Candida innate immunity at the mucosa

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We request that colour be used for Figure 1.
Abstract
The tremendous diversity in microbial species that colonise the mucosal surfaces of the human body is only now beginning to be fully appreciated. Distinguishing between the behaviour of commensal microbes and harmful pathogens that reside at mucosal sites in the body is a complex, and exquisitely fine-tuned process central to mucosal health. The fungal pathobiont Candida albicans is frequently isolated from mucosal surfaces with an asymptomatic carriage rate of approximately 60% in the human population. While normally a benign member of the microbiota, overgrowth of C. albicans often results in localised mucosal infection causing morbidity in otherwise healthy individuals, and invasive infection that often causes death in the absence of effective immune defence. C. albicans triggers numerous innate immune responses at mucosal surfaces, and detection of C. albicans hyphae in particular, stimulates the production of antimicrobial peptides, danger-associated molecular patterns and cytokines that function to reduce fungal burdens during infection. This review will summarise our current understanding of innate immune responses to C. albicans at mucosal surfaces.

Keywords
Candida albicans; innate immunity; microbiota; Candidalysin.
1. Introduction

In the past, mucosal surfaces were often considered to be merely a static barrier between the body and the external environment. However, recent research has now redefined mucosal barriers and the epithelial cells that comprise them as highly complex and dynamic structures capable of initiating and modulating several crucial host responses required to maintain tissue homeostasis during health and disease. The mucosal surfaces of the human body provide a varied and challenging niche for bacteria, fungi and viruses. The incredible diversity within the microbial communities that colonise the host mucosa makes differentiating between harmless commensalism and pathogenic behaviour difficult, particularly in the context of pathobionts, which can remain passive for extended periods of time before displaying pathogenic behaviour that is damaging to the host.

Fungi, particularly Candida species, are frequently isolated from the skin [1] and the mucosal surfaces of the body [2]. While C. albicans is often regarded as the most pathogenic of the Candida species, C. tropicalis, C. glabrata, C. parapsilosis, C. krusei and others contribute significantly to morbidity and mortality. Indeed, the recent emergence of fungal pathogens such as C. auris [3] highlight the prominence of Candida species and the unrelenting threat they pose to human health.

The morphological plasticity of C. albicans is a central virulence trait that facilitates mucosal pathogenesis. Overgrowth of C. albicans hyphae at mucosal surfaces occurs when host defences are diminished, resulting in localised mucosal infection. C. albicans infections of the oral cavity [2] present as creamy-white fungal plaques on the oral mucosa (pseudomembranous candidiasis), painful reddened lesions on the tongue (erythematous candidiasis) and smooth or nodular lesions on any mucosal surface within the mouth (chronic hyperplastic candidiasis). Growth of C. albicans on mucosal surfaces and abiotic
substrates results in the formation of biofilms, which are of increasing concern in medical settings [4, 5]. Regular contact between Candida biofilm and the hard palate of the oral cavity can result in Candida-associated denture stomatitis, and lifestyle choices (particularly smoking) can result in median rhomboid glossitis of the tongue. Candida species are frequently isolated from the gastrointestinal tract [6], which is widely regarded as the primary tissue from which the majority of systemic infections are acquired (usually following surgery or major abdominal trauma). Thus, mucosal colonisation by Candida species (and C. albicans in particular), is a major risk factor for potential life-threatening candidaemia.

The skin [1, 7] and mucosal surfaces of the body have evolved a number of incredibly sensitive and discerning mechanisms that allow appropriate immune defence to be initiated and controlled in response to changes in microbial behaviour. This article will review our current understanding of innate defences against C. albicans at mucosal surfaces.

2. The role of the mycobiome in relation to anti-fungal immunity

With the recent advances made in high-throughput sequencing technologies, researchers are now able to accurately characterise the complex microbial communities that develop within human habitats. The astonishing extent and complexity of these communities that has been revealed in these studies has suggested a level of importance in human health and disease that is only now beginning to be understood, with the “superorganism” or “holobiont” hypotheses being proposed [8]. However, despite the advances being made in this field, attention has largely focussed on the bacterial components of these rich microbial communities.

To date, the fungal communities, or mycobiome, have yet to be systematically investigated in the same way. There are several reasons for this. First, despite their
considerable biomass, fungi account for a relatively small percentage of the genes within the microbiome as compared to bacteria and archaea [9, 10]. Second, isolation of high-quality nucleic acids in sufficient quantities from fungal cells in a microbial community is challenging due to the complex cell wall structures that fungal cells possess, and requires a combination of enzymatic, chemical and mechanical lysis steps that vary with different fungi [11]. Finally, discrimination between the different fungal taxa is dependent on the databases and reference genome catalogues that are available. These databases are currently either incomplete or, in the case of reference genome catalogues, not available at present [12, 13]. However, the data that is available points to a critical role for the mycobiome and fungal components within it, in human health and disease [6, 14-16]. Indeed, microbiome analysis of HIV-positive and -negative individuals reveal long-term shifts in the mycobiome but not the bacteriome [17]. While some taxa, such as Candida and Penicillium appeared in all individuals, HIV-negative individuals were specifically associated with Pichia, Cladosporium and Fusarium species, while HIV-positive individuals showed the presence of Alternaria, Epicoccum and Trichosporon. Moreover, species common to both cohorts, including Candida, show differences in their relative abundance. These changes arise from complex interactions between individual taxa of the mycobiome and demonstrate that the Pichia spp associated with a healthy mycobiota produce factors that suppress the growth of pathogenic fungi such as Candida spp that show greater prevalence in HIV-positive individuals [17]. Thus, the loss of Pichia in HIV-positive individuals may in part, explain the prevalence of oral candidiasis associated with HIV-positive status, as a natural inhibitor of Candida is lost. In general, changes in the mycobiota are associated with modulation of immune responses and disease progression [6, 18], as well as maintenance of microbial population
architecture and host metabolic function [19]. Furthermore, components of the mycobiota interact with bacteria to impact on disease [15]. For example, the microbiota of Crohn’s disease patients contains an abundance of *Candida tropicalis* compared with healthy controls, and positively correlates with the production of anti-*Saccharomyces cerevisiae* antibodies; a diagnostic biomarker of Crohn’s disease [15]. Furthermore, both *Serratia marcesens* and *Escherichia coli* are elevated in Crohn’s patients, while “beneficial” bacteria (e.g. *Faecalibacterium prausnitzii*) show a significant decrease in their abundance.

Strikingly, the abundance of *S. marcesens*, *E. coli* and *C. tropicalis* in patients with Crohn’s disease positively correlate with one another; the biomass and thickness of these triple species biofilms are significantly greater than those of single and double species biofilms, with enrichment in the number of *C. tropicalis* hyphae that bind directly to both bacteria through fimbrial connections [15]. Thus, specific interkingdom microbial interactions may be key determinants in Crohn’s disease. Although there is mounting evidence that links changes in the micro/mycobiota with disease, there remains much to understand regarding the complex interactions between the mycobiota, the remaining microbiota, and the host. In order for *C. albicans* to cause disease at a mucosal surface, it must first interact with epithelial cells. The interactions that occur between *C. albicans* and epithelial cells are described below.

3. Interaction of *Candida albicans* with the host mucosa

3.1 Adhesion to epithelial cells

Attachment of *C. albicans* yeast and hyphae to epithelial cells is a prerequisite for colonisation and accordingly, a risk factor for the development of mucosal infection.

Attachment of *C. albicans* to mucosal sites of the body can be mediated either directly or
indirectly through association with bacterial and fungal components of the microbiota.

While *C. albicans* yeast cells are capable of interacting with and adhering to the host mucosa [20], the switch to hyphal growth invokes extensive transcriptional reprogramming of the fungus [21, 22], leading to changes in the composition of the fungal cell wall [23] that enable a more robust interaction with the epithelial surface to be established and maintained [24]. Two key *C. albicans* hypha-associated adhesins in this process are Als3p and Hwp1p, which mediate direct attachment to epithelial cells.

The Agglutinin-Like Sequence (Als) family of *C. albicans* adhesins contains eight members (Als1-7p and Als9p) which are GPI-linked to β-1-6 glucans in the fungal cell wall. Each member of the Als family contains an N-terminal substrate-binding domain, a highly variable central serine/threonine-rich domain comprised of numerous 36 amino acid tandem repeat sequences, and a C-terminal domain containing the GPI anchor [25]. The Als proteins of *C. albicans* exhibit a complex pattern of morphology-dependent and -independent expression that varies between individual clinical specimens, *in vivo* disease models, and fungal culture conditions *in vitro* [25, 26].

Als3p is strongly upregulated during epithelial infection [27], and disruption of ALS3 reduces epithelial adhesion *in vitro*. Similarly, an als2Δ/PMAL2-ALS2 mutant (where the one remaining wild type copy of ALS2 in a heterozygous ALS2 knockout is placed under the control of a regulatable promoter) also exhibited reduced epithelial adhesion [28]. In contrast, deletion of *C. albicans* ALS5, ALS6 or ALS7 increases fungal adhesion to the epithelium [29], indicating that the Als proteins have varied roles in mediating adherence to host mucosa. However, despite these findings, conflicting observations have been made regarding the precise role of ALS1, ALS2 and ALS4-6 in epithelial attachment [30-32].
The structural similarity that exists between specific *C. albicans* proteins and those of the host can in some instances be exploited to confer an advantage to the fungus. Hyphal Wall Protein 1 (Hwp1p) is a hypha-associated adhesin that is strongly expressed during colonisation and infection of the oral mucosa [27, 33]. The amino acid sequence of the Hwp1p N-terminal domain closely resembles that of natural substrates for mammalian transglutaminase enzymes [34]. By mimicking a natural host substrate, *C. albicans* exploits host transglutaminase activity, which covalently couples the N-terminal domain of Hwp1p to the epithelial surface [35]. Hwp1p has a greater affinity for terminally differentiated epithelial cells that display SPR3 and keratin 13 when compared with less differentiated cells [36], and deletion of *HWP1* results in reduced epithelial adhesion and virulence in a murine model of oropharyngeal candidiasis (OPC) [35, 37]. However, while the role of Hwp1p in mucosal infection is clearly established, it plays a minimal role in systemic infection and is therefore habitat specific [38].

*C. albicans* Int1p is an adhesin with structural similarity to human leukocyte integrin. Int1p is required for hyphal growth, intestinal colonisation in mice and virulence *in vivo* [39]. Heterologous expression of *INT1* in the non-adherent yeast *Saccharomyces cerevisiae* confers adhesion to epithelial cells [39, 40], confirming the role of Int1p as a primary adhesin. Phenotypes associated with *INT1* expression are complex and dose dependent, as reintegation of a single copy of wild type *INT1* into an *int1Δ/Δ* null mutant background restores hyphal growth but not epithelial attachment [39].

While only a handful of *C. albicans* proteins have been identified that mediate epithelial adhesion directly, numerous factors have been identified that exert an indirect influence on epithelial attachment. Collectively, these factors affect multiple pathways and processes including the expression and appropriate presentation of adhesins on the fungal
cell wall, and the activation of hypha-associated transcriptional circuitry required to induce the expression of morphology-dependent genes required for enhanced epithelial interaction. The factors that indirectly influence the interaction of *C. albicans* with host mucosa are presented in Table 1.

**Table 1. Factors that indirectly influence attachment of *C. albicans* to epithelial cells.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Epithelial Adhesion of Null Mutant</th>
<th>Epithelial Cell Type</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>BIG1</td>
<td>Endoplasmic reticulum protein</td>
<td>Decreased HeLa</td>
<td>[41]</td>
<td></td>
</tr>
<tr>
<td>BST1</td>
<td>Inositol deacylase</td>
<td>Decreased Caco-2 KB</td>
<td>[42]</td>
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<tr>
<td>CDC10</td>
<td>Septin protein</td>
<td>Decreased HeLa</td>
<td>[43]</td>
<td></td>
</tr>
<tr>
<td>CFL1</td>
<td>Ferric reductase</td>
<td>Decreased HeLa</td>
<td>[44]</td>
<td></td>
</tr>
<tr>
<td>CSF4</td>
<td>Putative glycosidase</td>
<td>Decreased FaDu</td>
<td>[45]</td>
<td></td>
</tr>
<tr>
<td>EAP1</td>
<td>GPI-anchored cell wall protein</td>
<td>Decreased HEK293</td>
<td>[46]</td>
<td></td>
</tr>
<tr>
<td>EFG1</td>
<td>Transcription factor</td>
<td>Decreased Caco-2</td>
<td>[47]</td>
<td></td>
</tr>
<tr>
<td>IFF4</td>
<td>GPI-anchored cell wall protein</td>
<td>Overexpression increases adherence</td>
<td>FaDu</td>
<td>[48]</td>
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<tr>
<td>IPT1</td>
<td>Sphingolipid biosynthesis</td>
<td>Decreased Gingival</td>
<td>[49]</td>
<td></td>
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<tr>
<td>IRS4</td>
<td>Cell wall integrity</td>
<td>Decreased HT-29</td>
<td>[50]</td>
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<tr>
<td>KRES5</td>
<td>Glucosyl transferase</td>
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<td>Decreased Buccal</td>
<td>[52]</td>
<td></td>
</tr>
<tr>
<td>MNT2</td>
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<td>[53]</td>
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<tr>
<td>MP65</td>
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<td>Decreased Buccal Caco-2</td>
<td>[54]</td>
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<tr>
<td>NOT5</td>
<td>Putative transcriptional complex</td>
<td>Decreased Buccal</td>
<td>[55]</td>
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<td></td>
<td>component</td>
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<tr>
<td>PDE2</td>
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<td>Decreased Buccal</td>
<td>[56]</td>
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<td>PHR1</td>
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<td>Decreased TR146 Caco-2</td>
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<td>Decreased Buccal</td>
<td>[61]</td>
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<tr>
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<td>[61]</td>
<td></td>
</tr>
<tr>
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<td>Gene Name</td>
<td>Expression</td>
<td>Tissue</td>
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<td>-----------</td>
</tr>
<tr>
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<td>Increased</td>
<td>Buccal</td>
<td>[61]</td>
</tr>
<tr>
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<td>Aspartic proteinase</td>
<td>Increased</td>
<td>Buccal</td>
<td>[62]</td>
</tr>
<tr>
<td>SAP10</td>
<td>Aspartic proteinase</td>
<td>Decreased</td>
<td>Buccal</td>
<td>[62]</td>
</tr>
<tr>
<td>SET1</td>
<td>Methyl transferase</td>
<td>Decreased</td>
<td>FaDu HT-29</td>
<td>[63]</td>
</tr>
<tr>
<td>SUN41</td>
<td>Putative glycosidase</td>
<td>Decreased</td>
<td>Caco-2</td>
<td>[64]</td>
</tr>
<tr>
<td>TUP1</td>
<td>Transcriptional corepressor</td>
<td>Decreased</td>
<td>SCC15</td>
<td>[65]</td>
</tr>
<tr>
<td>VAC1</td>
<td>Putative vesicle transport protein</td>
<td>Decreased</td>
<td>SK-LMS-1</td>
<td>[66]</td>
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</tbody>
</table>

### 3.2 Epithelial recognition of *C. albicans*

Physical recognition of *C. albicans* by host mucosa is achieved through the interaction of epithelial pattern recognition receptors (PRRs) with pathogen-associated molecular patterns (PAMPs). Fungal PAMPs include cell wall proteins which contain conserved structural motifs that are projected from the cell wall into the extracellular environment, and intracellular molecules such as nucleic acids. Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and nucleotide-binding domain leucine-rich receptors (NLRs) comprise the three main families of PRR. A successful PRR-PAMP interaction activates epithelial signalling pathways that contribute to innate defence against fungal infection.

While the involvement of PRRs in anti-fungal responses is well documented for myeloid cells [67] (and see sections 7 and 8), relatively little is known about the PRRs involved during epithelial recognition of *C. albicans*.

The epithelial ephrin type-A receptor 2 (EphA2) is a recently identified non-classical PRR that binds to exposed β-glucans of *C. albicans* yeast and hyphae [68]. β-glucan-induced phosphorylation of EphA2 is dependent upon fungal burden, and a yeast locked (efg1/cph1Δ/Δ) *C. albicans* mutant stimulates EphA2 phosphorylation within 15 min, indicating that epithelial recognition of fungus through EphA2 is morphology-independent.

Activation of EphA2 results in phosphorylation of MEK1/2 and p38 (leading to downstream
activation of c-Fos), phosphorylation of signal transducer and activator of transcription 3 (Stat3), and secretion of human beta defensin-2, chemokine (C-C motif) ligand 20 (CCL20), interleukin (IL)-1α, IL-1β and IL-8 [68]. Thus, epithelial recognition of C. albicans β-glucan via EphA2 results in antifungal and pro-inflammatory responses. The importance of EphA2-mediated innate recognition of C. albicans in vivo is highlighted by the observation that immune competent EphA2−/− knockout mice have higher fungal burdens in the oral cavity when compared to wild type mice [68].

All TLRs (except TLR7) are expressed by reconstituted human oral epithelium, and all except TLR5 and TLR7 are detectable in buccal epithelial cells from healthy donors [69]. Epithelial TLR4 in particular, plays a direct role in mucosal defence against C. albicans infection, a process that is dependent upon an intimate relationship with neutrophils. While infection of oral epithelial cells with C. albicans in vitro results in cellular damage and a robust pro-inflammatory response, expression of TLR4 remains unchanged compared to uninfected cells [69]. However, in the presence of polymorphonuclear leukocytes, epithelial cells secrete immune modulators that stimulate a robust PMN-mediated upregulation of epithelial TLR4, concomitant with a reduction in epithelial damage and protection against fungal invasion [69].

3.3 Invasion of mucosal barriers

Invasion of mucosal barriers by C. albicans is commensurate with localised infection. While yeast and hyphal morphologies of C. albicans are capable of adhering to the host mucosa, it is predominantly the hyphal form of the fungus that invades into the epithelial surface. C. albicans uses two temporally and mechanistically distinct mechanisms of invasion that together enable the fungus to access the superficial and underlying epithelial
tissues. These mechanisms are receptor-mediated induced endocytosis and active penetration.

3.4 Receptor-induced endocytosis

Once attached to the epithelial surface, the hyphae of *C. albicans* invade mucosal barriers by initiating a dynamic and complex host-driven process called receptor-induced endocytosis (RIE) [70, 71]. Indeed, the invading fungus remains completely passive during RIE, as hyphae that are rendered metabolically inviable by thimerosal treatment are still endocytosed by epithelial cells [71]. Endocytosis of *C. albicans* hyphae is triggered by epithelial recognition of fungal invasins expressed on the hypha cell wall, and occurs within 4 h of initial contact with epithelial cells [72].

While several host receptors are implicated in the process of fungal endocytosis, only two fungal invasins have been identified as triggers of internalisation: the adhesin Als3p, and Ssa1p (a member of the heat shock protein (HSP) 70 family of proteins) expressed on the *C. albicans* cell wall [73, 74]. Disruption of either ALS3 or SSA1 result in reduced invasion of epithelial cells and attenuated virulence in a murine model of OPC [73-75], with Als3p in particular playing a dominant role in this process. Als3p and Ssa1p are recognised by the epithelial receptor E-cadherin [73, 74], an interaction that stimulates a dynamic, hypha-induced reorganisation of epithelial clathrin, dynamin and cortactin that culminates in the formation of pseudopods that surround the fungus and facilitate internalisation [76]. Interestingly, inhibition of E-cadherin receptor activity results in a partial, but not complete block of fungal endocytosis [77], suggesting the involvement of additional host epithelial receptors and/or pathways in fungal internalisation. Indeed,
remodelling of the actin cytoskeleton in rabbit corneal epithelial cells is dependent on the small GTPases Cdc42, Rac1, RhoA and the tight junction protein ZO-1 [78].

Recognition of C. albicans Als3p and Ssa1p can also occur through interaction with a heterodimeric receptor complex comprised of the epidermal growth factor receptor (EGFR/HER1) and HER2 expressed on epithelial cells [77]. Latex beads coated with a recombinant N-terminal region of Als3p or recombinant Ssa1p are rapidly endocytosed by epithelial cells in vitro [73, 74], identifying both proteins as ligands that stimulate epithelial internalisation. During infection of oral epithelial cells in vitro, C. albicans activates the platelet-derived growth factor BB (PDGF BB) and neural precursor-cell-expressed developmentally down-regulated protein 9 (NEDD9) pathways in a cadherin-independent manner [79]. In contrast, analysis of samples obtained during a clinical study of vaginal candidiasis revealed that the PDGF BB, but not NEDD9 pathway is activated in response to infection [79], highlighting the tissue specific nature of epithelial responses to the fungus.

The aryl hydrocarbon receptor (AhR) is a cytoplasmic ligand-activated transcription factor that plays a central role in EGFR-mediated endocytosis of C. albicans in vitro and in vivo [80]. Infection of oral epithelial cells with C. albicans activates the AhR, which in turn leads to phosphorylation of Src family kinases, culminating in EGFR activation and fungal endocytosis. Inhibition of the AhR in immune-competent and immune-suppressed mice was found to reduce the severity of OPC [80], highlighting the contribution of AhR signalling to disease pathology. Given the intracellular location of the AhR, the mechanism of C. albicans-mediated receptor activation is likely to be indirect. However, the specific ligand that is induced by C. albicans to activate the AhR has yet to be identified. Small interfering RNA (siRNA)-mediated depletion of EphA2 also reduces RIE of C. albicans [68]. EphA2 knockdown
and chemical inhibition of receptor activity reduce EGFR phosphorylation, suggesting that EphA2 and EGFR function in the same endocytosis pathway [68].

Knockout studies investigating the role of the transcription factor Rim101p have provided further insight into the fungal proteins required to induce the uptake of *C. albicans* by epithelial cells. A *C. albicans rim101Δ/Δ* mutant was unable to efficiently initiate RIE by oral epithelial cells [81]. Notably, however, overexpression of Cht2p, Pga7p or Zrt1p in a *rim101Δ/Δ* mutant background restored the ability of *C. albicans* to trigger RIE efficiently, implicating these proteins in the process of epithelial internalisation. Despite the great advances made in our understanding of RIE, the molecular signalling events that drive this epithelial response are yet to be characterised in full and the extent of receptor and pathway redundancy between mechanisms remains to be explored in detail. The *C. albicans* factors involved in the processes of epithelial adhesion and RIE are presented in Figure 1A.

### 3.5 Active penetration of mucosal surfaces

Mucosal surfaces vary in their structure and cellular composition depending on location within the body. The stratified mucosa of the oral and vaginal lumen comprise several layers of epithelial cells, the outermost of which are terminally differentiated, whereas the epithelium that lines the gastrointestinal (GI) tract is composed of a single (non-stratified) layer of cells. Terminally differentiated epithelial cells are non-proliferative and considered less capable of supporting RIE. In order to invade a mucosal barrier that does not readily internalise hyphae, *C. albicans* uses the process of active penetration.

Unlike RIE, where the fungus remains passive during uptake [71], active penetration of host mucosa by *C. albicans* hyphae relies upon physical attributes of the fungus including turgor pressure, physical advancement of the hyphal tip, and secretion of hydrolytic
enzymes that facilitate fungal invasion through or between epithelial cells. Paradoxically, while only a single cell thick, the GI epithelium does not support fungal invasion by RIE, but by active penetration only, highlighting specific differences in epithelial responses to C. albicans. The mucosal barrier of the GI tract is coated with a layer of mucus. C. albicans hyphae secrete the aspartic proteinase Sap2p that degrades gastrointestinal mucins [82] and Sap5p that degrades E-cadherin [83], weakening the adherens junctions between epithelial cells, which may facilitate the translocation of fungus across the gut. Despite these contributions however, our understanding of the fungal proteins required for the process of active penetration remains incomplete.

Surprisingly, Als3p is not essential for active penetration as an als3Δ/Δ null mutant can still invade epithelial cells that are unable to endocytose hyphae [75]. However, Als3p may nevertheless provide points of anchorage on the mucosal surface which enable hyphal pressure upon the epithelial barrier to be sustained. Active penetration of C. albicans hyphae through mucosal barriers is delayed in onset compared with RIE and is also mechanistically distinct, as blocking the polymerisation of epithelial actin does not prevent the mucosa from being breached [75, 84]. In vivo, it is likely that a combination of active penetration and RIE are required for full invasion of stratified epithelial barriers. Active penetration across the outermost (terminally differentiated) epithelial layers is thought to occur first, and this is followed by RIE, when the invading fungus reaches the underlying (non-terminally differentiated) epithelial cells.

4. Candidalysin: epithelial damage and innate immunity

One of the hallmarks of C. albicans infection of mucosal surfaces is damage to the superficial epithelium. Damage to the oral and vaginal mucosa is mediated by Candidalysin,
a secreted dual function amphipathic peptide toxin encoded by the extent of cellular
elongation gene (ECE1) [85, 86]. Candidalysin adopts an alpha helical conformation in
solution and intercalates into the plasma membrane of epithelial cells, where it forms
heterogeneous and transient lesions that destabilise membrane structure, causing calcium
influx and release of intracellular contents (Figure 1B). Importantly, a C. albicans ece1Δ/Δ
null mutant and a mutant that does not express the Candidalysin-encoding region of ECE1
(ec1Δ/Δ+ECE1Δ184-279), form hyphae, adhere to and invade epithelial cells normally, but do
not cause damage or the secretion of pro-inflammatory cytokines. Moreover, both mutants
are attenuated in a murine model of OPC and a Zebrafish swim bladder model of mucosal
infection [85].

Epithelial recognition of Candidalysin triggers mucosal immunity predominantly
through MAPK signalling, activating the p38 and ERK1/2 pathways that in turn activate the
AP-1 transcription factor c-Fos and MAPK phosphatase 1 (MKP1) respectively, alerting the
host to the transition from colonising yeast to invasive, toxin-producing hyphae (Figure 1C).
Low concentrations of Candidalysin (<15 µM) trigger the release of pro-inflammatory
cytokines but do not cause epithelial damage, whereas higher concentrations (70 µM) of
toxin induce pro-inflammatory responses and cause extensive damage, demonstrating that
Candidalysin has dual functionality in a concentration-dependent manner.

Candidalysin-induced activation of epithelial cells culminates in the release of
cytokines including granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-
colon stimulating factor (GM-CSF), IL-1α, IL-1β and IL-6 (Figure 1D). C. albicans ECE1 and
Candidalysin mutants induce significantly lower levels of neutrophils to the site of infection
in vivo when compared to wild type controls [85, 86].
A host receptor for Candidalysin-induced signalling has yet to be identified; however, epithelial activation is not mediated through classical PRRs such as Toll-like receptors or C-type lectin receptors [87]. Intriguingly, Candidalysin can permeabilise synthetic lipid bilayers that do not contain an endogenous receptor [85], suggesting that the toxin may damage epithelial cells and activate innate immune responses through distinct mechanisms.

A model of mucosal infection has been proposed [85] in which the hyphae of C. albicans invade the epithelial surface, creating an invasion "pocket" [75] into which Candidalysin is secreted. During the initial stages of infection, the concentration of Candidalysin is insufficient to cause appreciable plasma membrane damage, but is nevertheless recognised by the epithelial cells of the host mucosa, stimulating an innate immune response. As infection progresses however, the concentration of Candidalysin within the invasion pocket increases to damage-inducing levels, causing membrane lesions that facilitate infection.

Vulvovaginal candidasis (VVC) is a disease of immune competent women. Approximately 75% of women will experience at least one episode of VVC in their lifetime [88], while almost 9% will suffer from recurrent infection [89]. Symptomatic VVC is characterised by itching, burning, and pain at the vulvovaginal mucosa, often accompanied by odorless vaginal discharge [90]. In contrast to the oral epithelium, recruitment of neutrophils to the site of infection during VVC does not result in fungal clearance but rather, causes an acute exacerbation of symptoms [91].

Similar to TR146 oral epithelial cells, treatment of A431 vaginal cells with Candidalysin causes damage, c-Fos/p-MKP1 signalling and cytokine secretion [86]. Notably, fungal burdens remain equivalent in mice that receive an intravaginal challenge of wild type C. albicans or strains unable to express and secrete Candidalysin. However, a significant
decrease in neutrophil recruitment, damage, and pro-inflammatory cytokine expression was observed in response to strains unable to produce the toxin, identifying Candidalysin as the driver of immune pathology in the vaginal environment [86]. Thus, Candidalysin plays a crucial role in the activation of innate defences against C. albicans hyphae at disparate mucosal sites in the body. The pathogenicity of natural C. albicans isolates is perhaps, unsurprisingly, correlated with the degree of epithelial damage caused, and release of the damage-associated cytokine IL-1α. Highly variable murine oral responses were observed from several isolates of C. albicans, including differences in neutrophil recruitment and the robustness of inflammatory responses [92]. Notably, from a panel of hypha-associated genes tested (ALS3, SAP4, SAP5, SAP6, HWP1 and ECE1), only significant differences in ECE1 expression (Candidalysin) was observed between isolates. However, ECE1 expression did not always correlate with a strains’ ability to damage epithelial cells [92], but this could be due to differences in adhesion and hypha formation in the individual strains tested and by the utilisation of a single ECE1 primer set to assess ECE1 gene expression in all strains.

5. Antimicrobial peptides, alarmins and cytokine responses

A multitude of host factors are known to be rapidly induced in response to C. albicans infection, including antimicrobial peptides (AMPs), which are among the first molecules to be released from the mucosal surface. The cathelicidins are a family of broad-spectrum AMPs that are expressed by a variety of epithelial and immune cells. Human cathelicidin (LL-37) and its murine equivalent (mCRAMP), are induced in response to C. albicans where they bind to the cell wall of the fungus, resulting in permeabilisation of the fungal plasma membrane [93]. Following secretion, LL-37 can be further processed into
shorter peptide fragments (designated RK-31 or KS-30) capable of killing *C. albicans* with equal or greater efficacy when compared to full-length LL-37 [94]. However, cathelicidin does not confer systemic or subcutaneous protection against *C. albicans* [93].

Lactoferrin (Lf) also possesses anti-fungal activity and, like LL-37, can be cleaved into distinct peptides to enhance functionality. Full-length Lf, together with two Lf-derived peptides (lactoferricin and lactoferrampin), exert candidacidal activity through mechanisms that involve disruption of the fungal plasma membrane [95-99]. Full-length Lf also induces apoptosis-like processes within the fungus [100] and sequestration of iron to induce fungistasis [101].

In contrast to Lf, the cationic salivary protein histatin 5 does not lyse *C. albicans* directly, but binds to β-glucans and cell wall proteins including Ssa1p and Ssa2p [102-105]. Once histatin 5 is bound to the *C. albicans* cell wall, it is transported into the fungus by two key polyamine transporters (Dur3p and Dur31p) [106, 107], where it exerts fungicidal activity by disrupting osmotic homeostasis and cell cycle control [102, 108].

The defensins are a family of cysteine-rich AMPs that exhibit anti-microbial activity against a wide range of organisms. Several members of the defensin family possess potent anti-*C. albicans* activity, including β-defensin 2 and 3 which kill *C. albicans* through a poorly defined mechanism that does not involve membrane disruption [109], as well as α-defensin 6 which blocks adhesion of *C. albicans* to intestinal epithelial cells [110]. The importance of defensins against *C. albicans in vivo* is demonstrated by the observation that mice lacking β-defensin 3 are highly susceptible to OPC [111].

Vitamin D is receiving increasing attention as a potent anti-microbial molecule involved in pro-inflammatory responses and cytokine regulation [112, 113] that can counteract pathogen-induced transcriptional changes in host monocytes [114]. Vitamin D3
exhibits direct anti-\textit{C. albicans} activity [115], and although the precise mechanism(s) of action \textit{in vivo} remain to be determined, 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), the hormonal form of Vitamin D, induces potent upregulation of LL-37 in tongue and lung epithelial cells, monocytes and neutrophils [116, 117].

Alarmins are endogenous intracellular proteins that are released from host cells following severe trauma and tissue damage. The S100A8 and S100A9 alarmins are released from the oral and vaginal mucosa in response to \textit{C. albicans} infection and contribute to (but are not essential for), recruitment of neutrophils that function to resolve the symptoms of infection in the oral, but not vaginal lumen in a strain specific manner [86, 92, 111, 118, 119].

In addition to AMPs and alarmins, numerous cytokines play a crucial role in the induction and regulation of innate immune defence against \textit{C. albicans}. Signalling mediated through IL-1 and its related cytokines is critical for the induction of effective anti-\textit{C. albicans} immunity. Absence of the IL-1 receptor significantly impairs expression of the neutrophil recruiting molecules CXCL1, CXCL2, CXCL5 and G-CSF and subsequent neutrophil migration [120]. Furthermore, IL-1 receptor deficient mice (\textit{Il1r1}$^{-/-}$) have significantly higher fungal burdens in the oral cavity at 3, 7, 14 and 21 days post-infection when compared to wild type controls [121].

IL-17 has emerged as one of the most important cytokines in mucosal anti-fungal immunity, with manifold functionality. Produced by a variety of lymphoid-derived cells (both innate and adaptive) IL-17 has a dramatic impact on innate immunity. While there are several isoforms of IL-17, each with distinct biological functions, it is IL-17A and IL-17F which play key roles in antifungal immunity. Although it has been suggested that neutrophils may produce IL-17, this does not appear to be the case during OPC [122]. However, one of the
more important roles of the IL-17 cytokines is to activate a neutrophil response. This involves secretion of chemokines that will recruit neutrophils (e.g. CXCL1/2 and CXCL5), as well as cytokines that activate neutrophils once they have been recruited to the site of infection (e.g. G-CSF) [123].

IL-17 is a potent inducer of anti-microbial peptide (AMP) secretion, notably β-defensins 1 and 3 in mice [111, 123], as well as histatins [124, 125]. Thus, given the importance of these AMPs during Candida infection [111, 126], the multiple roles of IL-17 in mediating protection against C. albicans are clearly established. IL-17A also affects the epithelial cells of the GI tract directly, where it regulates expression of occludins required for epithelial tight junctions and barrier integrity [127, 128]. Collectively, the multiple functionalities of IL-17 cytokines act to drive and regulate a number of essential and non-redundant host mechanisms required for effective defence against C. albicans infection at mucosal surfaces.

Collectively, AMPs, alarmins and cytokines exert numerous protective functions at mucosal surfaces in response to C. albicans infection, and although our understanding of these complex processes continues to improve, there is much still to learn, particularly with respect to fungi.

6. Mucosal inflammasome responses to C. albicans

Inflammasomes are cytosolic multi-protein complexes that play a critical role in immunity. They are comprised of a PRR (either a nucleotide-binding domain leucine-rich receptor (NLR), or an Absent In Melanoma-2 (AIM2) receptor), and are activated in response to innate recognition of exogenous and endogenous PAMPs and danger-associated molecular patterns (DAMPs). NLRs contain an N-terminal region comprised of either a PYRIN
or CARD domain, a central nucleotide-binding oligomerisation domain and a C-terminal leucine-rich repeat sequence [129, 130]. Physical association between a NLR and an adaptor protein (apoptosis-associated speck-like protein containing a CARD (ASC)), results in the recruitment and autocatalytic activation of pro-caspase-1, which subsequently cleaves the inactive cytokine zymogens pro-IL-1β and pro-IL-18 [131], to yield biologically active molecules.

While numerous inflammasomes exist [132], those most closely associated with innate defence against *C. albicans* at mucosal surfaces are the NLRP3 and NLRC4 complexes [121, 133]. Tissue specific roles have been ascribed to the NLRP3 and NLRC4 inflammasomes; the NLRP3 inflammasome is present in both haematopoietic and stromal compartments and plays a crucial role in the prevention of fungal dissemination during oral infection *in vivo* [121] while the NLRC4 complex is important for mucosal defence [133].

The production of active IL-1β from the oral mucosa in response to *C. albicans* is dependent upon both NLRP3 and NLRC4, and buccal mucosal tissues upregulate NLRP3 and NLRP4 expression within 72 h during the innate immune response to *C. albicans* infection [133]. Moreover, *Nlrp3*−/− and *Nlrc4*−/− knockout mice exhibit a significant reduction in the expression of IL-17A, IL-17F and antimicrobial peptides in the oral cavity, while the fungal burden of *Nlrc4*−/− mice is significantly higher during OPC when compared to wild type controls [133], highlighting the importance of inflammasome complexes, particularly NLRC4, for oral defence against *C. albicans* infection *in vivo*.

**7. Neutrophils in antifungal innate immunity**

Many protective innate immune responses at mucosal surfaces are mediated by myeloid cells, most notably neutrophils and macrophages, which rapidly infiltrate the site of
infection. Activation of neutrophils and macrophages is triggered by direct recognition of fungal PAMPs [134], by chemokines and AMPs, by cytokines released from epithelial cells in response to Candidalysin (e.g. IL-1α/β, G/GM-CSF, IL-8, CCL20, β-defensin-2/3 and S100A8/9) [85, 87, 123], and by IL-17 released from resident innate Th17 cells or γδ T cells [135]. Neutrophils and macrophages express a range of PRRs including TLR2, TLR4, DC-SIGN, Mincle, Dectin-2 and Dectin-3 that recognise C. albicans cell wall mannoproteins, TLR9 that responds to DNA, and Dectin-1 that interacts with cell wall β-glucans, leading to their full activation [134, 136-138]. 

Activation of PRRs by cognate PAMPs triggers the MAPK and NF-κB intracellular signalling pathways through the activation of MyD88, the inflammasome complex and SYK [134]. This signalling leads to downstream production of pro-inflammatory cytokines and antimicrobial factors, phagocytosis and a unique method of cell death termed NETosis [134, 139]. However, the PAMPs described above are not the only factors that stimulate neutrophil responses. Indeed, the secreted aspartic proteases (Saps) of C. albicans have also been shown to recruit neutrophils [140]. Furthermore, the production of IL-17 from innate type 17 cells (Figure 1E) and potentially, γδ T cells, in response to IL-1α/β released from epithelial cells will also lead to activation of infiltrating neutrophils [135], and a key role for IL-1 in coordinating responses to OPC has also been identified [120].

The classical method of neutrophil-mediated killing of C. albicans hyphae is phagocytosis followed by a lethal oxidative burst. The interaction between Dectin-1 and insoluble β-glucan leads to the formation of a phagocytic synapse that greatly improves the phagocytosis of C. albicans yeast and short hyphae [141]. However, hyphae that are too large to be phagocytosed by neutrophils remain a threat to mucosal tissues. To destroy these larger filaments, neutrophils can undergo NETosis, forming Neutrophil Extracellular
Traps (NETs) [142]. NETosis involves the cell “exploding” to release a web of chromatin coated with granule enzymes, antimicrobial peptides (e.g. calprotectin), and histones [142-144] (Figure 1F). Although both yeast and hypha morphologies of C. albicans trigger NETosis, the neutrophil response to hyphae is by far the most rapid.

Several C. albicans-associated triggers of NETosis have been identified, including reactive oxygen species (ROS) [145], fibronectin [146] and β-glucan, either through Dectin-1/SYK signalling [147], or through the complement receptor (CD11b/CD18) [146, 148]. The mechanisms of NETosis are now beginning to be elucidated, and appear to involve both autophagy and chromatin de-condensation through the ROS-activated peptidylarginine deiminase 4 (PAD4) [149, 150]. As well as killing fungi, NETs are also capable of slowing hyphal growth, potentially through the sequestration of micronutrients (e.g. zinc) [143].

8. Macrophages in antifungal innate immunity

Although neutrophils are the dominant myeloid cell recruited to the foci of infection, macrophages also infiltrate and perform similar protective functions, phagocytosing the fungus after PRR-PAMP interaction [151], albeit with a lower efficiency of killing when compared to neutrophils.

Intriguingly, C. albicans has been observed to survive within macrophages and even escape [152]. Often, this escape involves the formation of hyphae that physically pierce through the membrane of the phagolysosome and rupture the ingesting macrophage [153], although C. albicans can also escape macrophages in a non-lytic fashion [154] akin to the vomocytosis process described for Cryptococcus neoformans. Whatever the mechanism of escape, macrophages play a lesser role during disseminated C. albicans infection in mice when compared with neutrophils [134, 138].
Nevertheless, macrophages phagocytose *C. albicans* and respond through NLR-PAMP interactions that stimulate inflammasome assembly (resulting in the secretion of biologically active IL-1β), and yet another unique form of cell death termed pyroptosis [155]. Notably, the induction of macrophage pyroptosis by *C. albicans* is both a temporally and mechanistically distinct means of escape that precedes hypha-mediated exfiltration [156], and provides an important source of biologically active IL-1β (Figure 1G).

9. Innate immune memory

Conventional wisdom has it that while adaptive immunity ‘learns’ and has memory, innate immunity has no memory. However, recent findings have begun to overturn this paradigm. From the mid 1950’s onwards, numerous reports observed that administration of the *Bacillus* Calmette-Guérin (BCG) vaccine conferred improved resistance to other non-related pathogens, including *Staphylococcus aureus* [157], *Salmonella* [158] and *C. albicans* [159]. Similar observations have been made following infection of mice with an avirulent strain of *C. albicans*, which resulted in improved protection against virulent *C. albicans* and pathogenic bacteria, through a macrophage-dependent, but T cell-independent mechanism [160, 161]. Further to these findings is the landmark observation that mice which receive a sub-lethal infection of *C. albicans* are protected for up to 2 weeks against a subsequent lethal re-infection in a monocyte-dependent, but T and B cell independent manner [162].

Intriguingly, macrophages demonstrate marked plasticity in their effector responses [163]. The importance of macrophage plasticity was made evident by Quintin et al. [162], who demonstrated that pre-exposure of macrophages to *C. albicans* (and particularly to cell wall β-glucan), results in “functional reprogramming” through epigenetic changes to histones [162, 164]. Further studies have demonstrated that this functional reprogramming
is driven by a Dectin-1/Raf1/NF-κB signalling circuit [165]. In addition, reprogrammed monocytes show a metabolic shift in glycolysis, akin to that seen in the Warburg effect associated with cancer [166]. The net effect of macrophage reprogramming is an increased production of pro-inflammatory cytokines including TNFα, IL-1β and IL-6.

10. Innate type 17 cells

The critical role that IL-17, and particularly the specific lymphocytes that produce it, play in innate immunity to C. albicans has recently come to light in a series of landmark studies [111, 135, 167]. These studies demonstrate that mice with defects in the IL-17 receptor or downstream signalling components are highly susceptible to OPC [111]. Equally, the genetic basis for many cases of chronic mucocutaneous candidiasis (a condition characterised by perpetual C. albicans infection of the skin, nails and mucosal surfaces), has now been identified as a collection of loss-of-function mutations in IL-17 signalling [168-170], and auto-antibodies against IL-17 [171, 172].

Although IL-17 is produced by Th17 T cells of the classical adaptive immune response, it is a common mistake to regard IL-17 as a cytokine that functions purely in an adaptive capacity. Indeed, detailed and extensive research clearly demonstrates that IL-17 is produced by a broad selection of innate immune cells, including γδ-T cells, natural killer T cells (NKT), type 3 innate lymphoid cells (ILC3) and TCRβ+ ‘natural’ Th17 cells (nTh17) [173]. While ILC3s have been suggested to play a major role in protection against OPC [174], recent research has since indicated that nTh17 and γδ-T cells are the predominant source of IL-17 as Rag1-/- mice, which produce ILC3 cells but not nTh17 or γδ-T cells, have the same high susceptibility to OPC as IL-17R-/- mice [167].
The induction of innate type 17 immunity differs considerably from conventional adaptive immune responses to *C. albicans*, which are considered to be activated through CARD9 signalling [175]. Notably, CARD9 signalling is not required to drive innate type 17 responses [176], and innate type 17 cells are observed to be activated in response to the secretion of Candidalysin from invading *C. albicans* hyphae [135]. Indeed, oral infection of mice with an *ece1Δ/Δ* *C. albicans* mutant (does not produce Candidalysin), fails to induce IL-17 production and the characteristic activation of nTh17 cells associated with wild type responses to OPC. Furthermore, Verma et al. [135] identify a causal link between the production of IL-1 cytokines and the activation of innate type 17 responses, a finding that integrates with studies which demonstrate the key role of inflammasomes during murine OPC [121].

11. Conclusions

The tremendous diversity within the microbial populations that comprise the human microbiota provides a dynamic and ever-present immunological challenge for mucosal surfaces. Numerous complex and interconnecting mechanisms function during innate recognition and mucosal responses to *C. albicans*. The complex molecular events that transpire to enable disparate mucosal sites to distinguish between commensalism and pathogenicity are receiving more attention now than ever. Continued research will undoubtedly increase our understanding of the essential mechanisms of innate immunity required to control this most common of mucosal pathobionts.

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**Figure captions**

**Figure 1. Innate mucosal immune responses to** *C. albicans.*

A) Adhesion of *C. albicans* hyphae to the host epithelium is mediated through two key adhesins; Als3p and Hwp1p. The epithelial PRR EphA2 interacts with β-glucan independently of fungal morphology. EphA2, E-cadherin and EGFR/HER2 receptor complexes function during receptor induced endocytosis (RIE), most notably through the interaction of E-cadherin and EGFR/HER2 with the *C. albicans* invasins Als3p and Ssa1p. B) Candidalysin is secreted from the hyphae of *C. albicans* where it inserts into the host epithelial membrane, damaging the cell through the formation of pores that result in loss of intracellular contents (i.e. lactate dehydrogenase (LDH)), and influx of calcium. C) Epithelial detection of Candidalysin activates the MAPK signalling pathway (particularly p38) which activates the AP-1 transcription factor c-Fos, driving cytokine expression. Pathway activity is fine-tuned through the action of MKP1 on p38 and JNK. D) In response to *C. albicans* infection, epithelial cells secrete antimicrobial peptides (AMPs) and alarmins that combat the invading fungus, together with cytokines and chemokines that recruit and activate myeloid cells.
including innate type 17 cells, neutrophils and macrophages. E) Following infection with C. albicans, epithelial cells release IL-1 which activates innate type 17 cellular responses, leading to the secretion of IL-17. F) Neutrophils interact with C. albicans through PRRs (TLRs, CLR and NLR), then phagocytose and destroy yeast and short hyphae. Cytokine signals stimulate release of TNF-α from neutrophils which upregulates TLR4 expression on epithelial cells, conferring additional protection. Hyphae that are too large to be phagocytosed stimulate the production of neutrophil extracellular traps (NETs) through a process termed NETosis. G) C. albicans that is phagocytosed by macrophages may avoid destruction through the induction of pyroptosis and hyphal growth, allowing the fungus to escape from the macrophage. Adapted from [177].

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Figure 1

A

E-cadherin

EphA2

EGFR/Her2

Hypha

Als3p/Ssa1p

Hwp1p

β-glucan

Ssa1p/Als3p

Epithelial Cell

B

Hypha

Epithelial Cell

Ca^{2+}

Ca^{2+}

Ca^{2+}

Ca^{2+}

LDH

LDH

Candidalysin pore

C

JNK

p38

ERK1/2

MKP1

Il-1β

Neutrophil

Macrophage

Cytokines

Chemokines

AMPs/Alarmins

Recruitment

Clearance

E

IL-17R

Induction of cytokines chemokines AMPs

D

Upregulation of TLR4

F

NLRs

CTLR

TLR

NETosis

G

Pyroptosis

IL-1β