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RESEARCH ARTICLE

Anti-biofilm activity of antibody directed against surface antigen complement receptor 3-related protein—comparison of *Candida albicans* and *Candida dubliniensis*

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One sentence summary: Antibody directed against complement-receptor 3 related protein (CR3-RP) interfere with biofilm formation in Candida albicans and Candida dubliniensis.

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ABSTRACT

Candida albicans and *C. dubliniensis* are related yeasts that differ in the expression of virulence-associated proteins involved in adherence and biofilm development. CR3-RP (complement receptor 3-related protein) is one of the surface antigens expressed by *Candida* species. The main objective of this research was to elucidate the effect of the polyclonal anti-CR3-RP antibody (Ab) on adherence and the biofilm formed by *C. albicans* SC5314 and *C. dubliniensis* CBS 7987 and two clinical isolates *in vitro, ex vivo* and *in vivo*. A comparison of species, and of treated vs. non-treated with the anti-CR3-RP Ab showed a reduction in adherence (22%–41%) that was dependent on the time point of evaluation (60, 90 or 120 min), but did not prove to be species-dependent. Confocal microscopy revealed a decreased thickness in biofilms formed by both species after pre-treatment with the anti-CR3-RP Ab. This observation was confirmed *ex vivo* by immunohistochemistry analysis of biofilms formed on mouse tongues. Moreover, anti-CR3-RP Ab administration, 1 h post-infection, has been shown to promote larval survival compared to the control group in a *Galleria mellonella* infection model. Our data suggest a potential activity of the anti-CR3-RP Ab relevant to immunotherapy or vaccine development against biofilm-associated *Candida* infections.

Keywords: *Candida albicans*; *Candida dubliniensis*; CR3-RP; adherence; biofilm

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INTRODUCTION

Candida species (spp.) are a part of the normal human microbiota. *Candida dubliniensis* mostly colonizes the oral cavity and/or respiratory tract (Mahelová and Růžička 2017), especially in HIV-infected individuals (Coleman *et al.* 1997; Sullivan *et al.* 2004; Wahab *et al.* 2014), while *Candida albicans* is a common inhabitant of the gastrointestinal tract, urogenital tract and oral cavity (Sardi *et al.* 2013; Höfs, Mogavero and Hube 2016). Candidiasis is the most common global fungal infection (Sardi *et al.* 2013). *Candida albicans* has been isolated in more than 50% of candidiasis; however, the number of non-*albicans* spp. able to cause serious candidiasis has increased in recent years (Yapar 2014; Pu *et al.* 2015; Sandhu *et al.* 2017). Although *C. dubliniensis* is phylogenetically very similar to *C. albicans*, it differs in some genes, especially those coding for virulence-associated proteins. *Candida dubliniensis* lacks more than 168 genes characteristic of its 'yeast-cousin' *C. albicans* (Jackson *et al.* 2009), the majority of them encoding proteins related to the yeast-to-hyphae transition, tissue invasion or biofilm development (Moran *et al.* 2004; Jackson *et al.* 2009; Moran, Coleman and Sullivan 2012). Moreover, *C. dubliniensis* manifests a higher predisposition to develop resistance to fluconazole (Sullivan *et al.* 1995; Moran, Coleman and Sullivan 2012; Jordan *et al.* 2014). On the other hand, both *C. albincans and C. dubliniensis* are able to form a biofilm (Sullivan *et al.* 2004; Borghi *et al.* 2014). Adherence is the first and most crucial step in biofilm development, and various surface antigens participate in this process (Chaffin 2008; Gow and Hube 2012; Hebecker *et al.* 2014). CR3-RP (complement receptor 3-related protein) is one of the cell surface antigens of *Candida* spp. with functional and structural similarity to the human complement receptor 3 (CR3) expressed on neutrophils, macrophages and monocytes. CR3-RP has been demonstrated to bind human complement fragment iC3b and to mediate leukocyte diapedesis (Heidenreich and Dierich 1985; Bujdáková *et al.* 1997). Additionally, CR3-RP seems to be an important immunogenic mannoprotein participating in adhesion and biofilm development (Bujdáková *et al.* 2008, 2010). A fragment of CR3-RP was sequenced (DINGGGATLPQ), and according to this sequence, CR3-RP was categorized into the DING protein family (named after DINGGG N termini) (Bujdáková *et al.* 2008; Bernier 2013). Some other surface proteins contributing to biofilm development have been described, such as Eap protein, the Als protein family, the Hwp1 or MP65 proteins (Gomez *et al.* 1996; Nailis *et al.* 2010; Finkel and Mitchell 2011; Araújo, Henriques and Silva 2017). Additionally, antibodies generated after the immunization of animals with some of the above proteins seems to be promising in tools focused on fighting yeast infections (Fujibayashi *et al.* 2009; Mishra, Ali and Shukla 2015; Torosantucci *et al.* 2017). Recent studies showed that antibodies targeting Als3 (Coleman *et al.* 2009), MP65 (De Bernardis *et al.* 2007) or another 42.7 kDa unnamed surface antigen in the *Candida* cell wall (Mishra, Ali and Shukla 2015) decreased adhesion and biofilm formation.

This study is the continuation of a previous work (Bujdáková *et al.* 2008, 2010) investigating the role of CR3-RP in *Candida* adherence, the first stage of biofilm development, and provides new information about the therapeutic potential of the anti-CR3-RP antibody (Ab). This work tried to obtain knowledge about the role of CR3-RP and anti-biofilm properties of the anti-CR3-RP Ab against *C. albicans* and *C. dubliniensis*. The results also assessed the virulence power of *C. albicans* and *C. dubliniensis in vitro*, *ex vivo* on mouse tongue biofilms and *in vivo*, in the *Galleria mellonella* model of invasive candidiasis.

MATERIALS AND METHODS

Candida strains and growth conditions

Clinical isolates and reference strains of *C. albicans* and *C. dubliniensis* used in experimental research are included in Table 1. All strains were preserved at –80°C in 1 mL of yeast extract peptone dextrose broth (YPD broth, 1% yeast extract, 2% peptone, 2% D-glucose; all from Biolife, Milan, Italy) supplemented with 30% sterile glycerol (Centralchem, Bratislava, Slovakia). Afterwards, strains were subcultured on an YPD agar plate (YPD medium supplemented with 2% agar, Biolife, Milan, Italy). Prior to experimental work, strains were cultured on selective CHROMagar Candida (Becton, Dickinson, Germany). The correct identification of all strains was confirmed by specific PCR (Tamura *et al.* 2001) discriminating between *C. albicans* and *C. dubliniensis*. The protocol and results are in Supplementary material 1, Supporting Information. For some experiments, two reference strains (*C. albicans* SC5314 and *C. dubliniensis* CBS 7987) and two clinical isolates (*C. albicans* H2 and *C. dubliniensis* 29) were selected.

CR3-RP expression and quantification

The polyclonal antibody anti-CR3-RP (anti-CR3-RP Ab) was used (Bujdáková *et al.* 2008) in the experiments to block CR3-RP function. The antibody was diluted 1:100 in sterile phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4, all chemicals from AppliChem, Darmstadt, Germany) freshly prepared before each experiment.

The presence of CR3-RP was determined in yeast lysates by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and by western blot using an appropriate dilution

Table 1. List of tested strains and their origin.

Strain	Origin
Candida albicans SC5314	Reference strain (Gillum, Tsay and Kirsch 1984)
Candida albicans H1	Clinical isolate (HIV + patient, Slovakia[a])
Candida albicans H2	Clinical isolate (HIV + patient, Slovakia[a])
Candida albicans H3	Clinical isolate (HIV + patient, Slovakia[a])
Candida albicans H4	Clinical isolate (HIV + patient, Slovakia[a])
Candida dubliniensis CBS 7987	Reference strain (Centraal Bureau voor Schimmelcultures, Delft, The Netherlands)
Candida dubliniensis CD 36	Clinical isolate (HIV + patient, Dublin, Ireland, kindly provided by prof. Derek Sullivan)
Candida dubliniensis 29	Clinical isolate (HIV + patient, Slovakia[a])
Candida dubliniensis 9382/1	Clinical isolate (HIV + patient, Slovakia[a])
Candida dubliniensis 11663/2	Clinical isolate (HIV + patient, Slovakia[a])

[a]Clinical isolates were obtained from the Clinic of Infectology and Geographical Medicine, University Hospital Bratislava, Slovakia.

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of anti-CR3-PR Ab. CR3-RP expression was quantified using an Enzyme-linked immunosorbent assay (ELISA). These experiments were performed according to a previously described procedure (Bujdáková et al. 2008; Paulovičová et al. 2015). The detailed protocol and results are described in Supplementary material 2, Supporting Information.

Effectiveness of anti-CR3-RP Ab on adherence and biofilm formation *in vitro*

All strains were tested for biofilm-forming ability by XTT reduction assay (Ramage et al. 2001; Li, Yan and Xu 2003). The detailed protocol and results are provided in Supplementary material 3, Supporting Information. After the initial screening, the reference strains *C. albicans* SC5314 and *C. dubliniensis* CBS 7987 and two clinical isolates, *C. albicans* H2 and *C. dubliniensis* 29, were selected for further experiments.

One loopful of single colonies was transferred to 20 mL of YPD broth and cultivated for 16 h at 37°C with gentle shaking (100 rpm, Orbital Shaker OS-20, Biosan, Riga, Lithuania). Cells were harvested by centrifugation (5 min, 3000× g, 15°C, Universal 32 R Hettich Zentrifugen, Tuttlingen, Germany), followed by two-step washing with 20 mL of PBS. Pellets were then resuspended in 10 mL of PBS, counted using a hemocytometer and adjusted to a final concentration of 1×10^7 cells/mL. One milliliter of the obtained suspensions were centrifuged, and the pellets were first incubated with 100 μL of 10% gelatine (Oxoid, Hampshire, UK) in PBS (v/v) at room temperature (RT) with shaking (130 rpm). After 1 h, cells were washed twice with PBS and pre-incubated with the anti-CR3-RP Ab. The control sample was prepared by PBS treatment without antibody. All suspensions were incubated for 1 h at 4°C with shaking (130 rpm, 5 min), and then washed twice with 100 μL of PBS. After washing, cells were adjusted to a density of 1×10^6 cells/mL in fresh RPMI-MOPS medium—Roswell Park Memorial Institute 1640 medium (RPMI 1640 medium, Biowest, Nuaillé, France) without phenol red, supplemented with 2% D-glucose and buffered to pH 7.0 with 0.165 M morpholinopropane sulfonic acid (MOPS, Serva, Heidelberg, Germany).

The kinetics of adhesion were assessed in polystyrene 96-well plates (Sarstedt, Nümbrecht, Germany) at five selected time points (0, 30, 60, 90, 120 min), according to the protocol of Sohn et al. (2006) with some modifications. Briefly, 100 μL aliquots of suspensions (treated with Ab and untreated) were pipetted to 96-well plates and evaluated for adherence (at 37°C). At every time point, non-adherent cells were removed by two washing steps with PBS. The viability of adherent cells was evaluated by measuring their ability to reduce 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT, Sigma-Aldrich, St. Louis, USA) to water-soluble formazan—XTT reduction assay. Briefly, 100 μL of XTT solution (0.5 mg mL^{-1} of XTT; 1 μM Menadion, Sigma-Aldrich, St. Louis, USA) was added in each well and incubated for 1 h at RT in the dark. The metabolic activity of cells was measured at 490 nm with a microplate reader (MRX II, Dynex, USA). Results were calculated as a mean value ± standard deviation (SD) from at least five parallel wells and from two independent experiments.

Biofilm-forming ability was assessed according to the modified protocol described by Li, Yan and Xu (2003). Yeast cell suspensions (treated with Ab and untreated), prepared as described above, were adjusted to a final concentration of 1×10^6 cells/mL in fresh RPMI-MOPS medium. An aliquot of 100 μL/well was then seeded into 96-well plates (flat bottom, Sarstedt, Nümbrecht, Germany) and incubated for 90 min at 37°C. After the adherence phase, non-adherent cells were removed and adherent cells were washed twice with PBS. Adherent cells were then overlaid with 100 μL of fresh RPMI-MOPS medium and incubated at 37°C for a further 48 h. The viability of the mature biofilm was evaluated by XTT reduction assay as described above. Each experiment was performed in five parallel wells and performed twice. Data were expressed as mean values ± SD.

Confocal laser scanning microscopy

Fungal biofilms of *C. albicans* SC5314 and *C. dubliniensis* CBS 7987, developed from yeast suspensions treated or untreated with anti-CR3-RP Ab, were formed on 13-mm-diameter round coverslips (Sarstedt, Germany) placed in a 24-well plate. Biofilm-forming conditions were the same as for the *in vitro* biofilm assay. After incubation at 37°C for 48 h, coverslips were gently washed with PBS, stained with Calcofluor White (0.05% v/v; Sigma Aldrich, Germany), and mounted on a glass coverslip for confocal laser scanning microscopy (CLSM) visualization (TCS SP2, Leica, Wetzlar, Germany). Serial sections in the x-y plane were obtained along the z-axis. Three-dimensional reconstructions of the biofilm were obtained using the instrument's software.

Ex vivo biofilm assay on mouse tongue

Reference strains *C. albicans* SC5314 and *C. dubliniensis* CBS 7987 were selected for this experiment. Experiments were performed according to the protocol previously described by Černáková et al. (2015). The yeast cell suspension was pre-incubated with 100 μL of anti-CR3-RP Ab as described above. Afterward, biofilms were formed on the tongues of BALB/c female 7–8 weeks mice (Charles River, Germany) for 72 h. Mouse tongues from healthy animals were incubated, immediately after extraction, in a Petri dish in 4 mL of RPMI-MOPS medium for 90 min at 37°C with shaking (100 rpm) in the presence of the yeast suspension (1×10^7 cells/mL). After the adhesion phase, tongues were washed once with PBS to remove non-adherent cells. Finally, tongues were transferred to a wet chamber and incubated at 37°C in the presence of 5% CO_2 for a further 72 h. Afterward, the tongues were transferred into sterile 1.6 mL cryotubes (Sarstedt, Nümbrecht, Germany) overlaid with 1 mL of Cryomount medium (Histolab AB, Askim, Sweden), and stored in liquid nitrogen until use. Before staining, tongues were cut in a cryostat (Opticon, Carl Zeiss, Jena, Germany) and 14 μm sections were placed on Superfrost Plus slides (Gerhard Menzel GmbH, Brunswick, Germany) and stained with Periodic Acid Schiff (PAS, Diapath, Martinengo, Italy). Several sections from each tongue were evaluated using light microscopy (Carl Zeiss Microscope AxioLab.A1, Jena, Germany). Two independent experiments were performed with two replicates for each sample.

Galleria mellonella infection model

Sixth instar *G. mellonella* larvae were purchased from Allevamento Cirà (Como, Italy). Larvae were stored in wood shavings in the dark at 18°C until the experiment, and used within 1 week of being delivered. Sixteen randomly selected *G. mellonella* larvae (weight range 200–300 mg) were used per experimental group in all assays. Experiments were performed with the reference strains *C. albicans* SC5314 and *C. dubliniensis* CBS 7987 as well as with the clinical isolates of *C. albicans* H2 and *C. dubliniensis* 29. The tested anti-CR3-RP Ab was diluted 1:100 in PBS. Larvae

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were inoculated through the last left proleg using a Hamilton syringe (Hamilton Company, Reno, USA). Multiple injections were performed through different prolegs. The killing assay was performed as previously described (Fuchs *et al.* 2010; Borghi *et al.* 2014). The experimental groups were as follows: larvae inoculated with 2×10^5 yeast cell/larva and 1 h after infection with the anti-CR3-RP Ab; larvae inoculated with 2×10^5 yeast cell/larva and 1 h after infection with PBS; larvae inoculated with 10 μL of the anti-CR3-RP Ab (to evaluate toxicity); larvae inoculated with PBS (to monitor the injection trauma); untouched larvae (negative control). Caterpillars were then incubated at 37°C in plastic Petri dishes (Sarstedt, Nümbrecht, Germany) for 9 days and scored daily for survival. Larvae were considered dead when they displayed no movement in response to touch. Killing experiments were performed twice.

Statistical analyses

Results were evaluated by statistical analysis using one-way ANOVA (analysis of variance, free online statistical software available at http://in-silico.net/). Differences were considered statistically significant at $P < 0.05$(*), highly significant at $P < 0.01$ (**) and extremely significant at $P < 0.001$ (***).

The *G. mellonella* killing assay was analyzed using a log rank test, and Hazard Ratio (HR) was used as the descriptive measure (GraphPad Prism 7).

RESULTS AND DISCUSSION

Expression and quantification of CR3-RP protein

Candida albicans and *C. dubliniensis* express many antigens that are important virulence factors (Jackson *et al.* 2009; Hoyer and Cota 2016). CR3-RP is assumed to be one of the cell surface mimicry proteins participating in the immune response as well as in adherence during biofilm formation (Bujdáková *et al.* 2008, 2010). Moreover, its synthetically derived analog seems to represent a promising immunological tool not only for *Candida* serodiagnostics, but also an interesting target for antifungal therapy (Paulovičová *et al.* 2015). While the presence of CR3-RP in *C. albicans* has been already demonstrated, as well as in *C. glabrata*, *C. krusei* and *Saccharomyces cerevisiae* (Bujdáková et al. 1997; Paulovičová *et al.* 2015), data on *C. dubliniensis* are lacking. Magee *et al.* (2008) reported that *C. dubliniensis* harbors only 29 specific genes compared to *C. albicans*, which has 168 unique genes for this species. However, both species have been shown to be closely related in the expression of proteins involved in adhesion and biofilm development (Sullivan *et al.* 2004; Magee *et al.* 2008; Jackson *et al.* 2009).

In our study, the presence of CR3-RP was confirmed by SDS-PAGE followed by western blot in all analyzed *C. dubliniensis* strains (Supplementary data 2, Supporting Information; Fig. S2A and S2B, Supporting Information). The ELISA test revealed that CR3-RP expression is strain-dependent and not species-dependent (Supplementary data 2, Supporting Information; Fig. S2C, Supporting Information). The most significant expression was determined for the reference strain *C. albicans* SC5314; its expression was 50% higher ($P < 0.05$) than the *C. dubliniensis* CBS 7987 reference strain. The clinical isolates of *C. albicans* H2 and *C. dubliniensis* 29, used in the further experiments, were also selected on the basis of CR3-RP expression, and were found to be very similar to the one of *C. albicans* SC5314. Differences in protein expression among *Candida* isolates have already been reported (Bujdáková, Lell and Gruber 1999). Additionally, Alaei *et al.* (1993) suggested differences in the expression of the CR3-RP with respect to the morphological forms of *Candida* cultivated at 30°C, with higher expression of CR3-RP in the mycelial form. The observations were also later confirmed by Bujdáková *et al.* (1997).

Effect of the anti-CR3-RP Ab on adherence and biofilm development *in vitro*

Previous studies revealed that *C. albicans* pre-treated with the anti-CR3-RP Ab is not able to sufficiently adhere to a polystyrene surface, and to buccal epithelial cells, and to form a biofilm *in vitro*. It was postulated that this effect was achieved by an efficient CR3-RP blocking by polyclonal anti-CR3-RP Ab (Bujdáková *et al.* 2008, 2010). Taking into account this information, it was interesting to determine whether the anti-CR3-RP Ab is able to efficiently prevent adherence and biofilm development in both *C. albicans* and *C. dubliniensis*. At first, all strains were screened for the ability to form a biofilm (Supplementary data 3, Supporting Information; Fig. S3, Supporting Information). It was observed that *C. albicans* were stronger biofilm producers than *C. dubliniensis* isolates. These observations were also proved by many other studies comparing the adhesion and biofilm development of *C. albicans* and *C. dubliniensis* (Ásmundsdóttir *et al.* 2009; Kolecka *et al.* 2011; Jordan *et al.* 2014).

The kinetics of adhesion (Fig. 1), determined by incubating yeast cells with/without anti-CR3-RP Ab, revealed that the *C. albicans* SC5314 reference strain and *C albicans* and *C. dubliniensis* clinical isolates were able to occupy a polystyrene surface within 60 min (Fig. 1C and D). A prolonged incubation time (120 min) increased the saturation capacity of the *C. albicans* SC5314 reference strain (Fig. 1A). At this time point, no differences in adherence capability between clinical isolates and reference strains were observed. On the other hand, the *C. dublinienis* CBS 7987 reference strain (Fig. 1B) needed a longer time to achieve a similar saturation to that of the other strains. A comparison of the strains treated and non-treated with the anti CR3-RP Ab showed a maximal reduction in *C. albicans* SC5314 (30% in 90 min) and *C. dubliniensis* (41% in 90 min). For clinical isolates, the reduction was 22% (in 60 min) for *C. albicans* H2 (Fig. 1C) and 27% (120 min) for *C. dubliniensis* 29 (Fig. 1D). These results corresponded to an evaluation of the biofilm. The next research compared a biofilm developed by cells treated with anti-CR3-RP Ab with an untreated one. Significant differences ($P < 0.05$ for all samples) were observed in the biofilm formed by the cells from the suspension pre-incubated with anti-CR3-RP Ab in all tested strains (Fig. 2). The maximum reduction was 60% for both clinical isolates H2 and 29. Differences were also observed in the standard strains. The reduction was 30% and 36% for *C. albicans* SC5314 and for *C. dubliniensis* CBS 7987, respectively. It is of interest that the maximal reduction in biofilm viability was observed in the strains that expressed a higher amount of CR3-RP (*C. albicans* SC5314 < *C. albicans* H2 < *C. dubliniensis* 29, Fig. S2C, Supporting Information). *Candida dubliniensis* CBS 7987 expressed the lowest amount of CR3-RP (Supplementary data, Fig. S2C, Supporting Information), thus the reduction was not significant. As previously reported by Bujdáková, Lell and Gruber (1999), a difference in the expression of the CR3-RP is associated with the ability of a yeast to switch to the mycelial form. Indeed, this aspect was further investigated in terms of *C. albicans* vs. *C. dubliniensis*. The CLSM visualization of biofilms formed by both reference strains is shown in Fig. 3. The *C. albicans* SC5314 biofilm was thicker (around 28 μm thick) and characterized mostly by the presence of hyphae and pseudohyphae (Fig. 3A). In contrast, the *C. dubliniensis* CBS 7987 biofilm was thinner (20 μm) and

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Figure 1. Effectiveness of anti-CR3-RP Ab on adherence at the beginning of biofilm formation. Figures represent the ability of the tested strains to adhere onto a polystyrene surface after treatment with the anti-CR3-RP Ab, compared with control samples treated with PBS. (**A**) *Candida albicans* SC5314, (**B**) *C. dubliniensis* CBS 7987, (**C**) *C. albicans* H2, (**D**) *C. dubliniensis* 29.

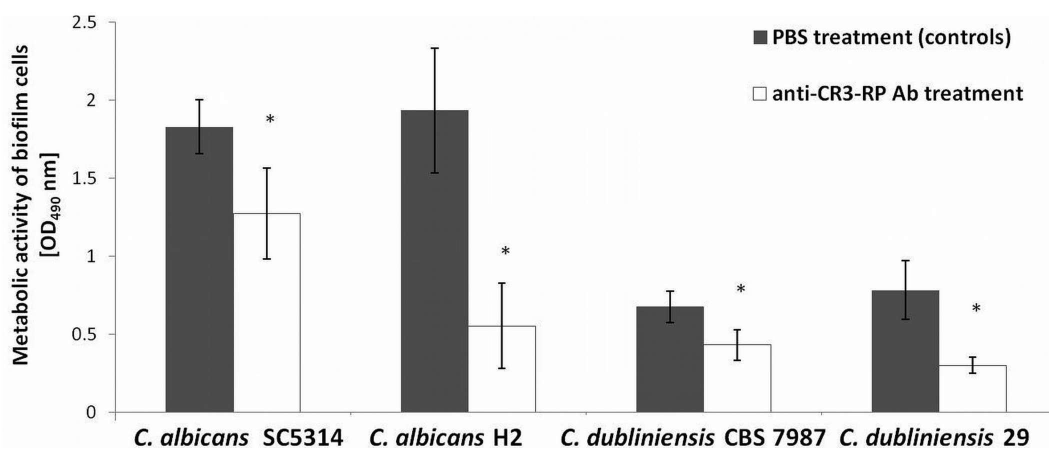

Figure 2. Effectiveness of anti CR3-RP Ab on development of biofilm formed by *C. albicans* SC5314, *C. albicans* H2, *C. dublinienis* CBS 7987 and *C. dubliniensis* 29. Yeast suspensions were pre-treated with the anti-CR3-RP Ab (light gray columns) and control samples with PBS (dark gray columns—controls) prior to the induction of biofilm formation. Differences were significant ($^*P \le 0.05$).

consisted mainly of yeast cells (Fig. 3B). Pre-incubation with the anti-CR3-RP Ab before biofilm formation resulted in a thickness reduction of about 36% and 40% for *C. albicans* SC5314 (Fig. 3C) and *C. dubliniensis* CBS7987 (Fig. 3D), respectively. Interestingly, despite differences in the morphological forms characterizing the biofilms, the proportional reduction was very similar. Previous studies on *in vitro* biofilms formed by *C. albicans* revealed that the addition of anti-CR3-RP Ab at the beginning of biofilm formation also significantly decreases biofilm viability (Bujdáková *et al.* 2008).

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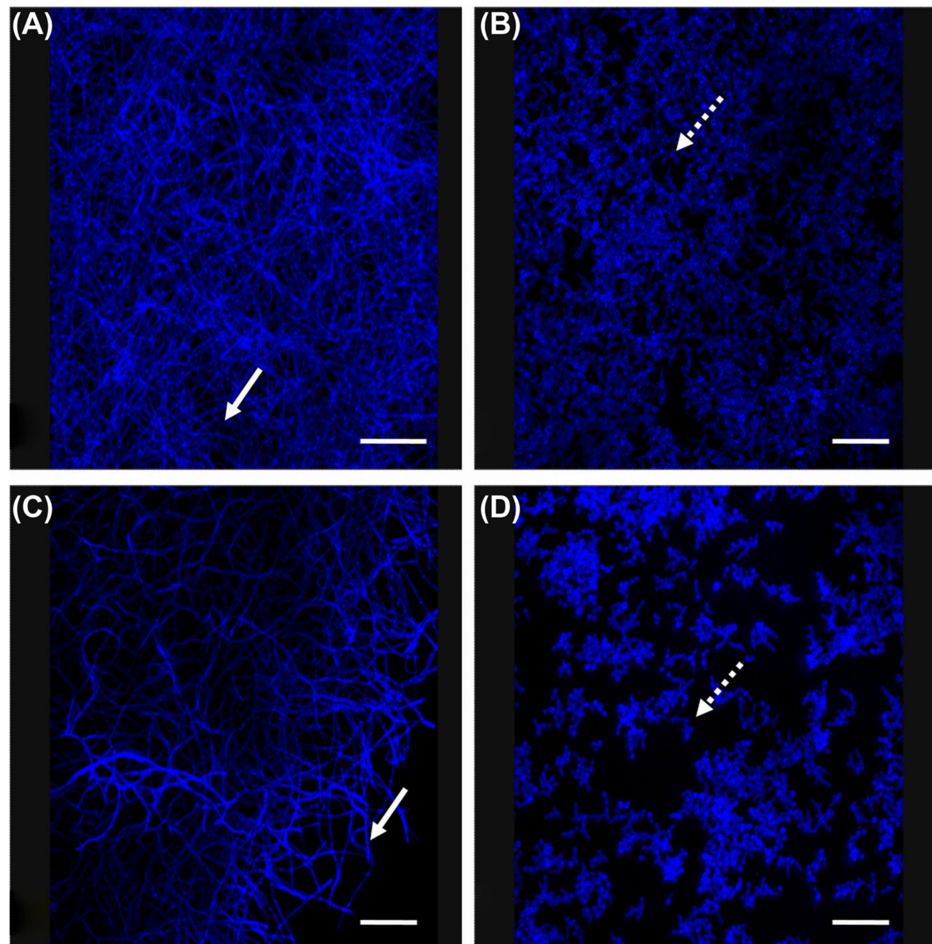

Figure 3. Confocal microscopy images of biofilms formed by *C. albicans* SC5314 (**A** and **C**) and *C. dubliniensis* CBS 7987 (**B** and **D**). *Candida albicans* suspensions were as follows: (A) PBS (control, thickness 28 μm); (C) anti-CR3-RP Ab (thickness 18 μm). *Candida dubliniensis* suspensions were pre-incubated with the following: (B) PBS (control, thickness 20 μm); (D) anti-CR3-RP Ab (thickness 12 μm). The full arrow points to the mycelial form and interrupted arrow points to the yeast form. Bars represent 20 μm.

A decrease in the adhesion of yeasts to human buccal epithelial cells after treatment with anti-CR3-RP Ab by about 35% was also observed (Bujdáková *et al.* 2010). The use of blocking antibodies targeting *Candida* surface proteins has been described in many studies as a promising tool for combating *Candida* infection (De Bernardis *et al.* 2007; Coleman *et al.* 2009; Torosantucci *et al.* 2017). The study by Gomez *et al.* (1996) described the cell wall mannoprotein MP65 of *C. albicans* to be an important protein participating in adhesion. Experiments using specific anti-MP65 antibodies demonstrated a decreased adhesion of *C. albicans* to vaginal epithelial cells (De Bernardis *et al.* 2007). Fujibayashi *et al.* (2009) also described the blocking of cell wall antigens involved in adhesion with three anti-*Candida* IgY antibodies produced in egg yolk. The authors also suggested a possible cross-reaction of antibodies with Als3 and Hwp1 proteins, resulting in a decrease in adhesion and biofilm formation (Fujibayashi *et al.* 2009). Additionally, Torosantucci *et al.* (2009) described the protective properties of the IgG2b isotype of an anti-β-glucan monoclonal antibody that was able to inhibit *in vitro* hyphal growth and adherence to human epithelial cells. It is of interest that CR3-RP was originally detected through an interaction with the monoclonal antibody OKM1, isotype IgG2b (Alaei *et al.* 1993; Bujdáková *et al.* 1997), also used for purifying the fragment of this protein that was subsequently administrated for the immunization of rabbits and collection of the polyclonal anti-CR3-RP Ab (Bujdáková *et al.* 2008).

Participation of CR3-RP in biofilm formed on mouse tongues

Results from *in vitro* investigations revealed the participation of CR3-RP in the adhesion and biofilm development of *Candida* spp. This was also confirmed by a newly developed model on mouse tongues (Fig. 4). This *ex vivo* model for biofilm studies was first described by Černáková *et al.* (2015). *Candida albicans* SC5314 and *C. dubliniensis* CBS 7987 were used for biofilm formation on tongues. As for *in vitro* experiments, the biofilm of *C. albicans* SC5314 (Fig. 4A) was thick and composed of hyphae or pseudohyphae, whereas the *C. dubliniensis* CBS 7987 biofilm was thinner and formed of yeasts. We observed a decrease in biofilm thickness after anti-CR3-RP Ab pre-treatment (Fig. 4C and D). The two species exhibited differences in invasiveness; with only *C. albicans* able to penetrate the inner layers of tongue tissue. This observation is in agreement with previous studies (Kolecka *et al.* 2011; Jordan *et al.* 2014). In particular, by using an *ex vivo* RHE 're-constituted human epithelium' model, Kolecka and coworkers showed that *C. dubliniensis* is unable to penetrate deeper into the tissue, and has a lower tendency to filament. The lower ability of

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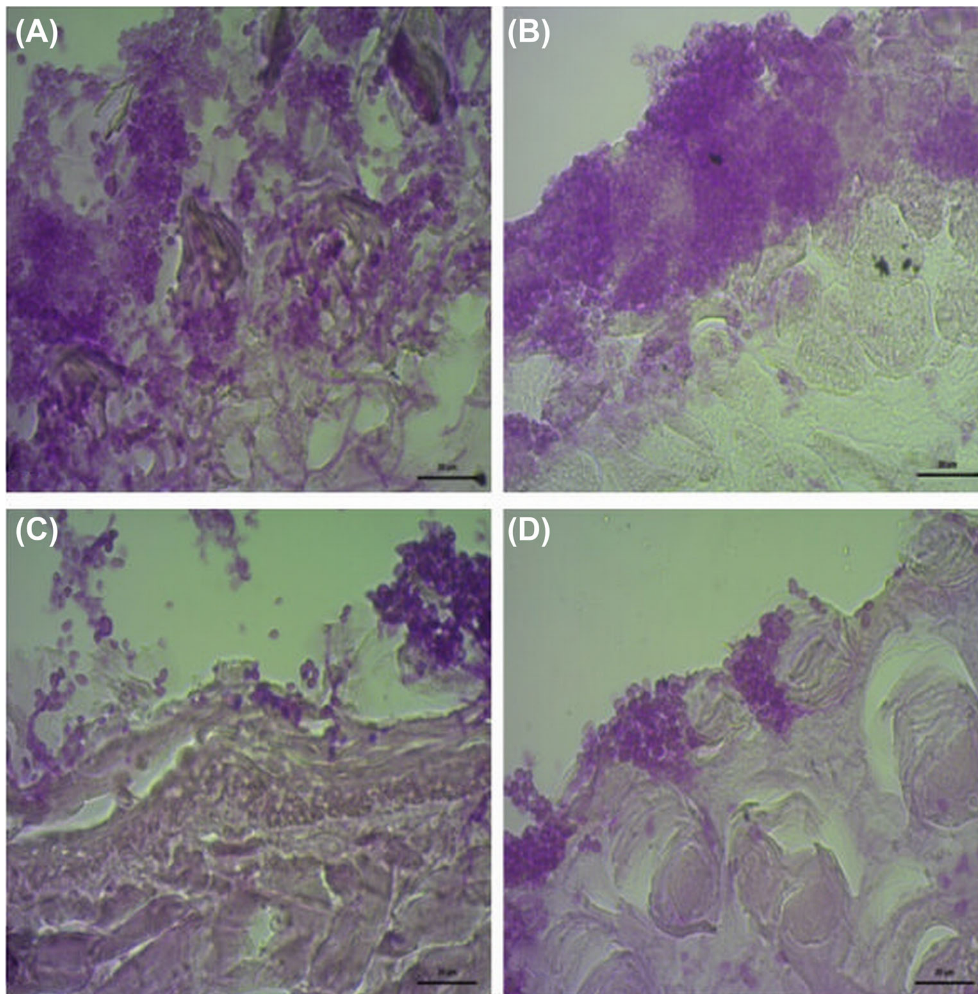

Figure 4. Cryo cut sections of mouse tongues after 72 h post biofilm-formation by *C. albicans* SC5314 (**A** and **C**) and *C. dubliniensis* CBS 7987 (**B** and **D**). *Candida albicans* suspensions were pre-incubated with the following: (A) PBS (control); (C) anti-CR3-RP Ab. *Candida dubliniensis* suspensions were pre-incubated with the following: (B) PBS (control); (D) anti-CR3-RP Ab. Bars represent 20 μm.

C. dubliniensis to form a biofilm could be mainly associated with the expression level of genes encoding the morphological transition. *Candida* albicans SC5314 exhibited the upregulation of hypha-specific genes within 30 min post-inoculation, resulting in a rapid induction of filamentation and increased RHE damage. *Candida* dubliniensis CD36 did not exhibit a detectable upregulation of hypha-specific genes, grew in yeast form and caused limited RHE damage (Spiering *et al.* 2010). Besides the differences in biofilm morphology, our study also confirmed the significant reduction in biofilm biomass after anti-CR3-RP Ab treatment.

Galleria mellonella survival assay

The use of alternative ethical issue-free models, like the invertebrate *G. mellonella*, for studying *Candida* pathogenesis and the effectiveness of antimicrobial compounds, seems to be very promising, because of the low cost, easy manipulation and the potential to provide a great deal of information, as evidenced by experiments (Borghi *et al.* 2014; Jacobsen *et al.* 2014; Rajendran *et al.* 2015; Maguire, Duggan and Kavanagh 2016). We initially tested whether the anti-CR3-RP Ab could be toxic for *G. mellonella* larvae. No differences in larval survival were observed compared with both PBS-injected and untouched control group larvae. After excluding any toxic effects, the anti CR3-RP Ab was further used as therapeutic treatment 1 h post-infection with *C. albicans* and *C. dubliniensis*. Killing curves demonstrated that anti-CR3-RP Ab treatment slightly improved the survival of larvae infected with both *C albicans* (HR 1.49, 95% confidence interval [CI] 0.7237–3.06) and *C. dubliniensis* (HR 1.41, 95% CI 0.6867–2.896) reference strains (Fig. 5A and B, respectively). The effectiveness of anti-CR3-RP Ab administration was also shown in larvae infected with the clinical isolates *C. albicans* H2 (HR 1.501, 95% CI 0.6474–3.481) (Fig. 5C) and *C. dubliniensis* 29 (HR 2.132, 95% CI 0.7449–6.1) (Fig. 5D).

The increase in survival correlated with strain-dependent differences in adhesion and biofilm formation capabilities. In agreement with the previous observation describing a decreased virulence of *C. dubliniensis* (Henriques, Azaredo and Oliveira 2006; Jordan *et al.* 2014), a higher survival of larvae was observed after infection with *C. dubliniensis*. We hypothesize that the increase in larvae survival, after treatment with the anti-CR3-RP Ab, could be caused by negating the functionality of CR3-RP via its blocking. Vilela *et al.* (2002) and Ásmundsdóttir (2009) used an *in vivo* model of systemic candidiasis in mice; microscopy analyses of autoptically collected organs revealed that *C. albicans* was more virulent and its biofilm was characterized by the presence

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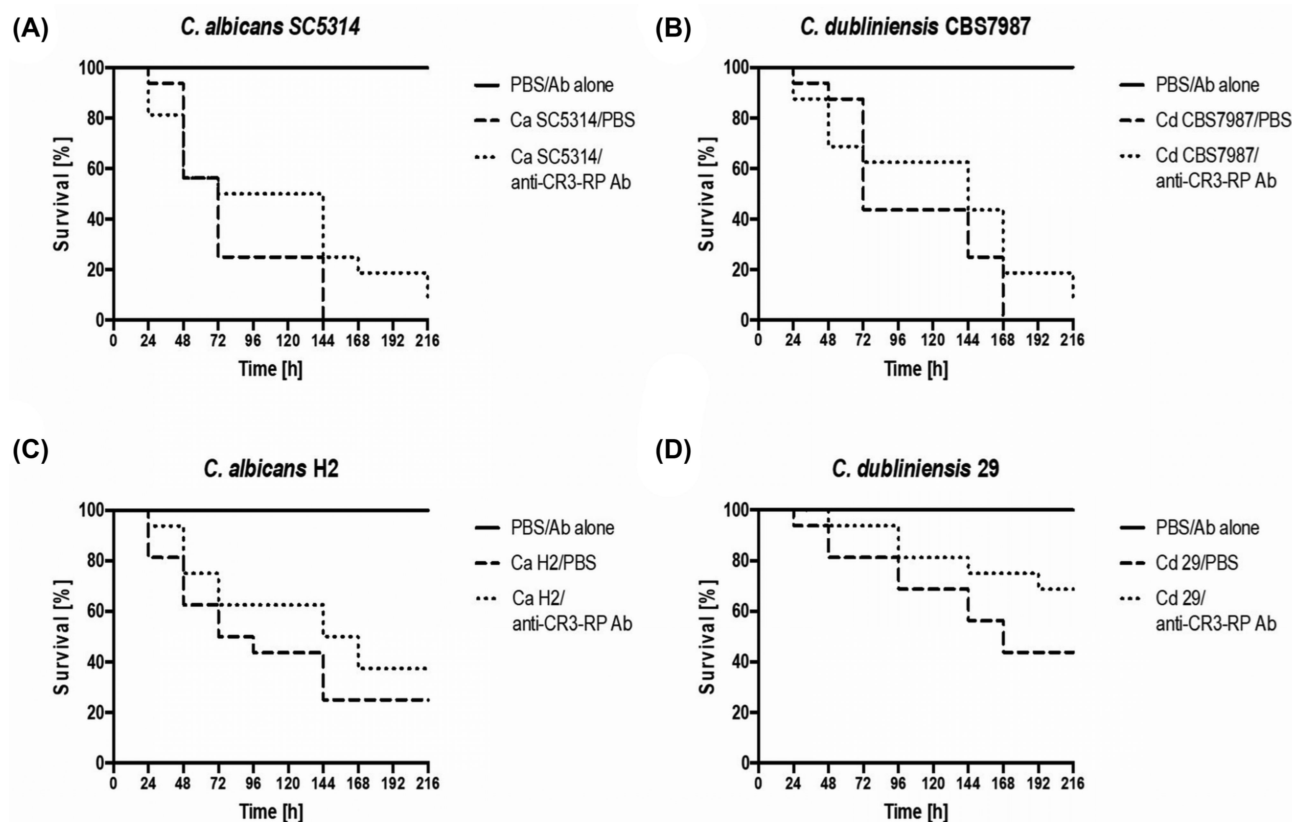

Figure 5. Kaplan–Meier curves showing the effect of anti-CR3-RP Ab-treatment on *G. mellonella* larval survival after *C. albicans* or *C. dubliniensis* systemic infection. (**A**) *C. albicans* SC5314, (**B**) *C. dubliniensis* CBS 7987, (**C**) *C. albicans* H2 and (**D**) *C. dubliniensis* 29. Larvae were primary inoculated with the yeast suspension and 1 h later treated with (i) PBS (control), (ii) anti-CR3-RP Ab.

of hyphae and pseudohyphae; in contrast, in the *C. dubliniensis* biofilm, only yeast cells were visible.

In conclusion, our results confirmed a lower virulence of *C. dubliniensis* compared with the phylogenetically similar *C. albicans*. The *Candida* surface protein CR3-RP was shown to actively participate in adhesion and biofilm development. The effectiveness of the anti-CR3-RP Ab on biofilm formation through a decrease in adhesion in both *Candida* spp. is evident. The precise mechanism of action is unfortunately still unclear. We hypothesize that the anti-CR3-RP Ab could covalently bind the CR3-RP protein in the *Candida* cell wall, affecting its function. Additionally, the obtained results with the anti-CR3-RP Ab in all models, and mainly *in vivo* in *G. mellonella*, suggest its possible use in immunotherapy or vaccine development against biofilm-associated *Candida* infections.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSPD online.

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Conflict of interest. None declared.

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