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Vaginal Clinical Isolates of *Candida albicans* Differentially Modulate Complosome Activation in Vaginal Epithelial Cells

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Abstract: In this in vitro study, we compared two clinical vaginal strains of *C. albicans*, a Colonizing strain from a healthy woman and a strain from a VVC patient, for their ability to activate the complosome and release anaphylatoxins in vaginal epithelial cells (VECs). The complosome controls different activities in innate immune cells and epithelial cells; however, its role in the response of VECs to *Candida* remains untested. Our results show that: i) both strains triggered cleavage of C3 into C3a and C3b within VECs, while infection with the Colonizing strain led to greater release of the anaphylatoxin C3a; ii) infection with the VVC isolate led to a strong reduction of both C5 and C5a in VECs, while no increase in C5a release was observed after infection with either strain; iii) Cathepsin-family gene expression and Cathepsin D activity were reduced in VECs infected with the VVC strain, but not in those infected with the Colonizing strain; iv) infection with the Colonizing strain induced a significant increase in intracellular C5aR1 while intracellular C3aR levels remained unchanged. Collectively, our data suggests the propensity of VVC strain to inactivate the C5/C5aR1 axis and to reduce the C3/C3aR axis, dampening the activity of the complosome in VECs. These effects exerted by the VVC strain suggest a novel strategy of immune evasion by *C. albicans* and may open new perspectives to find new therapeutic targets against vaginal fungal infections.

Keywords: complement; complosome; vulvovaginal candidiasis; *Candida albicans*; vaginal epithelial cells

1. Introduction

Vulvovaginal candidiasis (VVC) is an infection that affects approximately 75% of women worldwide, with no specific predisposing factors. Under certain specific circumstances, around 5-8% of women with VVC may experience recurrent forms (RVVC), consisting in 3-5 episodes of VVC per year [1]. *C albicans* is part of the human microbiota, and it normally dwells in the oral, gastrointestinal and genital tract [2]. VVC is predominantly caused by *C. albicans*, which plays a primary role in the development of the disease. However, non-*albicans Candida* species, such as *C. parapsilosis*, *C. glabrata*, *C. tropicalis* and *C. krusei* also trigger the disease [3]. Symptoms associated with VVC include vulvar itching, swelling, or redness, but they may progress to vulvar edema, fissures, excoriations, or thick, lumpy discharge. Symptoms cause stress and discomfort in affected women, influencing both their personal relationships and work life [4].

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A different immune response occurs in symptomatic patients with VVC compared to asymptomatic women, namely an increased neutrophilic infiltration with more fungal hyphae detectable in the vaginal swabs and with an increased exposure of β-glucan on such hyphal fragments [5]. This suggests that the immune response may polarize differently when the vaginal epithelium is in contact with pathogenic VVC-associated *C. albicans* strains versus Colonizing *C. albicans* strains [6]. By using an *in vitro* model including a monolayer of VECs, routinely used to mimic vaginal mucosa [6], our studies revealed a stronger tendency to induce fungal shedding and epithelial cell exfoliation in VVC-associated *C. albicans* strains compared to Colonizing *C. albicans* strains. Interestingly, our data also suggested that a selected Colonizing *C. albicans* strain (Ca 14314) differentially activates integrins, ferroptosis and type I interferon pathways in comparison with the VVC *C. albicans* strain (Ca 01887) [6]. However, the major mechanisms behind the differential responses in VECs induced by VVC-associated or Colonizing strains remain elusive.

The complement system is known to mediate immune responses to disseminated candidiasis, but its role in VVC onset has not been explored in depth. It has been recently demonstrated that following the estrogen-induced overexpression of the fungal cell surface protein Gpd2, the complement regulatory protein Factor H accumulates on the surface of fungal cells [7]. These results are in line with work showing that *C. albicans* can inhibit the complement system and its downstream effector functions by exposing FH-binding molecules (such as the moonlighting protein Hgt1), or by releasing Pra1 [8–12].

The complement system is a complex network of more than 30 proteins that can induce the killing of microorganisms by the formation of the terminal complement complex [11]. Activation of the complement system occurs via three main pathways: classical, lectin, and alternative [13–15]. These three pathways have a common purpose, which is the induction of C3 and C5 cleavage into the powerful anaphylatoxins C3a and C5a [16,17]. This cleavage is believed to take place largely in the extracellular space and the generated anaphylatoxins play pivotal roles in activating inflammation and acute immune response. Moreover, complement activation leads to the induction of the opsonin C3b and downstream products (C5b, C6 and C9). The latter form the membrane attack complex (MAC), that ultimately leads to microbial lysis [17,18]. Since the fungal cells are protected from the lysis by MAC thanks to the composition of their cell wall, C5, C3a and C3b become the main complement effectors in the context of antifungal defenses. The idea that these complement components play a pivotal role in antifungal defenses is strengthened by the increased susceptibility to systemic fungal infections in C3- and C5-deficient mice [19]. In addition, it has been demonstrated that during systemic candidiasis an efficient antifungal response is provided by C5a through the engagement of C5aR1 receptors [19].

Complement proteins are mainly produced in the liver, but they can also be secreted by circulating monocytes, macrophages, and neutrophils, as well as in tissues by endothelial cells, airway epithelial cells and fibroblasts [20–23], although in lesser amounts compared to hepatic production [14]. Recent work has highlighted the role of intracellular activation of the complement components C3 and C5 in the "complosome" [20,21,24]. The complosome has been shown to regulate cellular activities such as mitochondrial activity, glycolysis, and oxidative phosphorylation, which orchestrate and support cellular vitality [20]. Dectin-1, a CARD9-coupled C-type lectin receptor that recognizes *C. albicans*, has been demonstrated to trigger intrinsic C5a production in macrophages and to synergize with this complement component in mediating fungal killing [25]. In addition, the activation of intracellular C3aR and C5aR1 (located on mitochondria) by C3a and C5a complement fragments has been demonstrated to increase the levels of reactive oxygen species (ROS), thus leading to the activation of Nlrp3 inflammasome [26]. The C5a/C5aR1 axis can also modulate the immune response, promoting a more pronunced pro-inflammatory tendency in cells such as macrophages and making these cells active against pathogens [19,27,28].

In contrast to the established role of complement in systemic antifungal responses, the interplay between complement and fungi at mucosal surfaces is not completely understood. However, it has been shown that lung epithelial cell-derived C3 provides protection from lung injury during bacteria

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pneumoniae [29]. In this context, we analyzed the ability of *C. albicans* to activate complosome in vaginal epithelial cells (VECs), and to respond differentially to an infection with either a VVC or a Colonizing *C. albicans* strain. Here, we demonstrate that complosome activation, anaphylotoxin production and the presence of anaphylotoxin receptors occur in VECs, in the context of an in vitro vulvo-vaginitis model. Overall, these events suggest that *C. albicans* is responsible for complement activation, for C3a and C3b extracellular secretion, and for cell-associated C5a production by vaginal epithelium. Our data suggest that, following their activation, such complement components may directly activate epithelial cells through the anaphylotoxin receptors C3aR and C5aR1.

2. Materials and Methods

2.1. Fungal Strains and Cell Culture Conditions

The Colonizing strain of *C. albicans* (Ca 14314) and the VVC-associated strain of *C. albicans* (Ca 01887), referred throught the manuscript as Colonizing and VVC strain respectively, were isolated from vaginal swabs of a healthy colonized woman and a symptomatic VVC woman, as previously described [5]. These strains are already part of our *C. albicans* strain library. The isolated strains were kept in frozen stocks at –80 °C and every month were defrosted and maintained by a weekly passage onto Sabouraud Dextrose Agar (SDA) plates (Oxoid, Milan, Italy). Before each experiment, Colonizing and VVC strains were inoculated in Yeast Extract (1%)-Peptone (2%)-Dextrose 2% (YPD) broth (Condalab, Madrid, Spain) and incubated overnight at 37°C under agitation.

2.2. Vaginal Epithelial Cells (VEC)

The human A-431 cell line, from vaginal epithelial squamous cell carcinoma (ATCC CLR-1555™), was selected because it is routinely used to represent the vaginal epithelium [30]. The A-431 vaginal epithelial cells (VEC) were mantained in Dulbecco's Modified Eagle's Medium (DMEM) (Sial, Rome, Italy), supplemented with 10% of heat-inactivated fetal bovine serum (iFBS) (Capricorn Scientific, Ebsdorfergrund, Germany), Gentamicin (50 mg/mL) (Bio Whittaker, Verviers, Belgium), Streptamycin (2 mg/mL) (Sial), and L-glutamine (2 mM) (EuroClone, Milan, Italy). Before the experiments, the cells were seeded in a 24-well plate (Corning, Glendale, AZ, USA) (5x105 cells/well/mL) in DMEM plus 10% iFBS then incubated for 24 hr at 37 °C, with 5% CO_2 to promote the monolayer generation.

2.3. Antibodies

The monoclonal antibodies mouse anti-human complement component-5 (C5) (clone 10B6), mouse anti-human C5a/C5a des-arg (clone 2042) and the mouse anti-human C3b/iC3b/C3d (clone 1H8) were kindly provided by Hycult Biotech (Uden, Netherlands). The polyclonal antibody, goat anti-human complement component-3 (C3) was purchased from BioRad (Hercules, CA, USA). The monoclonal anti-human C3a/C3a des/arg antibody (clone K13/16) was obtained from MilliporeSigma (Merck KGaA, Darmstadt, Germany). The antibodies anti-C5 and anti-C3 were labelled using a FITC-Conjugation Kit (Abcam, Cambridge, UK). The antibodies anti-C5a/C5a des-arg, anti-C3b/iC3b/C3d and anti-C3a/C3a des/arg were labelled by an APC-Conjugation Kit (Abcam). The anti-C5aR1 antibody was purchased from Biolegend (San Diego, CA, USA). The anti-C3aR antibody was obtained from Miltenyi Biotech (Bergisch Gladbach, Germany).

2.4. Flow Cytometry Analysis

Flow cytometry analysis was performed to analyze the expression of the proteins C3, C5, C5a, C3a, C3b and the receptors C5aR1 and C3aR within VECs. The target proteins were detected in different flow cytometry panels. The VECs (5x105 cells/well/mL) were incubated in DMEM + 10% iFBS with Colonizing and VVC strains (MOI 1:10) for 4 hr at 37 °C, with 5% CO_2. The cells were then washed with PBS to remove unbound *C. albicans*, harvested and centrifuged to remove the residual fungi in the culture. Next, the cells were incubated with the Fixable Viability Dye eFluor 780 (1:6000)

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(Invitrogen, Waltham, MA, USA) and fixed by Fixation Buffer (Biolegend) for 20 min in the dark at Room Temperature (RT). Subsequently, the cells were permeabilized with True-Phos Perm Wash (Biolegend) for 20 min at RT, then incubated, for each flow cytometry measurement, in Perm Wash buffer including anti-human C5 (1:250), anti-human C5a/C5a des-arg (1:250), anti-human C3 (1:250), anti-human C3a/C3a des/arg (1:250), anti-human C3b/iC3b/C3d (1:250), anti-human C5aR1 (1:100) and anti-human C3aR (1:100) for additional 20 min at RT in the dark. The cells were then washed and resuspended in PBS and recorded by FACSsymphony A1 (Becton Dickinson, Franklin Lakes, NJ, USA). In order to exclude the presence of C5aR1 and C3aR on the cell membrane, in selected experiments, the above-described staining procedures were performed without a permeabilization step. The data analysis was carried out by means of FlowJo software (Becton Dickinson); in each measurement, 8000 events corresponding to single and living cells were included in the analysis. Due to the non-specific binding observed in intracellular staining using isotype controls, we alternated FMO (Fluorescence Minus One) controls or isotype antibody controls to identify the correct gating strategy for the detection of true positive populations. With this approach, we minimized artifacts and ensured an accurate identification of positively labelled cells.

2.5. ELISA Test for the Detection of C3a and C5a Detection in the Supernatant

The C3a and C5a levels were measured in cell culture supernatants from VECs stimulated with either Colonizing or VVC strain, as well as those from unstimulated cells. Supernatants were collected after 24 hr and centrifuged to remove all cells and cell debris. For detection of C3a and C5a, the BD OptEIA Human C3a ELISA Kit and BD OptEIA Human C5a ELISA kit were employed, respectively (BD Biosciences, Franklin Lakes, NJ, USA), in accordance with the Manufacturer's instructions and published elsewhere [31].

2.6. RNA Seqencing and Analysis

The RNA-seq analysis was performed as described by Sala et al [6] and using the already-published datasets. Briefly, VEC monolayers were infected with either Colonizing or VVC strain (MOI 1:1), as described above. Cell pellets were sent for RNA preparation and NGS analysis using Illumina paired-end sequencing at Eurofins (Eurofins, LLC) [32–38]. Gene expression data and sequence data are accessible at the NCBI Gene Expression Omnibus under accession number GSE207081. DEseq2 comparison of VECs infected with Ca 14314 versus with Ca 01887 was used to determine differential expression of cathepsin-family genes.

2.7. Cathepsin D Activity Analysis

Cathepsin D activity within VECs, infected with the Colonizing or VVC strains, was evaluated by the Cathepsin D Activity Assay Kit (Fluorometric) (Abcam), following the Manufacture's instructions. Briefly, VECs (5x105 cells/well/mL) were incubated with Colonizing or VVC strain in a ratio of 1:10 (VECs: C. albicans) for 4 hr or 24 hr at 37 °C, with 5% CO2 in DMEM + 10% iFBS. After incubation, the cells were harvested and washed with PBS, to remove C. albicans. Next, the cells were lysed by lysis buffer, and the lysates were incubated for 2 hr at 37 °C in the dark with a reaction buffer containing Cathepsin D substrate. The fluorescence emission, corresponding to the Cathepsin D activity, was recorded by means of a microplate reader (Thermo Fisher Scientific, Fluoroskan FL) using Ex/Em 328/460 nm filter. The data were analyzed by SkanIt software 5.0 (Thermo Fisher Scientific) and espressed as a percentage (%) of activity, as compared to uninfected VECs.

2.8. Statistical Analysis

Statistical analyses were carried out using GraphPad Prism 10 software (GraphPad, Boston, MA, USA). The Shapiro-Wilk test was used to analyze data distribution within each experimental group. Differences between experimental groups were analyzed by One-way ANOVA test followed by the uncorrected Fisher's LSD multiple comparison's test or by Kruskal Wallis test followed by the

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uncorrected Dunn's multiple comparison's test. Cathepsin D activity was analyzed by paired Student's t-test. Significance throughout the figures is indicated as follows: ns: not significant; significant increment: * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$; significant reduction: # $p \leq 0.05$; ### $p < 0.001$; #### $p < 0.0001$.

3. Results

A monolayer of VECs was incubated with cell culture medium, or with Colonizing or VVC *C. albicans* strains for 4 hr at 37 °C with 5% CO_2. Analysis of the complosome was performed by flow cytometry. The gating strategy was set to analyze only VECs, removing interference from *C. albicans* and considering only living single cells (Fig. S1).

First, we analyzed the presence of C3 and its cleavage fractions C3a and C3b within VECs. Our results show that appoximately half of the VECs analyzed were positive for C3 (49,8%) with a non-significant increase after infection with either Colonizing (63,2%) or VVC (63,8%) strains (Fig. 1a). The C3a-positive VEC cell fraction was low in uninfected cells (5,4%), and this fraction significantly increased after infection with both Colonizing (21,7%) and VVC (27,7%) strains. Interestingly, no differences in the fraction of C3a positive infected cells was observed relative to the strain used for the infection (Fig. 1b). Similarly, the fraction of C3b-positive VECs was low in uninfected cells (8,5%) and significantly increased after infection with both the Colonizing (29,9%) and VVC (20,3%) strain. However, a significantly lower fraction of C3b-positive VECs was detected in VECs infected with the VVC strain as compared to those infected with the Colonizing strain (Fig. 1c).

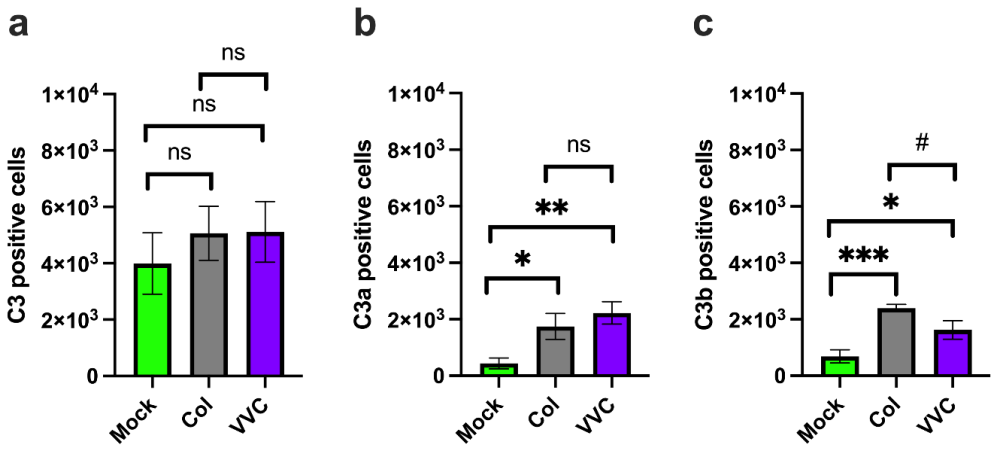

Figure 1. C3, C3a and C3b analysis. A monolayer of VECs was infected or not (Mock) with the Colonizing strain (Col) or the VVC strain (VVC) for 4 hr. After incubation, levels of C3 (a), C3a (b) and C3b (c) within VECs were evaluated by cytofluorimetric analysis. Data in the graphs show the mean ± SEM of C3, C3a and C3b positive cells (cell counts from 8000 live cells) obtained from 4 differents experiments. * $p \leq 0.05$; # $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Next, we measured C5 and C5a within VECs. Here, approximately half of the VEC analyzed were positive for C5 (54%), and this level was maintained upon infection with the Colonizing strain (47,6%). However, the amount of C5-positive VECs was significantly reduced upon infection with the VVC strain (17,9%), as compared to uninfected or Colonizing strain-infected VECs (Fig. 2a). In parallel, a significantly lower number of C5a-positive VECs were observed upon infection with the VVC strain, as compared to infection with the Colonizing strain (Fig. 2b).

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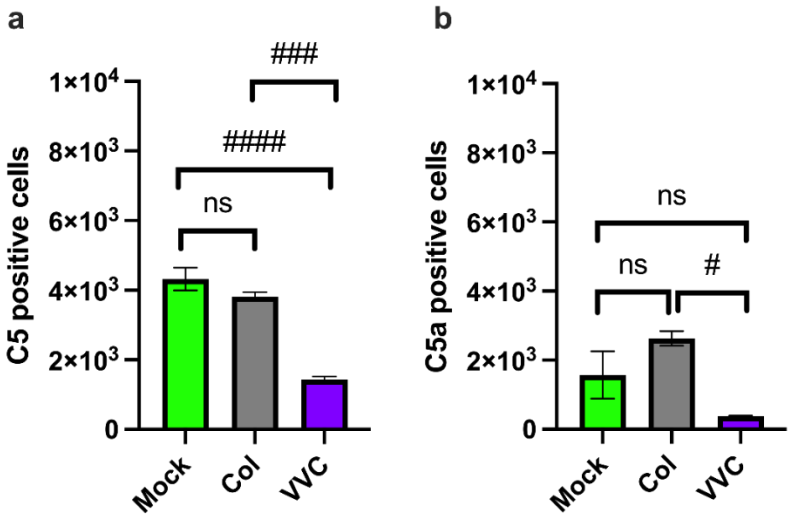

Figure 2. C5 and C5a analysis. A monolayer of VECs was infected or not (Mock) with the Colonizing strain (Col) or the VVC strain (VVC) for 4 hr. After incubation, the levels of C5 (a) and C5a (b) within VECs were evaluated by cytofluorimetric analysis. Data in the graphs show the mean ± SEM of C5 and C5a positive cells (cell counts from 8000 live cells) obtained from 3 differents experiments. # $p \leq 0.05$; ### $p < 0.001$; #### $p < 0.0001$.

An ELISA assay was performed on the supernatants from VECs uninfected or infected for 24 hr with the Colonizing or the VVC strain to assess the C3a and C5a release upon infection. The results showed a significant increase in the release of the anaphylatoxin C3a from VECs infected with Colonizing strain compared to uninfected cells or those infected with VVC strain. The analysis did not show any difference between uninfected VECs or VVC strain-infected VECs (Fig. 3a). No differences in C5a release were observed in the context of infection, either (Fig. 3b).

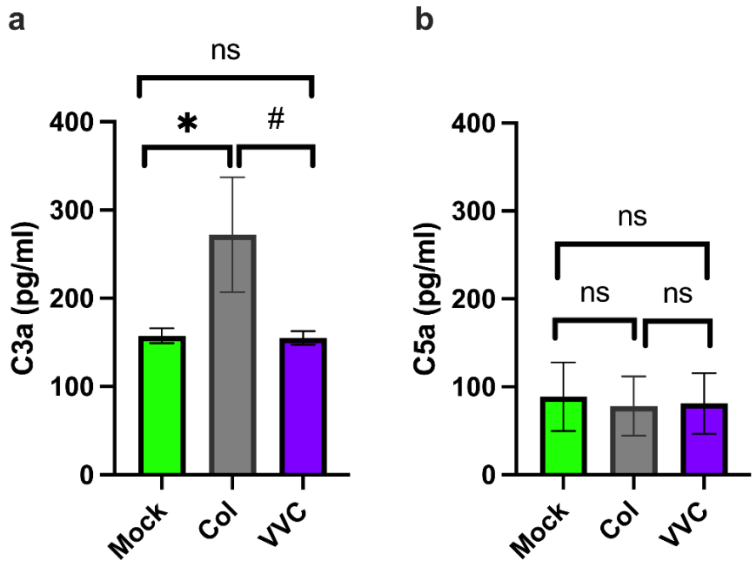

Figure 3. Extracellular release of C3a and C5a. A monolayer of VECs was infected or not (Mock) with the Colonizing strain (Col) or VVC strain (VVC) for 24 hr. After incubation, supernatants were collected and tested for extracellular release of C3a (a) and C5a (b) by specific ELISA kit. Data in the graphs show the mean ± SEM of extracellular C3a and C5a from 5 differents experiments. * $p \leq 0.05$; # $p \leq 0.05$.

To explain the tendency of the Colonizing strain to induce greater cleavage of C3 and C5 in VECs upon infection, RNAseq analysis of an already published dataset was performed [6]. The analysis

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was conducted on VECs infected with either the Colonizing or the VVC strain. We focused on the Cathepsin family, which is known to participate in the cleavage of intracellular complement components [14]. We found an overall trend towards down-regulation of the whole Cathepsin family, with significantly lower gene expression of Cathepsins B, D, K and S in VECs infected with the VVC strain compared to those infected with Colonizing strain (Fig. 4a). We then tested if lower Cathepsin expression is correlated with lower activity; in this context, Cathepsin D activity was directly measured in infected VECs [27]. Consistent with the gene expression results, we found a significant reduction of Cathepsin D activity in VECs infected with the VVC strain compared to cells infected with Colonizing strain after 4 hr and 24 hr (Fig. 4b).

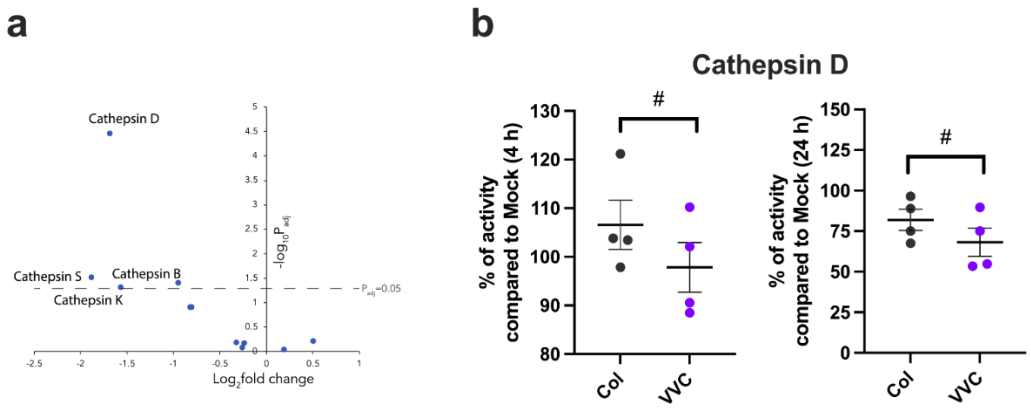

Figure 4. RNAseq analysis of Cathepsins genes and Cathepsin D activity. (a) Volcano plot of the $\log_2$ ratio of gene expression between VVC strain and Colonizing strain (x-axis) against the -$\log_{10}$-transformed adjusted p-value, as calculated in DESeq2, for each Cathepsin gene expressed above background (Cathepsins A, B, C, D, F, H, K, L, S, V and Z). (b) Cathepsin D activity in VECs infected with the Colonizing strain (Col) or VVC strain (VVC) after 4 hr or 24 hr. Data in the graphs in (b) show the mean ± SEM of the % of Cathepsin D activity in infected VECs as compared to uninfected, obtained from 4 different experiments. # $p \leq 0.05$.

The presence of cell-associated C3a, C3b, C5a and C5b suggests that there is activation of the complosome in infected VECs. To determine if the VECs can respond to elevated levels of intracellularly-produced complement components, we also analyzed the expression and the potential modulation of C3a and C5a intracellular receptors: C3aR and C5aR1. Our results show that the number of C3aR-positive VECs did not significantly increase upon infection with either Colonizing and VVC strains, as compared to uninfected VECs (Fig. 5a). In contrast, a significant increase of C5aR1 expression was observed after the infection with the Colonizing strain but not with the VVC strain (Fig. 5b). No VECs positive for membrane bound C3aR and C5aR1 were found (data not shown).

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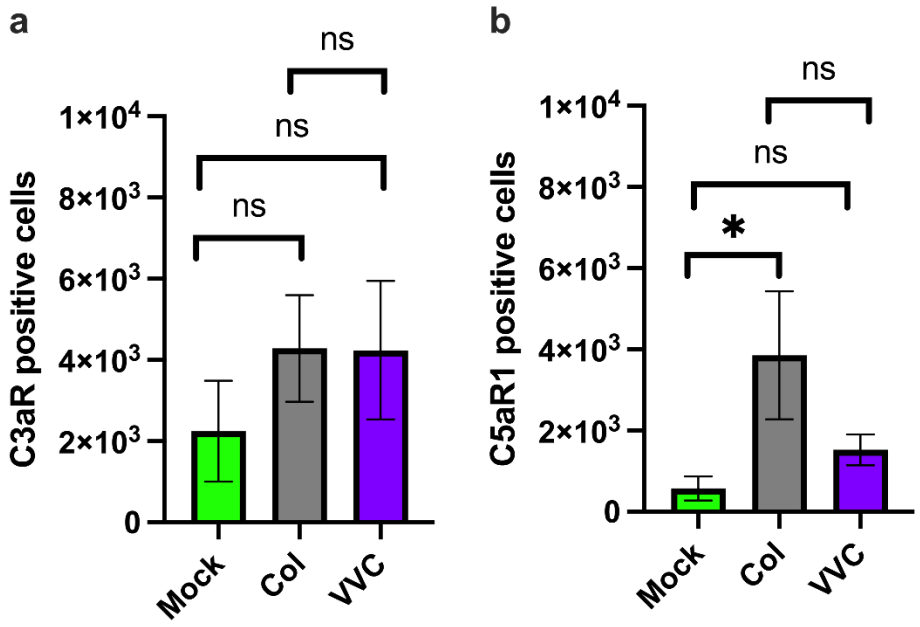

Figure 5. Intracellular C3aR and C5aR1 analysis. A monolayer of VECs was infected or not (Mock) with the Colonizing strain (Col) or VVC strain (VVC) for 4 hr. After incubation, intracellular C3aR (a) and C5aR1 (b) were analyzed by cytofluorimetric analysis. Data in the graphs show the mean ± SEM of C3aR and C5aR1 positive cells (cell counts from 8000 live cells) obtained from 4 different experiments. * $p \leq 0.05$.

4. Discussion

In this comparative study, we have used a Colonizing strain and a VVC strain of *C. albicans* that had been previously characterized and employed in an *in-vitro* infection model of VECs. These strains had been demonstrated to differentially activate specific VECs intracellular pathways (such as type I IFN pathway), fungal shedding and epithelial exfoliation [5].

In fungal infections, several components of fungal cell wall, such as β-glucans and galactomannans, have been reported to activate complement [39]. Nonetheless, several evasion strategies are adopted by fungal cells to avoid complement, such as binding to Factor H and secretion of proteases (such as Secreted Aspartyl Proteases by *C. albicans*) that cleave complement components, thus impairing complement function [39,40].

In the present work, the evaluation of VECs-associated C3 and of its cleavage products (i.e., the anaphylatoxin C3a and the opsonin C3b), in response to *C. albicans* infection, shows that the number of infected VECs positive to C3a is significantly higher than uninfected controls, irrespective of the Colonizing or VVC strain. These data indicate that VECs perceive *C. albicans* as a threat, and they respond by cleaving the complement component C3 to C3a that acts as a signal for the recruitment of neutrophils and other cells involved in the innate immune response. However, it appears that only the infection by the Colonizing strain enables VECs to trigger such recruitment, as demonstrated by the significantly higher levels of C3a detected in cells supernatant, compared to the levels of C3a in uninfected or VVC-infected VECs supernatants. Differently, the secretion of C3a is similar in VVC-infected and uninfected VECs, notwithstanding the significantly higher number of C3a positivity in VVC-infected cells. It follows that the VVC strain might alter the VECs perception of the threat represented by *C. albicans* infection, or alternatively it might block their response to such threat by degrading intracellular C3a or preventing its release. This effect could be due to the higher capacity of the VVC strain to release hydrolytic enzymes and will be investigated in future studies.

Moreover, the relatively low gene expression of some Cathepsins, which are closely related to the cleavage of intracellular complement components [27], together with the lower levels of intracellular C3b in VVC strain-infected VECs, is in line with this hypothesis.

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The number of infected VECs positive for the opsonin C3b (the second product of C3 cleavage), is higher in the VECs infected with either the Colonizing or VVC strains when compared to uninfected controls, even though the C3b+ fraction is significantly lower in VECs infected with the VVC strain as compared to those infected with the Colonizing strain. Since C3b is crucial for opsonization and fungal killing [9], this result could indicate that VECs retain their ability to counteract intracellular fungal invasion, upon infection with a Colonizing strain, whereas such capacity might be reduced upon infection with the VVC strain. Interestingly, given the lack of difference in the number of C3a positive infected VECs, we hypothesize that, in the VVC-infected VECs, C3b might be degraded more quickly. Indeed, as already mentioned and better detailed below, the secretion of Pra-1 and Secreted Aspartyl Proteases (Saps) enable *C. albicans* to degrade the complement proteins, suggesting that the VVC strain could release more of these fungal proteins, thus allowing increased degradation of complement fractions.

Concerning C5 (the complement component that generates the anaphylotoxin C5a once cleaved), a significant reduction of positive VECs to C5 is evident in the VVC strain-infected VECs. Interestingly, in the VVC-infected VECs, the number of C5a positive cells is significantly lower when compared to C5a positive VECs infected by the Colonizing strain. This result mirrors the observed down-regulation in the gene expression of several members of the Cathepsins family, particularly Cathepsin D but also Cathepsins A, B, C, F, H, K, L, S, V and Z in VVC-infected VECs. Moreover, in such cells a marked reduction in Cathepsin D activity was observed. Since the role of these enzymes in the cleavage of the complement components C3 and C5 is well established, our data point to the ability of the VVC strain to impair the cleavage of C5, likely achieved through the impairment of Cathepsin gene expression and activity. In addition, the levels of C5a in the supernatants of VECs does not change with infection with either strain. This finding could suggest a greater involvement of C5a in the intracellular compartment, while suggesting the pivotal role of C3a as an anaphylatoxin in promoting immune cell recruitment in response to fungal infection [41]. The differential activation of complosome is consistent with the enhanced ability of the VVC strain to cause epithelial exfoliation and block type I IFN activation [6]. Specifically, the Colonizing strain elicits more extracellular C3a release and more cell-associated C5a production, both of which are associated with greater activation and control of infection.

Finally, by analyzing the intracellular levels of the anaphylotoxins C3a and C5a receptors (C3aR and C5aR1), we showed that only the Colonizing strain significant increase intracellular C5aR1 while no significant increase in intracellular C3aR was observed after the infection with either strain. These results strenghten our idea that the Colonizing strain is more prone to stimulate the complosome and that the C5a/C5aR1 and C3a/C3aR axes seem to be partly compromised in VECs infected with the VVC strain. Figure 6 summarizes our hypothesis of complosome modulation in VECs by the Colonizing and VVC-associated strain.

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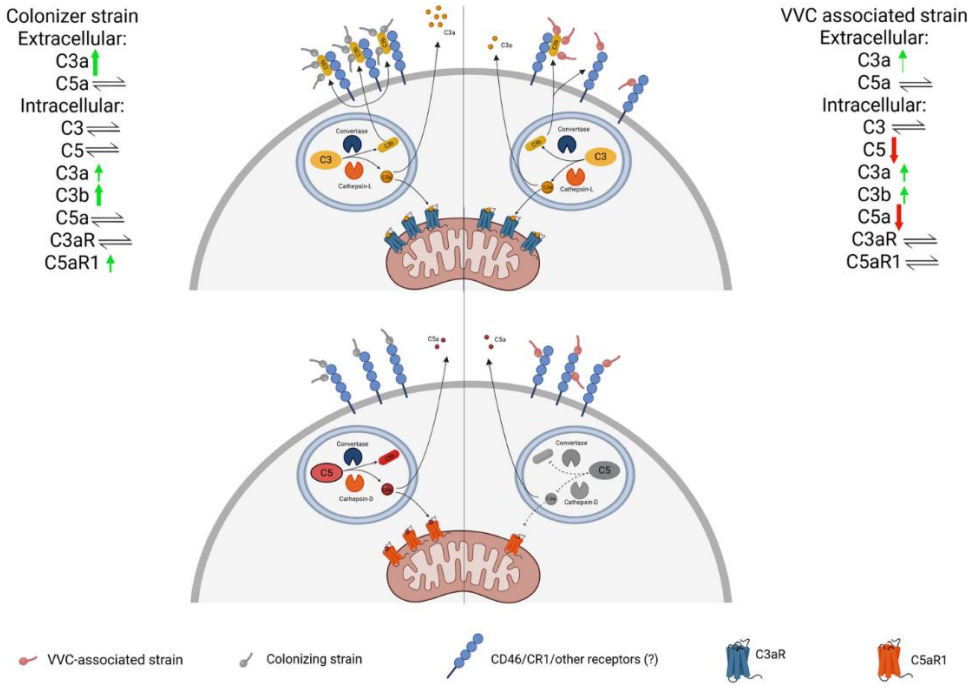

Figure 6. Activation of complosome in VECs. Mechanism of activation of C3, C3a and C3b (upper panel) and C5, C5a (lower panel) and intracellular C3aR and C5aR1 involvement in VECs infected with the Colonizing and VVC-associated strain. Created with Biorender.

5. Conclusions

Taken together, our results highlight cell-associated complement modulation in VECs by *C. albicans* infection and two distinct immune responses prompted by Colonizing and VVC strains. By setting off a more effective cleavage of intracellular complement components, the Colonizing strain persists in the host by establishing a mutual interaction that provides reciprocal advantages. In contrast, by reducing complosome activity in VECs, the VVC strain seems to act by dysregulating the complement response, thus evading the immune mechanisms through the limitation of immune cell recruitment and ultimately promoting the progression from infection to disease. In line with this data, literature reports have linked several *C. albicans* virulence traits to complement system dysregulation. The β-glucan masking by cell wall mannans blocks complement components impairing the lectin pathway [42,43]; the secretion of Pra-1 and Secreted Aspartyl Proteases (Saps) enable *C. albicans* to degrade the complement proteins C3b, C4b and C5, thus inhibiting the formation of MAC and blocking the classical and alternative pathways [9,44]; specific sequence variations in *C. albicans* Sap2 (such as the V273L substitution) increase degradation of C3 and C3b, thus reducing complement activation [45]. Therefore, according to the literature and our present results, we suggest that the regulation of complosome could be a potential therapeutic target in the context of VVC, where specific therapies are currently unavailable. Indeed, our data support the idea that by targeting the complement system, for example through the modulation of C5a/C5aR1 and/or C3a/C3aR axes, may provide an effective novel approach to counteract fungal infection of the vaginal mucosa.

More studies are needed to further elucidate the tissue-specific immune regulatory functions of complement and the contribution of complosome activation in mucosal fungal diseases. These studies will include an analysis of how intracellular C3aR and C5aR1 activation regulates *C. albicans* adhesion and invasion to the epithelium, modifies epithelial integrity and modulates epithelial functions relevant to fungal infection. Given that intracellular complement receptors are located on mitochondria [46] their activation may regulate mithocondrial function, through mtROS production and/or mtDNA release. This ultimately could lead to a different activation of type I IFN pathway through several mechanisms, including cGAMP-STING system activation.

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Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/doi/s1, Figure S1: Representative gating strategy applyed for VECs discrimination from *C. albicans*.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, S.K., and E.P.; methodology, S.K., N.P., L.S., and R.T.W.; software, S.K., R.T.W.; validation, S.K., R.T.W., and E.P.; formal analysis, S.K., A.A., R.T.W., and E.P.; investigation, S.K., N.P., L.S., and R.T.W.; resources, M.C., S.P., and E.P.; data curation, S.K., A.A., M.C., W.P., R.T.W., and E.P.; writing—original draft preparation, S.K., A.A., R.T.W., and E.P.; writing—review and editing, S.K., A.A., M.C., W.P., R.T.W., and E.P.; visualization, S.K., and E.P.; supervision, S.K., and E.P.; project administration, E.P.; funding acquisition, M.C., S.P., and E.P. All authors have read and agreed to the published version of the manuscript." Please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

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