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Candida albicans-epithelial interactions and induction of mucosal innate immunity

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Abstract

Candida albicans is a human fungal pathogen that causes millions of mucosal and life-threatening infections annually. *C. albicans* initially interacts with epithelial cells, resulting in fungal recognition and the formation of hyphae. Hypha formation is critical for host cell damage and immune activation, which are both driven by the secretion of Candidalysin, a recently discovered peptide toxin. Epithelial activation leads to the production of inflammatory mediators that recruit innate immune cells including neutrophils, macrophages and innate Type 17 cells, which together work with epithelial cells to clear the fungal infection. This review will focus on the recent discoveries that have advanced our understanding of *C. albicans*-epithelial interactions and the induction of mucosal innate immunity.

Keywords

Candida; Candidalysin; pathogenicity; virulence; hyphae; epithelium; mucosal; adhesion; invasion; damage; innate immunity; neutrophil; macrophage; dendritic cell; Type 17 immunity; IL-17

Introduction

Candida albicans is normally a harmless commensal organism within the normal microbiota in approximately half the world's population. In the commensal phase, *C. albicans* most

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likely resides in the mucus layer of mucosal surfaces. However, occasionally and under certain circumstances, *C. albicans* may encounter host cells directly, which may result in the attachment of the fungus to epithelial cells. Depending on the strain of *C. albicans* and the physiological and immune status of the host, this interaction event can lead to superficial overgrowth and epithelial invasion, followed by disease and immune activation. *C. albicans* is the most prevalent *Candida* species causing infections in humans and is the causative agent of oral and vaginal candidiasis (e.g. thrush), giving rise to severe morbidity in millions of individuals worldwide. Given that potentially fatal systemic infections can arise from breaches of the mucosal barrier (predominantly from the gut) it is of paramount importance to understand how *C. albicans* interacts with cells of the innate immune system and how this fungus is restricted to the mucosal surface in health. Critical to this is an understanding of how epithelial cells are able to discriminate between harmless (commensal) and dangerous (pathogenic) *C. albicans* cells, which determines whether a mutually beneficial commensal relationship or immune activation takes place.

C. albicans interaction with epithelial cells: adhesion and invasion

Epithelial cells at mucosal surfaces are the first point of contact with *C. albicans* and constitute the first line of defence. Although fungal pathogenicity depends on the type of mucosal tissue, there are common virulence mechanisms and principles. *C. albicans* adhesion to epithelial cells is mediated through the interaction of fungal cell wall moieties and surface proteins with host receptors (Table 1). *C. albicans* yeast cells are recognized by oral epithelial cells (in the TR146 cell line) and induce three signalling pathways within 15 min; the nuclear factor-kappaB (NF-κB) pathway, the phosphatidylinositol-4,5-bisphosphate 3-kinase (Pi3K), and all three mitogen-activated protein kinase (MAPK) pathways (p38, JNK (c-Jun N-terminal kinase) and ERK1/2 (extracellular signal-regulated protein kinase)). This results in the activation of the p65/p50 transcription factor *via* NF-κB, the c-Jun transcription factor *via* JNK and ERK1/2, and AKT (protein kinase B) and mTor (mammalian target of rapamycin) *via* Pi3K signaling [1,2]. Initial binding may constitute recognition of fungal cell wall mannans and β-glucans but this does not fully activate epithelial cells as proinflammatory cytokines were not induced [2]. Lack of activation by *C. albicans* cell wall polysaccharides was also found in skin keratinocytes [3], suggesting that fungal polysaccharides play a limited role in inducing epithelial/keratinocyte immune responses. While many other yeast-associated secreted/cell-surface proteins (e.g. Sap1-3/9/10, Als1/3/4/9, Mp65, Phr1, Iff4, Sun41, Pra1, Eap1, Utr2 and Ecm33), cell wall processing proteins (e.g. Big1, Mnt1/2, Mnn9), and protein trafficking/vesicle transport proteins (e.g. Vps11) are thought to promote epithelial adhesion, this is likely to be *via* indirect mechanisms given that these proteins possess complex, multi-factorial functions that contribute to cell wall integrity and hypha formation [4–7].

Adhesion of *C. albicans* to an epithelial cell is a strong inducer of hypha formation. The formation of hyphae occurs within 30 – 60 min and this is accompanied by the expression of hypha-associated proteins, which are known to possess critical roles in adhesion, invasion, damage induction and immune activation/evasion. The two key hyphal proteins that promote epithelial adhesion are Hwp1 (hyphal wall protein 1) [8] and Als3 (agglutinin-like sequence 3) [9,10]. Hwp1 is highly expressed in human oral infections [11] and acts as a substrate for

epithelial transglutaminases, enabling strong covalent links with other epithelial proteins [12]. Als3 is both an adhesin and an invasin, and together with Ssa1 (heat shock protein) promotes the endocytosis of *C. albicans* into epithelial cells *via* E-cadherin [13–15] and the EGFR/Her2 (epidermal growth factor receptor/human epidermal growth factor 2) complex [16]. Endocytosis is an entirely host driven process and does not require viable hyphae [17]. Other pathways that promote *C. albicans* endocytosis include the PDGF BB (platelet-derived growth factor BB) and NEDD9 (neural precursor-cell-expressed developmentally downregulated protein 9) pathways, which both require hypha formation and Als3 expression [18•]. However, despite possessing adhesion/invasin activities, Als3 does not directly induce epithelial cell damage or cytokine production [19]. The AhR (aryl hydrocarbon receptor) also contributes to the endocytosis of *C. albicans via* Src family kinase phosphorylation of EGFR, but AhR is not involved in epithelial damage or cytokine induction by *C. albicans* and it is unknown how AhR is activated [20•]. Currently, the level of redundancy between these different pathways (E-cadherin, EGFR/Her2, AhR, PDGF BB and NEDD9) and how they communicate to promote C. *albicans* endocytosis is unclear. It is important to note that induced endocytosis is not the only invasion route of *C. albicans.* Indeed, active penetration, which does not require host activities, seems to be the dominant invasion route depending on the type of epithelial cell [21].

Epithelial damage and immune activation by Candidalysin

While *C. albicans* adhesion and invasion leads to fungal recognition and signal pathway activation, surprisingly this does not translate into epithelial damage or innate immune activation [2,17]. Recently, it was discovered that *C. albicans* hyphae induce both epithelial damage and innate immunity through the secretion of a cytolytic peptide toxin called Candidalysin, which is encoded by the hypha-associated *ECE1* gene [22••]. Candidalysin is an amphipathic peptide that adopts an α-helical structure and is the first peptide toxin to be identified in any human fungal pathogen. In oral epithelial cells, Candidalysin induces calcium ion influx and lactate dehydrogenase (LDH) release, which are characteristics of cell damage and membrane destabilization (Figure 1). Notably, *C. albicans* mutants where the entire *ECE1* gene or the Candidalysin-encoding region has been deleted, have full invasive potential *in vitro* but are incapable of inducing tissue damage or cytokine release, and are highly attenuated in a murine model of oropharyngeal candidiasis and a zebrafish swimbladder mucosal model [22••].

Candidalysin induces epithelial immunity predominantly *via* MAPK signalling, specifically (i) the p38 pathway, resulting in the activation of the AP-1 transcription factor c-Fos, and (ii) the ERK1/2 pathway, resulting in the activation of MKP1 (MAPK phosphatase 1) that regulates immune responses [22••]. Together, these pathways lead to the production of pro-inflammatory cytokines including IL-1α/β, IL-6, GM-CSF and G-CSF. Importantly, p38/c-Fos and MKP1 is also activated in human vaginal epithelial cells [23] and by other hypha-forming *Candida* species [24]. Therefore, these signalling pathways may enable different mucosal tissues to detect fungal hyphae, thereby potentially identifying when certain *Candida* species have become pathogenic. Notably, epithelial activation by Candidalysin is not mediated *via* C-type lectin receptors (CLRs) or Toll-like receptors (TLRs) [2], suggesting that epithelial cells utilise different sensing mechanisms than myeloid cells;

whereby myeloid cells respond to *C. albicans* cell wall moieties (β-glucan and mannans) (see below) and epithelial cells respond to damage-inducing *C. albicans* through p38/c-Fos/MKP1 by detecting Candidalysin activity [25–27]. Similar p38 activation has been observed in murine intestinal epithelial cells with bacterial pathogens (*Citrobacter rodentium*) [28] and in *C. elegans* (nematode worm) with *C. albicans* [29], indicating that p38 signalling may be a common epithelial mechanism for the detection of pathogenic microbes.

Innate immunity at mucosal surfaces: neutrophils and macrophages

C. albicans, predominantly through Candidalysin activity, induces proinflammatory cytokines, chemokines and antimicrobial peptides (e.g. IL-1α, IL-1β, IL-8, G-CSF, GM-CSF, β-defensin 3, CCL20 and S100A8/9) from epithelial cells that are required for immune cell recruitment [2,22••,30]. The key myeloid cells that are initially recruited to the site of infection include neutrophils and macrophages (Figure 1). These immune cells recognize *C. albicans* cell wall mannans and DNA *via* TLR2, 4 and 9, and fungal β-glucan *via* CLRs including Dectin-1/−2, DC-SIGN or Mincle [31–33]. Activation of TLRs and CLRs leads to the induction of NF-κB, MAPK and Syk signaling and the production of pro-inflammatory cytokines and further downstream immune effector functions. Nod-like receptors (NLRs) can also be activated by danger signals or internalized fungal compounds and this leads to inflammasome activation and the secretion of IL-1β and IL-18, which help protect against superficial and disseminated *C. albicans* infection [34].

Upon *C. albicans* infection, neutrophils are rapidly recruited to the site of entry. Even without physical contact to invading hyphae, neutrophils respond to epithelial derived chemokines and growth factors and release TNFα, which in turn triggers a protective effect in epithelial cells *via* the upregulation of TLR4 [35]. Neutrophils also inhibit hyphal formation without direct contact [36,37]. Neutrophils can also be recruited by responding directly to *C. albicans*-derived factors such as the secreted aspartic proteases (Saps) [38•]. Furthermore, neutrophils phagocytose (e.g. *via* CLRs) and kill *C. albicans* yeast cells and short hyphae intracellularly predominantly via oxidative burst mechanisms. *C. albicans* hyphae that are too large to be phagocytosed are either growth-inhibited or killed extracellularly through the formation of neutrophil extracellular traps (NETs or NETosis), *via* the release of granule enzymes and through secretion of antimicrobial peptides such as calprotectin [39,40,41•]. Indeed, the zinc binding properties of calprotectin inhibits *C. albicans* growth during NET formation [40]. *C. albicans* hyphae trigger NETosis more effectively and rapidly than yeast cells, but both morphologies can induce NETs *via* autophagy and oxidative mechanisms [42•]. Reactive oxygen species [43], fibronectin [44] and Dectin-1 signaling [45] have also been implicated in NET formation. However, the role of Dectin-1 is controversial as other studies indicate that NET release by β-glucan is mediated via complement receptor 3 (CD11b/CD18) and not Dectin-1 [44].

Macrophages are also recruited to the site of infection and ingest non-opsonized *C. albicans* after recognition by TLRs and CLRs [47]. While macrophages phagocytose and kill *C. albicans* intracellularly in the phagolysosome through oxidative and nitrosative mechanisms, their activity and efficiency of killing is lower than that of neutrophils. Thus, *C. albicans* is readily able to survive within and escape from macrophages *in vitro* [48] and macrophages

play a more minor role *in vivo* during murine disseminated infections [32,34]. Macrophages also recognize *C. albicans via* intracellular NLRs, which activates the NLRP3 inflammasome, leading to production of pro-inflammatory IL-1β and IL-18 as well as pyroptotic host cell death [49,50]. Although immune cell death was originally thought to be hypha-dependent, hypha-independent triggers of pyroptosis have also been described [51,52•]. Therefore, filamentation alone may not be sufficient to trigger NLRP3 inflammasome-mediated pyroptosis [52•,53]. Intracellular hypha formation is driven by active alkalinization of the phagosome [54] and causes macrophage cell death by at least two different mechanisms: pyroptosis and physical piercing of the macrophage membrane [49,50,54]. Notably, NLRP3 inflammasome activation is not necessarily coupled with pyroptosis and the fungal trigger that activates the inflammasome still remains unknown. Finally, while inflammasome activation can lead to IL-1β and IL-18 production, IL-1β has been implicated with Th17 responses whereas IL-18 appears to promote Th1 activity [55].

Innate immunity at mucosal surfaces: innate Type 17 cells

A key insight into requirements for host defense against mucosal candidiasis came from the recognition that mice lacking the IL-17 receptor or its key downstream signaling adaptor Act1 are highly susceptible to oropharyngeal candidiasis (OPC) [30,56,57]. Even more strikingly, when humans were subsequently identified with loss-of-function mutations in the same genes, their dominant disease susceptibility was chronic mucocutaneous candidiasis (CMC) [58,59,60••]. IL-17 is the eponymous cytokine of the Th17 lineage, and a common misconception is that this cytokine functions mainly in the adaptive immune response. However, a variety of innate cells of lymphoid origin produce IL-17, including γδ-T, natural killer T (NKT), innate lymphoid cell type 3 (ILC3) and TCRβ+ 'natural' Th17 cells (nTh17) [61]. In the context of OPC, IL-17 is produced mainly by γδ-T and nTh17 cells, and mice lacking a TCR (e.g., Rag1−/− or IL-7Rα−/− mice) are highly susceptible to infection [62]. Although ILC3s have also been reported in this context [63], Rag1−/− mice have ILC3 cells but still show the same high susceptibility to OPC as IL-17R-deficient mice [62]. The role of neutrophils in producing IL-17 is controversial, but data in the murine OPC model argues against neutrophils as a source of this cytokine [64].

Surprisingly, activation of innate Type 17 cells appears to be quite distinct from activation of adaptive Type 17 immunity. A Dectin-1-Syk-CARD9 pathway was shown to be important for activating immunity to systemic candidiasis [65]. Consistently, CARD9 is essential for the adaptive Th17 recall response in oral candidiasis. However, this adaptor was largely dispensable for induction of the acute innate IL-17 response [66], a finding that was also recently verified for Dectin-1 and TLR2 (A Verma *et al.*, unpublished). This new study finds that Candidalysin production by *C. albicans* hyphae is the triggering factor for innate IL-17 production in the murine model of OPC (Figure 1). Mice infected with *ECE1*-deficient strains show only minimal induction of IL-17 or activation of nTh17 cell production of this cytokine. Additionally, IL-1R signaling is required for activation of the innate Type 17 response, with contributions from both hematopoietic and non-hematopoietic compartments (A Verma *et al.*, unpublished). A vital role for IL-1 in defense against OPC was shown previously in studies of the inflammasome in mouse OPC [67] and was recently verified in contributing to neutrophil activation in this setting [68]. Collectively, these data indicate that

the early, innate response to *C. albicans* in the oral mucosa depends on sensing of tissue damage through Candidalysin, and not simply the presence of β-glucan components revealed upon fungal filamentation.

Although the IL-17 receptor is expressed ubiquitously, we found that the essential responder cell in the context of oral candidiasis is the superficial oral epithelial cell [69•]. Mice with a conditional deletion of the IL-17 receptor in Keratin 13+ cells (including oral and buccal epithelial cells, but not skin, gut or other tissues) show a similar fungal susceptibility as mice with a full knockout of this receptor. Moreover, gene pathway signatures induced in the oral mucosa during acute infection were highly conserved with genes induced by *C. albicans* and IL-17 in human oral keratinocytes [69].

The anti-fungal functions of IL-17 are multi-fold. First, IL-17 is a potent activator of the neutrophil response, which it triggers by inducing expression of neutrophil-recruiting chemokines and cytokines such as G-CSF, CXCL1/2 and 5 in oral tissue [30]. It should be noted that the extent to which IL-17 drives neutrophil signals may be variable [30,64,70]. Second, IL-17 potently induces anti-microbial peptides (AMPs), particularly β-defensins-1 and −3. Mice lacking these defensins show markedly increased susceptibility to OPC [30,69•,71]. IL-17 may also act on salivary gland cells, contributing to the production of antifungal AMPs such as histatins [72,73]. The combined action of IL-17 signaling promotes effective, non-redundant host defense to mucosal candidiasis.

As noted above, several human kindreds were identified with inherited mutations in the IL-17 receptor signaling pathway that cause CMC [58–60]. Additionally, other gene defects that predispose to CMC are associated with defective IL-17 production or function, including mutations in *STAT3* (Hyper-IgE Syndrome, HIES), *STAT1* and *AIRE* (APECED) [74]. In the latter case, neutralizing antibodies against Type 17 cytokines are found in affected patients, raising the possibility that disease is associated with reduced IL-17 signaling. Of course, in all these cases, IL-17 is likely produced by both innate cells and conventional (adaptive) Th17 cells. In this regard, HIV patients with low CD4 T cells counts are highly prone to OPC, and this has been particularly associated with Th17 loss [75]. Finally, in 2016 the first biologic drugs (Secukinumab, Ixekizumab) targeting IL-17 (specifically, IL-17A and the IL-17A/F heterodimer) directly came to the market to treat psoriasis, a strongly IL-17-driven autoimmune disease [76]. Surprisingly, the incidence of OPC is quite low, in the range of 4–8% of patients [77]. This may simply mean that blockade is incomplete, due either to dose effects or access of anti-IL-17 antibodies to the oral mucosal tissue. Alternatively, these biologics spare IL-17F, which has been shown to cooperate with IL-17A in promoting resistance to OPC [63,78]. Cumulatively, these findings all support a central role for IL-17 receptor signal transduction in mucosal host defense, at both innate and adaptive levels.

Conclusion

The epithelial cell plays a fundamental role in the host response to *C. albicans* (Figure 1). Both *C. albicans* yeast and hyphae are recognized, but only hyphae are able to invade epithelial cells by induced endocytosis and/or active penetration, causing activation of

epithelial cells. Endocytosis of *C. albicans* is mediated *via* multiple epithelial receptors and the fungal invasin Als3, but epithelial cells are predominantly activated by the hypha-associated peptide toxin Candidalysin. Candidalysin damages epithelial membranes and activates danger response pathways mediated *via* p38/cFos and ERK/MKP1, which results in immune activation and the secretion of cytokines and chemokines. These effector molecules recruit innate immune cells such as neutrophils, macrophages and innate Type 17 cells. Neutrophils (and macrophages) directly kill or restrict the fungus through phagocytosis mechanisms and/or NET formation, and innate Type 17 cells secrete IL-17 and other inflammatory effectors to further recruit neutrophils and promote mucosal barrier function. These innate immune responses work in conjunction with epithelial cells to control the fungal infection. It is clear that *C. albicans* hypha formation is critically important for both fungal pathogenicity and the host response. Additional advances into these epithelial-hyphal interaction events will no doubt provide valuable insights into our understanding of *C. albicans* infections in the future.

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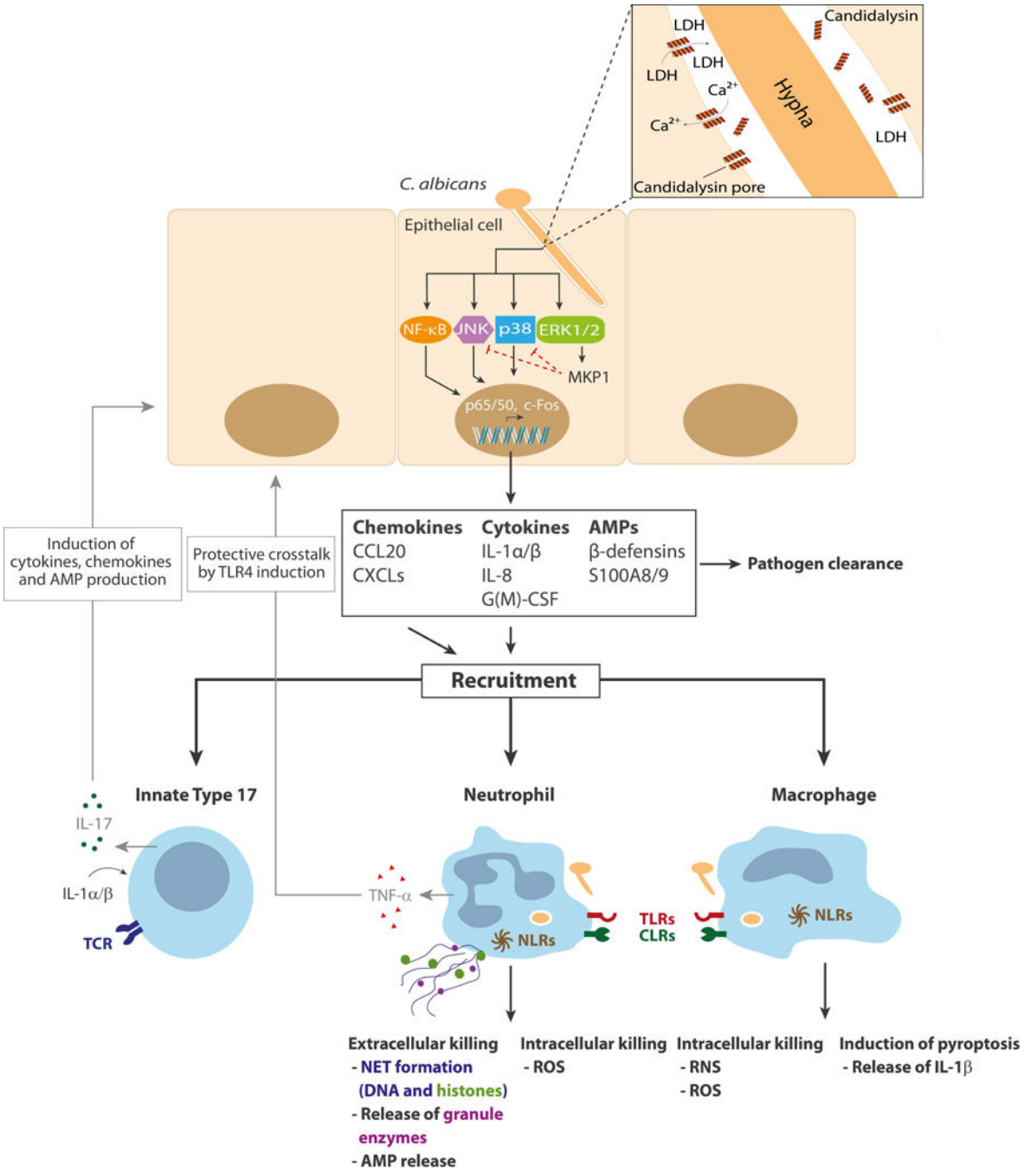

Figure 1.

Innate immunity against C. albicans at the oral mucosa. C. albicans hypha formation induces the formation of an invasion pocket and the production of Candidalysin (helical peptide; top right panel). At high concentrations (>15 μM) Candidalysin forms pores that result in membrane damage (LDH release), calcium influx, and the activation of the epithelial cell, predominantly via the MAPK signalling pathways and the transcription factor c-Fos. MKP1 activation (via ERK1/2) contributes to the regulation of the epithelial immune response. Epithelial activation leads to chemokine, cytokine and antimicrobial peptide (AMP) release

and the subsequent recruitment of innate immune cells, including macrophages, neutrophils and TCRβ+ type 17 cells. Macrophages and neutrophils recognise and phagocytose the fungus through traditional pattern recognition receptors such as TLRs and CLRs. This results in death of the fungus via oxidative or nitrosative (ROS/RNS) killing or the induction of pyroptosis in macrophages (and the release of IL-1β) or NET formation in neutrophils. Neutrophils also release TNFα, which induces the upregulation of TLR4 in epithelial cells. IL-1α/β released by epithelial cells, macrophages and potentially other cell sources activate TCRβ+ type 17 cells, which in turn release IL-17 that subsequently induces the release of additional chemokines, cytokines and antimicrobial peptides (AMP) from epithelial cells, further promoting fungal clearance and barrier function.

Table 1

C. albicans genes involved during interactions with epithelial cells

Fungal component/gene	Epithelial function or target receptors	Reference
Structural polysaccharides		
β-glucan	Induces epithelial signalling. Recognised by Epha2.	[2]. M Swidergall *et al* (abstract)
Mannans	Induces epithelial signalling. Receptors not identified.	[2]
Chitin	Induces epithelial signalling. Receptors not identified.	[2]
Adhesins		
HWP1	Adhesion to epithelial cells via transglutaminase activity. Specific host receptors unknown.	[8]
ALS1-9	Adhesin family. Structural studies indicate this family has multiple epithelial targets.	[14,79–82]
INT1	Interaction with epithelial integrins.	[83]
Toxins		
ECE1	Parent protein of Candidalysin. Induces c-Fos and MKP1 signalling. Receptor activation indicated but not identified.	[22••]
Endocytosis		
ALS3	Activation of or interaction with E-cadherin, EGFR/Her2, AhR, NEDD9 and PDGF BB	[13,15,16,18•,20•]
SSA1	HSP70 family member. Activation of or interaction with EGFR/Her2	[15]
Active Penetration/hydrolysis		
SAP1-8	Secreted aspartic proteases – digestion of epithelial tissues. Sap5 degrades E-cadherin	[84,85]
PLB1	Phospholipase B1 – digestion of epithelial tissues	[86]
LIP1-10	Lipase family – digestion of epithelial tissues	[87]