Title: Candidalysin drives epithelial signaling, neutrophil recruitment, and immunopathology at the vaginal mucosa.

Running title: Candidalysin and vaginitis

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Unlike other forms of candidiasis, vulvovaginal candidiasis, caused primarily by the fungal pathogen *Candida albicans*, is a disease of immunocompetent and otherwise healthy women. Despite its prevalence, the fungal factors responsible for initiating symptomatic infection remain poorly understood. One of the hallmarks of vaginal candidiasis is the robust recruitment of neutrophils to the site of infection, which seemingly do not clear the fungus, but rather exacerbate disease symptomatology. Candidalysin, a newly discovered peptide toxin secreted by *C. albicans* hyphae during invasion, drives epithelial damage, immune activation and phagocyte attraction. Therefore, we hypothesized that Candidalysin is crucial for vulvovaginal candidiasis immunopathology.

Anti-*Candida* immune responses are anatomical site specific, as effective gastrointestinal, oral, and vaginal immunity is uniquely compartmentalized. Thus, we aimed to identify the immunopathologic role of Candidalysin and downstream signaling events at the vaginal mucosa. Microarray analysis of *C. albicans*-infected human vaginal epithelium *in vitro* revealed signaling pathways involved in epithelial damage responses, barrier repair, and leukocyte activation. Moreover, treatment of A431 vaginal epithelial cells with Candidalysin induced dose-dependent pro-inflammatory cytokine responses (including IL-1α, IL-1β and IL-8), damage, and activation of c-Fos and mitogen activated protein kinase (MAPK) signaling, consistent with fungal challenge. Mice intravaginally challenged with *C. albicans* strains deficient in Candidalysin exhibited no differences in colonization as compared to isogenic controls. However,
significant decreases in neutrophil recruitment, damage, and pro-inflammatory cytokine expression were observed with these strains. Our findings demonstrate that Candidalysin is a key hypha-associated virulence determinant responsible for the immunopathogenesis of \textit{C. albicans} vaginitis.

**INTRODUCTION**

Vulvovaginal candidiasis (VVC), caused primarily by the polymorphic fungal pathogen \textit{Candida albicans}, remains a serious worldwide health concern leading to significant quality of life issues for immunocompetent women (1). Symptomatic VVC is manifested by itching, burning, and pain sensation at the vaginal and vulvar tissue, often accompanied by odorless vaginal discharge (2). Globally, VVC is estimated to be the most prevalent human fungal infection, with over 75% of women experiencing at least one episode in their lifetime and 5-8% suffering from idiopathic recurrent infection (3). In recent years, VVC has been described as an immunopathology, in which the host neutrophil and associated cytokine response actually exacerbates disease symptoms, yet fails to adequately control the fungus (4-7). While much effort has been placed on defining host immunological mechanisms contributing to VVC protection, the fungal virulence factors that dictate conversion from asymptomatic colonization to fulminant infection remain poorly understood.

Using model systems, several laboratories have collectively begun to unravel this complex host-pathogen interaction. Studies revealed that vaginal (as well as oral) epithelial cells can discriminate between colonizing yeast and invasive hyphae by activation of the MAPK/c-Fos/MKP1 pathway and this "sensing" is concomitant with
cellular damage (8-10). This was largely recapitulated in the murine model of VVC, where hypha-deficient strains of *C. albicans* (e.g. *efg1Δ/Δ*, *efg1Δ/Δ/cph1Δ/Δ*, *NRG1* overexpression) colonized equally well or better than wild type strains, yet failed to induce hallmark immunopathology (e.g. PMN/neutrophil attraction, S100A8 production, IL-1β release) or mucosal damage (lactate dehydrogenase (LDH) release). It was fairly unsurprising that fungal burden alone was not sufficient for symptomatic infection, as the vaginal mucosa is often colonized by *C. albicans* without clinical presentation of disease. Similarly, a live challenge study in women volunteers demonstrated that fungal burden was not solely sufficient to explain VVC susceptibility, as women that were highly colonized did not always develop symptoms and *vice versa* (4). Therefore, these collective findings suggest that the yeast-to-hypha transition itself or downstream hypha-associated effectors are likely required for tissue damage and subsequent immunopathological inflammation at the vaginal mucosa. However, the precise fungal factors and mechanisms that contribute to neutrophil recruitment, induction of immunopathology, and mucosal damage remained elusive.

Recently, the *C. albicans ECE1* (extent of cell elongation) gene product was demonstrated to be crucial for cellular damage, innate cytokine production, and neutrophil recruitment during murine oropharyngeal candidiasis (OPC). *ECE1*, a highly expressed, hypha-associated gene encodes a protein (Ece1p) that is processed into eight distinct peptides by the fungal protease Kex2p (11, 12). Genetic, biochemical and functional assays determined that amino acids 62-92 of Ece1p encode a fungal toxin termed Candidalysin, which possesses both lytic and immunostimulatory activity (including MAPK signaling) on oral epithelial cells (12). Importantly, an *ece1Δ/Δ* null
mutant retains the capacity to form hyphae yet is unable to induce an inflammatory response. Given these observations, we hypothesized that Candidalysin may comparably activate vaginal epithelial cells and govern VVC immunopathology in vivo. This study demonstrates that a single fungal factor, Candidalysin, is responsible for inducing vaginal cellular damage and pro-inflammatory responses during C. albicans infection in vitro and in vivo. As such, the identification of a secreted toxin as the factor responsible for driving symptomatic vaginal inflammation may offer novel treatment modalities for arresting symptomatic disease.

RESULTS

Differential gene expression and pathway induction in reconstituted human vaginal epithelium following C. albicans challenge. The reconstituted human vaginal epithelium (RVE) model is an excellent in vitro surrogate to study epithelial-specific responses of vaginal candidiasis, as the tissue layer is sufficiently differentiated, supports robust hyphal invasion, and infected RVE tissue largely resemble in vivo dynamics (13, 14). In order to elucidate global host transcriptomic changes in vaginal epithelium in response to challenge with C. albicans (as compared to PBS sham control), total epithelial RNA was selectively isolated from three independent RVE at 6 and 24 h post-challenge and subjected to microarray analysis. As with oral epithelium, the intermediate (6 h) time point is associated with initial fungal adherence and microbial recognition, while the late (24 h) time point is associated with fungal invasion and cellular damage (10, 15). Approximately 800 and nearly 4,000 genes were differentially expressed (p <0.001) at 6 and 24 h, respectively in response to C. albicans.
Comparatively, few genes were regulated in response to PBS-sham treatment at the same time points (Fig. S1). At the intermediate stage of infection (6 h post-infection), the majority of differentially expressed genes were up-regulated (Fig 1A), with only 65 genes strongly up-regulated (> 4-fold) and none showing strong down-regulation (> 4-fold). However, by late stages of infection (24 h), an increase in the proportion of genes showing down-regulated expression was observed (Fig 1B).

Approximately 320 genes were strongly up-regulated at 24 h (> 4 fold) and over half of the genes showing up-regulation at 6 h were also strongly up-regulated at the later time point (Fig 1C). Surprisingly, relatively few genes were strongly (>4 fold) down-regulated in response to fungal challenge at either time point.

Gene ontology, pathway, and network mapping revealed profiles from C. albicans infected cells as consistent with MAPK, NF-κB, PI3K, ErbB receptor, and TNF signaling pathways (Fig. 2). Pathways involving extracellular matrix remodeling, including proteoglycans in cancer, focal adhesion, adherens junctions, and tight junctions were also significantly enriched during C. albicans infection. Pathways involved in responses to infection by other microbes, including Epstein-Barr virus, Shigella, Hepatitis B, Influenza A, Herpes virus, Salmonella, and trypanosome infection were also predicted to be activated, suggesting conservation of epithelial responses against a broad array of pathogens. Pathways predicted to be activated were generally conserved at 6 h and 24 h time points. A list of individually expressed genes may be found in Table S1.

Genes involved in innate inflammatory signaling were strongly induced by C. albicans, including those encoding cytokines IL-8 (100-fold), IL-1A (18-fold), IL-1B...
(3.8-fold), CXCL1 (19-fold), CXCL2 (26-fold), GM-CSF (10-fold), and prostaglandin synthase PTGS2 (7.3-fold), many of which play critical roles in recruiting inflammatory cells (particularly neutrophils) to the site of infection. Similar to previous findings, there was clear induction of genes associated with MAPK activity: MAP3K2 (6.8-fold), MAP2K3 (4-fold), MAP3K9 (4-fold), MAP4K4 (2.7-fold). Additionally, C. albicans infection led to epithelial induction of c-FOS (32-fold) and c-JUN (17.7-fold), which encode members of two families that form the heterodimeric transcription factor AP1, a major effector of MAPK activation. The dual specificity phosphatase 1 (DUSP1) gene, encoding a regulator of MAPK signaling, was also elevated (6.7-fold) in response to C. albicans.

A number of genes involved in tissue repair, wound healing, or dampening of active inflammation were also up-regulated during C. albicans infection, including the genes coding for IL-24 (2.3-fold) and IL-1RN (4-fold) (16, 17). Interestingly, a number of other related genes were also induced, including genes coding for HBEGF (heparin binding EGF-like growth factor, 39.5-fold) and EREG (epiregulin, 6-fold) that are members of the epidermal growth factors (EGFs). They exert their function by binding to their cognate receptors EGFR or v-erb-b2 oncogene homolog (ERBB) to induce cellular proliferation and healing of skin and epidermal tissues (18, 19).

**Candidalysin damages and activates vaginal epithelial cells.** As we observed an up-regulated expression of genes encoding several pro-inflammatory cytokines (e.g. IL-1A, IL-1B, IL-8, GM-CSF) and chemokines during RVE challenge with C. albicans at time points when hyphae invaded the vaginal tissue, we sought to determine whether
the hypha-associated peptide toxin Candidalysin similarly elicited these effector and damage responses. Indeed, there was a dose-dependent release of lactate dehydrogenase (LDH) when Candidalysin was applied to A431 cells (Fig. 3A). Significant levels of cellular damage were observed with doses above 15 μM as compared to treatment with the vehicle control.

Vaginal epithelial cells respond to *C. albicans* hyphae by activating the p38-MAPK and ERK1/2-MAPK signalling pathways, resulting in the regulated secretion of proinflammatory cytokines (8). To assess whether Candidalysin is capable of activating these pathways, epithelial cells were exposed to Candidalysin in vitro, and c-Fos production/MKP1 phosphorylation was assessed by Western blotting (Fig. 3B). The c-Fos/p-MKP1 response was induced strongly by 15 and 70 μM Candidalysin, whereas the vehicle was unable to activate signalling. Concomitant with damage, treatment with Candidalysin caused a dose-dependent increase in the release of IL-1α, IL-1β, G-CSF, GM-CSF and IL-8 in spent culture supernatants (Fig. 3C-H). The lone exception was IL-6, which was only significantly elevated at the highest Candidalysin concentration (70 μM). With the exception of IL-6, all cytokines assayed were significantly induced at Candidalysin doses above 3 μM; however, this dose was insufficient to cause significant damage (Fig. 3A), suggesting that Candidalysin exhibits dual functionality, serving both immunostimulatory and lytic roles against vaginal epithelial cells, similar to that observed in oral epithelia (12).

Candidalysin is required for vaginitis immunopathology. We next questioned whether Ece1p and/or Candidalysin contribute to immunopathology in an established
estrogen-dependent mouse model of vulvovaginal candidiasis (VVC). Therefore, we utilized strains of *C. albicans* that had been deleted for both copies of ECE1 (*ece1Δ/Δ*) and restored with one full-length allele (*ece1Δ/Δ+ECE1*) or one mutant allele lacking the Candidalysin-encoding region of ECE1 (*ece1Δ/Δ+ECE1Δ184-279*), along with the appropriate parental isogenic control (BWP17+Clp30: from here referred to as “WT”).

Somewhat surprisingly, recovered fungal burdens from the vaginal lavage fluid were not significantly different between strains at either d 3 (Fig. 4A) or d 7 (Fig. 4B) post-inoculation. However, there was a significant reduction in the number of neutrophils recruited into the vaginal lumen during challenge with either *ece1Δ/Δ* or *ece1Δ/Δ+ECE1Δ184-279* strains, which was restored to WT levels during infection with the *ece1Δ/Δ+ECE1Δ184-279* re-integrant strain (Fig. 4C,D,G, yellow arrows). Consistent with this phenotype, levels of the damage biomarker LDH were significantly reduced with these same mutants as compared to infection with WT or *ece1Δ/Δ+ECE1* re-integrant (Fig. 4E,F). Given our previous data using hypha deficient strains, a morphogenesis defect may account for this phenotype (6). However, *ece1Δ/Δ* and *ece1Δ/Δ+ECE1Δ184-279* strains robustly formed hyphae at the vaginal mucosa, as did WT and *ece1Δ/Δ+ECE1* strains (Fig. 4G, green arrows). Thus, these results demonstrate that Candidalysin is required for vaginal immunopathogenesis *in vivo* and that hypha formation alone is insufficient to elicit hallmark immunopathology.

*Candidalysin-dependent innate cytokine expression is conserved between mouse and human.* We also wanted to determine whether the Candidalysin-induced innate immune response observed in human vaginal epithelial cells paralleled cytokine
expression in the murine vaginal mucosa in vivo. RNA was isolated from whole vaginas of mice challenged with WT, ece1Δ/Δ, ece1Δ/Δ+ECE1, ece1Δ/Δ+ECE1Δ184-279 and PBS sham and gene expression assessed by qPCR. Overall, cytokine gene expression patterns were similar between in vitro and in vivo samples, including Candidalysin-induced expression of the genes Il-6, Cxcl2, Il-1a, and Il-1b (Fig. 5A,C-E). There was a similar trend for expression of the genes Cxcl1 and Gm-csf, although only the ECE1 null mutant (ece1Δ/Δ) demonstrated a statistically significant reduction in cytokine gene induction (Fig. 5B,F). Unexpectedly, G-csf gene expression was not increased during challenge with any of the fungal strains, unlike that observed with Candidalysin treatment (Fig. 5G). In the oral cavity, C. albicans induces expression of the antimicrobial peptide (AMP) cathelicidin, of which the murine equivalent is the cathelicidin related AMP (CAMP) (20). Interestingly, the gene encoding for CAMP was not induced in the vagina by Candidalysin, and in fact was down-regulated similarly by all strains as compared to sham treatment (Fig. 5H). However, induction of the antimicrobial peptide β-defensin 3 (mBD3) gene was Candidalysin-dependent (Fig. 5I).

We also sought to determine if two inflammatory markers previously identified as associated with VVC immunopathology were regulated in a Candidalysin-dependent manner. Expression of the gene coding for S100a8, a calcium-binding protein with important functions in antifungal defense and danger responses and strongly induced during C. albicans infection, was almost completely absent during infection with Candidalysin deletion strains (Fig. 5J) (21, 22). Similarly, the gene encoding serum amyloid A3 (Saa3), an inducible acute phase apolipoprotein capable of recruiting...
immune cells to inflammatory sites was similarly increased in a Candidalysin-dependent fashion (Fig. 5K) (23, 24).

Finally, we validated whether production of cytokines at the protein level (at both d 3 and d 7 p.i.) was Candidalysin-dependent. Indeed, *C. albicans*-mediated secretion of IL-1α, IL-1β, CXCL2, and S100A8 into the vaginal lavage fluid required expression of a functional Candidalysin (Fig. 6A-H). Despite increased expression of the genes encoding for IL-6, CXCL1, and GM-CSF in vaginal tissue, we were unable to demonstrably quantify these cytokines at the protein level in the lavage fluid of mice inoculated with any of the *C. albicans* strains tested (data not shown).

**DISCUSSION**

In recent years, vulvovaginal candidiasis has been identified as an immunopathology, in which the host immune response, orchestrated by a series of pro-inflammatory cytokines and chemokines, actually exacerbates symptomatic disease. A landmark live-challenge study conducted by Fidel and colleagues led to this paradigm shifting hypothesis, as the presence of neutrophils in the vaginal lavage fluid of women intravaginally inoculated with *C. albicans* was tightly correlated to disease symptomatology (e.g. vaginal itching, burning, discomfort) (4). Activation of innate immune signaling results in the recruitment of neutrophils to the vaginal mucosa and experimental evidence suggests that these cells then amplify the inflammatory cascade, seemingly without reducing fungal burden (22, 25). The *in vitro* and *in vivo* data presented in this study identify Candidalysin as the crucial virulence factor that drives both *C. albicans*-induced neutrophil recruitment and vaginal immunopathogenesis.
The vaginal epithelial response to *C. albicans* infection provides new insight into immunopathological signaling. Microarray data derived from *C. albicans*-infected human vaginal epithelial cells strongly paralleled what had been observed previously using targeted multiplex cytokine assays to determine host response to vaginal infection (8). Unsurprisingly, many of the genes uncovered by RNA-Seq as differentially regulated during murine vaginitis were not found in our human microarray datasets (26).

The first explanation of this is that the human response (microarray data) is not strictly homologous with the murine response. However, given the similarity and linkage of immunopathology with neutrophil influx to the vaginal lumen in both human and murine vaginal infections, this explanation seems less likely. A more plausible explanation is that the microarray data presented here provide an epithelial-specific response that is independent of hormonal modulation and the presence of other cell types. Murine RNA-Seq data were derived from whole vaginal tissue, thus hematopoetic and stromal compartments were similarly represented (26). While each strategy offers its own unique strengths and weaknesses, direct comparison between datasets must be made with caution. However, despite these methodological differences, there was relatively strong conservation between pro-inflammatory responses in both datasets, including eicosanoid signaling (*PLA2GB4*, *PTGS2*), S100 alarmin expression, and strong CXCL2 chemokine up-regulation (25-27). Additionally, increased expression of *IL-1β* and the IL-1 receptor antagonist (*IL1-RN*) genes was also identified in both datasets, suggesting that the IL-1 circuit is activated in a *C. albicans*-specific manner at the vaginal epithelium.
Recently, RNA-seq analysis of human cells collected by longitudinal vaginal swabs during healthy and symptomatic vaginitis states revealed that v-erb-b2 erythoblastic leukemia viral oncogene homolog 2, (ERBB2) and platelet-derived growth factor (PDGF-BB) signaling may have important function during vaginal candidiasis (28). Interestingly, our microarray dataset (human vaginal epithelium) did not directly demonstrate increased expression of genes encoding ERBB2 or PDGF-BB, but several genes coding for downstream targets of these factors were highly induced, including JUNB, FOS, DUSP5, NR4A1, IL-8, IL-1B, TNFAIP3, and EGR1, among others. Therefore, in vitro infection of A431 cells largely mimics responses observed during clinical vaginitis.

Another striking observation was the strong transcriptional up-regulation of the gene encoding EGF ligand heparin-binding EGF-like growth factor (HB-EGF, ~40-fold) and the gene coding for the enzyme heparin sulfate 3-O-sulfotransferase 1 (HS3ST1), suggesting the presence of heparin sulfate at the vaginal mucosa. Indeed, recent work by Yano et al. demonstrated that heparin sulfate can be recovered from the vaginal lavage fluid of mice and that its presence is enhanced by exogenous estrogen administration (29). Interestingly, treatment of recovered vaginal fluid with heparinase restored the capacity of PMNs to kill C. albicans in vitro, suggesting that heparin sulfate may phenotypically alter or physically inhibit neutrophil-fungus interaction. One potential mechanism was presented whereby heparin sulfate outcompetes the fungal surface antigen Pra1 for its natural ligand Mac1 present on the surface of neutrophils to prevent killing of the fungus (29, 30). Although the precise role of heparin sulfate at the vaginal mucosa remains unclear, it is often up-regulated in other epithelial or epidermal tissues.
in response to damage, functioning in the tissue repair process (31). Therefore, the
capacity of *C. albicans* hyphae and Candidalysin to damage epithelia and potentially
elevate free vaginal heparin sulfate may indirectly contribute to a fungal fitness strategy
to defend against PMN-mediated clearance at the mucosal surface. Collectively, these
results may help to explain why neutrophils are ineffective at reducing fungal burden
during vaginitis, despite being robustly recruited to the vaginal lumen indirectly by
Candidalysin.

**Candidalysin: the key fungal factor driving damage and vulvovaginal
immunopathogenesis.** Previous data generated from our laboratories have
demonstrated that both oral and vaginal epithelial cells can differentially sense and
respond to yeast and hyphal forms of *C. albicans* with modestly different signaling
mechanisms, cytokine secretion, and hyphal burden activation thresholds (6, 8, 10).
These differences in responses may represent site specific fine-tuning of mucosal
immunity—a hypothesis supported by the observed differences in transcription factors
activated in the two cell types (8, 10). Regardless, it is now clear that Candidalysin plays
a crucial role in activating epithelial responses at disparate mucosal sites. Interestingly,
some cytokines (e.g. IL-6) were induced by treatment with Candidalysin alone but not
during infection with *C. albicans*. Thus, it is likely that Candidalysin concentration plays
a major factor in its lytic and immunostimulatory function and currently it is unclear what
general or microniche concentrations of Candidalysin are present in an *ex vivo* or *in vivo*
setting or what other host or fungal factors (e.g. secreted aspartyl proteinases, cell wall
components) it may interact or synergize with during infection.
The hypothesis that epithelial surfaces discriminate between yeast and hyphal forms of *C. albicans* has been established by linking hypha formation with the capacity to damage epithelial surfaces *in vitro* and *in vivo* and the subsequent release of danger-associated molecular patterns (DAMP) to activate the cellular inflammasomes, including NLRP3 (6, 8, 10, 32-34). The use of NLRP3-/- mice during VVC has demonstrated that neutrophil migration and pro-inflammatory signaling is reduced in these animals, presumably due to a defect in the ability to recognize and respond to DAMP signals (26, 35). Furthermore, a population-level genetic study revealed that the 12/9 genotype was significantly associated with high levels of NLRP3 effector cytokines found in the vaginal lavage fluid of women with recurrent VVC (RVVC), suggesting the recognition of DAMP signals is important in disease immunopathogenesis (36). Given that the presence of Candidalysin is sufficient to induce damage at the vaginal mucosa and elicits inflammasome effector responses (i.e. IL-1β), it is possible that Candidalysin serves as a fungal DAMP capable of inflammasome activation. Investigations are currently underway to address these possibilities.

This concept of linking fungal pathogenicity to damage was further supported by findings from Schönherr et al. in which the virulence of *C. albicans* clinical isolates was directly correlated with their capacity to induce oral mucosal insult (43). Notably, only the expression of *ECE1*/Candidalysin was strongly correlated with damage and pathogenesis in several (but not all) *C. albicans* isolates. However, it is likely that simultaneous and combined expression of several attributes (e.g. hyphae and Candidalysin) is required for full virulence. The interplay of colonization, host response, and damage was elegantly summarized by Casadevall and Pirofski into “The Damage-
Response Framework” (DRF), a rubric describing a pathogen’s ability to cause disease on a continuum of host immune status and damage capacity (37). Recently, Jabra-Rizk and colleagues revisited DRF in the context of C. albicans pathogenesis specifically, concluding that VVC fits into class 6 of the DRF, in which a pathogen only causes damage in the context of an aggressive immune response (38). Based on these and previous findings, this appears to hold true, given that robust PMN and cytokine levels are strongly associated with disease symptomatology and centrally dependent on the capacity of Candidalysin to cause damage. However, neutrophil depleted mice (anti-Ly6G) exhibit equivalent vaginal LDH levels as isotype treated controls during experimental VVC, suggesting that mucosal damage still occurs in the absence of robust classical immunopathology (6). Moreover, PMN recruitment in humans is highly associated with disease onset, but has not been extensively evaluated as a requirement or worsening criterion for immunopathology. Therefore, it is arguable that VVC may be better categorized in class 5 of the DRF, by which the pathogen causes damage across the spectrum of immune responses but damage may be enhanced by strong immune responses.

In summary, this study demonstrates that Candidalysin is critical for the induction of immunopathological signaling at the vaginal mucosa, and that these responses are largely conserved at both human and murine epithelial surfaces. Furthermore, our findings decouple hypha formation per se from disease symptomatology and clearly link vaginitis immunopathogenesis with Candidalysin production and its capacity to directly damage the vaginal mucosa. In light of these findings, studies designed to determine the mechanistic interaction of Candidalysin with the vaginal epithelium are warranted.
Therapeutic strategies to either neutralize Candidalysin itself, inhibit its expression, or block downstream host signaling pathways may offer a unique opportunity to more quickly arrest symptomatology of this most prevalent human fungal infection.

**MATERIALS AND METHODS**

**Ethics statement.** The animals used in this study were housed in AAALAC-approved facilities located at the University of Tennessee Health Sciences Center (UTHSC) in the Regional Biocontainment Laboratory (RBL). The UTHSC Animal Care and Use Committee approved all animals and protocols. Mice were given standard rodent chow and water *ad libitum*. Mice were routinely monitored for signs of distress, including noticeable weight loss and lethargy.

**Cell lines, strains and primers.** The A431 human vulvar epidermoid carcinoma cell line was used in this study. All *C. albicans* strains used, including Candidalysin deletion mutants, are those described by Moyes, et al (12, 39). All primers used for quantitative PCR (qPCR) are listed in Table S2.

**Microorganism growth.** *C. albicans* strains were maintained as glycerol stocks stored at -80°C. A small amount of stock was spread onto yeast peptone dextrose (YPD) agar and incubated at 30°C for 48 h to obtain isolated colonies. A single colony was transferred to 10 mL of YPD liquid medium and incubated at 30°C with shaking at 200 rpm for 16 h prior to vaginal infection.
Microarray analysis. Reconstituted human vaginal epithelia (RVE: 5-day) created using the A431 cell line were purchased from SkinEthic Laboratories (France) and used as previously described (8). RNA was isolated from three independent RVE infected with C. albicans SC5314 for 6 and 24 h or an equal volume of PBS using the GenElute total mammalian RNA miniprep kit (Sigma, UK) and trace genomic DNA removed using the Turbo DNase-free kit (Ambion, UK). For microarray analysis, RNA was amplified using the MessageAmp Premier RNA Amplification Kit (Ambion, UK) and hybridized onto U133a 2.0 gene chips (Affymetrix, UK) after fragmentation by metal-induced hydrolysis into 35-200 nucleotide fragments according to standard protocols. Chips were scanned (Affymetrix GeneChip Scanner 3000) and assessed using Affymetrix Command Console (AGCC) software suite. This data was statistically analyzed using the Bioconductor R package, PIANO. Genes were considered to be differentially up- or down-regulated when their expression was changed by at least 2-fold with an fdr-adjusted p-value of less than 0.01. Gene Ontology and pathway analysis was performed on the generated gene list using both PIANO and DAVID (40, 41).

Cytokine release. A431 vaginal epithelial cells were cultured in Dulbecco’s Modified Eagle Medium Nutrient Mixture + L-glutamine (Life technologies) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Life technologies) and 1% (v/v) penicillin-streptomycin (Sigma) at 37°C, 5% CO₂. Candidalysin peptide (SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK) was purchased from Peptide Protein Research Ltd (UK). Prior to Candidalysin challenge, confluent A431 epithelial cells were serum-starved overnight and all experiments were carried out in serum-free DMEM.
medium. Cells were incubated with Candidalysin (prepared as a 10 mg/mL stock in sterile water) at doses of 1.5, 3, 15 and 70 μM for 2 h at 37°C in 5% CO₂. Sterile water (vehicle only) controls were also included. Culture supernatants were then isolated and human IL-1α, IL-1β, IL-6, IL-8, GM-CSF, and G-CSF quantified by Magnetic Luminex Performance Assay (Biotechne) and Bio-Plex 200 System (BioRad) according to the manufacturer’s instructions.

**Epithelial cell damage assay.** Damage to epithelial monolayers following a 24 h challenge with Candidalysin was determined by quantification of lactate dehydrogenase activity in cell culture supernatants using a CytoTox 96 non-radioactive cytotoxicity assay (Promega) according to the manufacturer’s instructions as previously described (12). Porcine lactate dehydrogenase (Sigma) was used to create the standard curve.

**Preparation of protein extracts.** Epithelial cells were lysed using a modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease (Sigma-Aldrich) and phosphatase (Perbio Science) inhibitors. Crude lysates were cleared by centrifugation at 4°C and protein concentration estimated by BCA assay (Thermo Scientific) according to the manufacturer’s instructions.

**SDS PAGE and Western blotting.** Proteins were resolved by electrophoresis on 12% SDS PAGE gels using a mini-protean tetra cell system (BioRad). Electrophoresed
proteins were transferred to nitrocellulose membrane (BioRad) using a mini-transblot
electrophoretic transfer cell (BioRad). p-DUSP1/MKP1 (S359) and c-Fos rabbit
monoclonal antibodies were purchased from Cell Signaling Technologies. Actin (clone
C4) mouse monoclonal antibody was purchased from Millipore. Peroxidase-conjugated
Affinipure Goat anti-mouse and anti-rabbit IgG secondary antibodies were purchased
from Jackson Immune Research. Membranes were blocked in 1 x TBS (Severn
Biotech) containing 0.001% (v/v) tween-20 (Acros Organics) and 5% (w/v) fat free milk
powder (Sainsbury’s). Primary antibodies diluted (1:1000 or 1:10,000 as suggested by
manufacturer) in TBS-tween and 5% milk (c-Fos), or TBS-tween and 5% bovine serum
albumin (p-DUSP1/MKP1) were added and membranes incubated overnight at 4°C with
gentle shaking. Following incubation, membranes were washed with 1 x TBS containing
0.01% (v/v) tween-20, diluted (1:10,000) HRP-conjugated secondary antibody added
and membranes incubated for 1 h at room temperature. Membranes were washed as
described and exposed to Immobilon Western Chemiluminescent HRP substrate
(Millipore) prior to visualisation by exposure to film (GE Healthcare). Alpha-actin was
used as a loading control.

Murine model of vaginal candidiasis. A murine model of Candida vaginitis was
utilized as described previously (6, 26, 42). Female 6-8 week old C57BL/6 mice were
purchased from Charles River laboratories and housed in isolator cages mounted onto
ventilated racks. Mice were administered 0.1 mg of estrogen (β-estradiol 17-valerate;
Sigma) dissolved in 0.1 mL sesame oil subcutaneously 72 h prior to inoculation with C.
albicans. Stationary-phase cultures of C. albicans strains were washed three times in
sterile, endotoxin-free phosphate-buffered saline (PBS) and resuspended in a 0.2 × volume of PBS. Cell suspensions were diluted, counted on a Neubauer hemocytometer, and adjusted to 5 × 10^8 CFU/mL in sterile PBS. Estrogen-treated mice were intravaginally inoculated with 10 μL of the standardized blastoconidial cell suspension, generating an inoculum size of 5 × 10^6 blastoconidia. At d 3 and/or d 7 p.i. mice underwent vaginal lavage with 100 μL of PBS. Resultant lavage fluids were spiked with 1 μL of 100 × EDTA-free protease inhibitors (Roche) and kept on ice until processing for immunopathological markers. After sacrifice, vaginal tissue was surgically excised and stored for downstream analyses. All animal experiments were conducted with n=4 mice per group, repeated, and data combined unless noted otherwise.

Assessment of fungal burden and vaginitis immunopathology. All immunopathological markers were assessed as described previously (6). (i) Lavage fluid was serially diluted 10-fold using the drop-plate method, plated onto YPD agar containing 50 μg/mL chloramphenicol, plates incubated for 24 h at 37°C, and the resulting colonies enumerated. CFU/mL values per group are reported as medians. (ii) Lavage fluid (10 μL) was smeared onto glass slides and stained by the Papanicolaou technique to assess polymorphonuclear leukocyte (PMNs) recruitment (small, blue, cells with multi-lobed nuclei). PMNs were counted in 5 non-adjacent fields by standard light microscopy using a 40X objective and values reported as mean + standard error of the mean (SEM). (iii) Murine IL-1α, IL-1β, CXCL2, and S100A8 was assessed in clarified, diluted (1:20-1:100) vaginal lavage fluid using commercial enzyme-linked immunosorbent assays (eBioscience, R&D Systems) according to manufacturer's
protocol. Results are reported as the mean + SEM. (iv) Lactate dehydrogenase (LDH) activity was measured in clarified, diluted (1:100) lavage fluid using the commercial available CytoTox 96 nonradioactive cytotoxicity assay (Promega). Results are reported as the mean + SEM.

**Isolation of RNA from vaginal tissue.** RNA was extracted from whole vaginas as described previously (26). At d 3 p.i., vaginal tissue was surgically excised, immediately placed into RNALater (Thermo Fisher), and incubated at 4°C overnight. The following day, tissues were transferred to TRI Reagent (Sigma), finely minced with scissors, mechanically homogenized (Pro Scientific), and centrifuged at 12,000 × g for 10 min at 4°C. RNA was isolated by chloroform-ethanol precipitation and the pellet resuspended in nuclease-free water according to TRI Reagent instructions. RNA concentration was measured by spectroscopy at A260/280 and integrity verified by 3-(N-morpholino)propanesulfonic acid (MOPS) gel electrophoresis to visualize intact 18s and 28s rRNA bands.

**qRT-PCR analysis.** RNA from vaginal tissue was isolated as described above. RNA concentrations were equalized amongst samples and 200 ng aliquots were treated with RNase-free DNase according to the manufacturer’s instructions (Thermo Scientific). RNA was reverse transcribed using random hexamers and the RevertAid kit according to the manufacturer’s protocol (Thermo Scientific). Proprietary primer sets spanning exon-exon junctions were ordered from IDT for murine *Il-6, Cxcl1, Cxcl2, Il-1a, Il-1b, Gm-csf, G-csf, Camp, S100a8, Saa3, Defb3, and Act1b* (Table S2). All primers were
used at the manufacturer’s recommended concentrations along with 2 × Maxima Sybr
Green mix (Bio-rad) to amplify 20 ng of cDNA. qPCR reactions were monitored and
analyzed with the Applied Biosystems 7500 platform and associated software.
Expression levels of target genes in infected mice were compared to a reference gene
(\textit{ACT1B}) and naïve controls using the \textit{\(\Delta\Delta\text{Ct}\)} method as described previously (43).

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FIGURE LEGENDS

Figure 1. Global differential gene expression induced during *C. albicans* infection of Reconstituted Vaginal Epithelium (RVE). Three independent RVE were challenged with *C. albicans* or mock infected with PBS for 6 or 24 h and differential gene expression (≥ 2-fold change) was assessed by microarray analysis. Volcano plots depicting log2 fold expression (blue dotted lines) at p<0.001 (red dotted line) changes of genes between (A) 6 h and (B) 24 h time points following challenge with *C. albicans* or PBS sham. (C) After adjusting for p-value and false discovery rate (fdr, p< 0.01), Venn-diagram plot depicts absolute number of genes expressed between *C. albicans* at 6 h (red) and 24 h (green).

Figure 2. Host signaling pathways predicted to be activated during *C. albicans* infection of RVE. Based on differential gene expression (≥ 2-fold change), KEGG pathway analysis using the DAVID web-based package revealed several host pathways predicted to be significantly (p<0.001) activated at (A) 6 h or (B) 24 h. Pathways are listed in order of highest probability of activation. Significance was assessed using DAVID statistical package via ANOVA analysis.
Figure 3. Candidalysin is sufficient to induce cellular damage and proinflammatory responses in vaginal epithelial cells. (A) A431 vaginal epithelial cells were exposed to Candidalysin (1.5, 3, 15, and 70 μM) for 24 h and cellular damage quantified by LDH assay. Data are presented as fold change relative to vehicle control. Statistics are applied relative to the vehicle control (n=3 biological repeats). (B) Western blot analysis of the vaginal epithelial response to different concentrations of Candidalysin. Epithelial cell lysates (20 μg total protein) were probed with anti c-Fos and anti p-MPK1 antibodies. One representative blot presented (from n=3 biological repeats). (C-H) Quantification of cytokines (IL-1α, IL-1β, G-CSF, GM-CSF, IL-6, and IL-8) secreted from vaginal epithelial cells in response to different concentrations of Candidalysin. Statistics are applied relative to the vehicle control (n=3 biological repeats). Graphs are plotted as the mean + SEM. A and C-H: Statistical significance was calculated using one-way ANOVA and Dunnet’s post-test. *** p < 0.001, ** p < 0.01, * p < 0.05.

Figure 4. Candidalysin is required for neutrophil recruitment and mucosal damage in a murine model of vulvovaginal candidiasis. Groups of estrogen-treated C57BL/6 mice (n=4) were intravaginally challenged with WT (black bars), ece1Δ/Δ (dark gray bars), ece1Δ/Δ+ECE1Δ184-279 (white bars), and ece1Δ/Δ+ECE1 (light gray bars) strains of C. albicans and vaginal lavage fluid assessed longitudinally at d 3 and d 7 for (A, B) fungal burden, median; (C, D) PMNs, mean + SEM; (E, F) the damage biomarker LDH, mean + SEM. (G) Papanicolaou staining was performed on smears made from vaginal lavage fluid to assess PMN influx (yellow arrows) and hypha formation (green...
arrows) at d 3 and d 7 p.i. and are representative images. All inoculation groups were performed in duplicate and data combined. A-F: Statistical significance was calculated using a one-way ANOVA and Tukey’s post-test. *** p < 0.001, ** p < 0.01, * p < 0.05.

Figure 5. Candidalysin is required for pro-inflammatory cytokine expression in the murine vagina. Groups of estrogen-treated C57BL/6 mice (n=4) were intravaginally challenged with WT (black bars), ece1Δ/Δ (dark gray bars), ece1Δ/Δ+ECE1Δ184-279 (white bars), and ece1Δ/Δ+ECE1 (light gray bars) strains of C. albicans, whole vaginal tissue excised at d 3 p.i., and extracted RNA processed for qPCR analysis. Genes chosen for qPCR included those previously identified as being induced by Candidalysin or C. albicans during in vitro or in vivo challenge, including: (A) Il-6, (B) Cxcl1, (C) Cxcl2, (D) Il-1a, (E) Il-1b, (F) Gm-csf, (G) G-csf, (H) Camp, (I) Defb3, (J) S100A8, and (K) Saa3. All genes were internally compared to the Actb housekeeping gene and to mock-infected controls using the ΔΔCt method. Graphs are plotted as the mean normalized fold expression + SEM. Statistical significance was calculated using a one-way ANOVA and Tukey’s post-test. ** p < 0.01, * p < 0.05.

Figure 6. Candidalysin is required for release of hallmark proinflammatory cytokines and chemokines into the vaginal lavage fluid during murine vaginitis. Groups of estrogen-treated C57BL/6 mice (n=4) were intravaginally challenged with WT (black bars), ece1Δ/Δ (dark gray bars), ece1Δ/Δ+ECE1Δ184-279 (white bars), and ece1Δ/Δ+ECE1 (light gray bars) strains of C. albicans and vaginal lavage fluid assessed longitudinally by ELISA at d 3 and d 7 p.i. for inflammatory markers, including (A, B) IL-
All inoculation groups were performed in duplicate and data combined. Graphs are plotted as the mean ± SEM. Statistical significance was calculated using a one-way ANOVA and Tukey’s post-test. *** p < 0.001, ** p < 0.01, * p < 0.05.