play a protective role against *C. albicans* infection in the oral cavity (Conti & Gaffen, 2010). PDL cells also secrete cytokines such as IL-10 and transforming growth factor-β (TGF-β), which are anti-inflammatory and necessary for tissue repair during the healing process (Colic et al., 2009; Deschner et al., 2000; Pinkerton et al., 2008). Therefore PDL cells may play an integral role in the resistance to *C. albicans* infection.

In conclusion, the present studies have shown that PDL cells limit the formation of biofilms by *C. albicans*. Furthermore, PDL cells were able to change the environment and inhibit migration of *C. albicans* to form biofilm. The current *in vitro* model presented will allow the conduction of further studies to understand the pathogenicity of *C. albicans* biofilms formation, as well as the host response.

Conflict of interest

The authors deny any conflicts of interest.

Ethical approval

None.

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