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1	Title: Candidalysin drives epithelial signaling, neutrophil recruitment, and	
2	immunopathology at the vaginal mucosa.	
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3031 ABSTRACT

32

33 Unlike other forms of candidiasis, vulvovaginal candidiasis, caused primarily by 34 the fungal pathogen Candida albicans, is a disease of immunocompetent and otherwise 35 healthy women. Despite its prevalence, the fungal factors responsible for initiating 36 symptomatic infection remain poorly understood. One of the hallmarks of vaginal 37 candidiasis is the robust recruitment of neutrophils to the site of infection, which 38 seemingly do not clear the fungus, but rather exacerbate disease symptomatology. 39 Candidalysin, a newly discovered peptide toxin secreted by C. albicans hyphae during 40 invasion, drives epithelial damage, immune activation and phagocyte attraction. 41 Therefore, we hypothesized that Candidalysin is crucial for vulvovaginal candidiasis 42 immunopathology.

43 Anti-Candida immune responses are clearly anatomical site specific, as effective 44 gastrointestinal, oral, and vaginal immunity is uniquely compartmentalized. Thus, we 45 aimed to identify the immunopathologic role of Candidalysin and downstream signaling 46 events at the vaginal mucosa. Microarray analysis of *C. albicans*-infected human 47 vaginal epithelium in vitro revealed signaling pathways involved in epithelial damage 48 responses, barrier repair, and leukocyte activation. Moreover, treatment of A431 vaginal 49 epithelial cells with Candidalysin induced dose-dependent pro-inflammatory cytokine 50 responses (including IL-1 α , IL-1 β and IL-8), damage, and activation of c-Fos and mitogen activated protein kinase (MAPK) signaling, consistent with fungal challenge. 51 52 Mice intravaginally challenged with C. albicans strains deficient in Candidalysin 53 exhibited no differences in colonization or hyphal burdens as compared to isogenic

controls. However, significant decreases in neutrophil recruitment, damage, and proinflammatory cytokine expression were observed with these strains. Our findings
demonstrate that Candidalysin is a key hypha-associated virulence determinant
governing the immunopathogenesis of *C. albicans* vaginitis.

58

59 **INTRODUCTION**

60 Vulvovaginal candidiasis (VVC), caused primarily by the polymorphic fungal 61 pathogen Candida albicans, remains a serious worldwide health concern leading to 62 significant quality of life issues for immunocompetent women (1). Symptomatic VVC is 63 manifested by itching, burning, and pain sensation at the vaginal and vulvar tissue, 64 often accompanied by odorless vaginal discharge (2). Globally, VVC is estimated to be 65 the most prevalent human fungal infection, with over 75% of women experiencing at 66 least one episode in their lifetime and 5-8% suffering from recurrent infection (3). In 67 recent years, VVC has been described as an immunopathology, in which the host 68 neutrophil response actually exacerbates disease symptoms, yet fails to adequately 69 control the fungus (4, 5). While much effort has been placed on defining host 70 immunological mechanisms contributing to VVC protection, the fungal virulence factors 71 that dictate conversion from asymptomatic colonization to fulminant infection remain 72 poorly understood.

Using model systems, several laboratories have collectively begun to unravel this complex host-pathogen interaction. Studies revealed that vaginal epithelial cell damage and immune activation is dependent on *C. albicans* hypha formation, with epithelial immunity being mediated via the MAPK transcription factor c-Fos and regulated by the

MAPK phosphatase MKP1 (6). This MAPK/c-Fos/MKP1 pathway is able to discriminate
between *C. albicans* yeast and hyphal cells and is retained between both vaginal and
oral epithelia (7, 8). The activation of this signaling pathway is also coupled with lactate
dehydrogenase (LDH) release, a marker of epithelial damage (6). Therefore, the ability
of *C. albicans* to form hyphae and breach the epithelial barrier governs innate cytokine
release and pro-inflammatory responses.

83 Using a clinically relevant murine model of VVC, it was also determined that 84 genetic blockade of hypha formation (e.g. $efg1\Delta/\Delta$, $efg1\Delta/\Delta/cph1\Delta/\Delta$, NRG1 85 overexpression) failed to induce hallmark immunopathology at the vaginal mucosa, 86 including polymorphonuclear leukocyte (PMN/neutrophil) recruitment, S100A8 release, 87 IL-1β production, and LDH release confirming a crucial role for hyphae in this disease 88 (9). Moreover, depletion of neutrophils *in vivo* failed to reduce LDH release from mice 89 infected with hypha-competent strains of C. albicans (9, 10). Although, depletion of 90 PMNs did significantly reduce several hallmark cytokines and chemokines during 91 infection. These data suggest that C. albicans directly damages the vaginal mucosa 92 which elicits migratory neutrophils as secondary effectors that amplify symptomatic 93 vaginal inflammation. However, the fungal factors and mechanisms that contribute to 94 neutrophil recruitment and induction of immunopathology remain elusive. In general, 95 these experiments recapitulated findings previously observed using human vaginal 96 epithelial cell lines (6). Using a combination of RNA-Seq and targeted knockout studies, 97 it was demonstrated that the NLRP3 inflammasome, a host signaling complex activated 98 in response to cellular damage, is crucial for IL-1 β release and PMN recruitment to the 99 vaginal mucosa during C. albicans infection, consistent with candidal infection at other

anatomical sites (11). However, an important question remained: is hypha formation *per* se sufficient for damage-induced inflammation or are distinct fungal factors associated
 with the morphogenetic transition involved in these processes?

103 Until recently, individual gene deletions in *C. albicans* have demonstrated only 104 modest phenotypic alterations with respect to fungal colonization or cytokine signaling 105 at the vaginal mucosa; the greatest reported effects being with deletion of the secreted 106 aspartyl proteinase genes, SAP2 and SAP5 (11-14). However, recent work has 107 revealed that the C. albicans ECE1 (extent of cell elongation) gene product is crucial for 108 cellular damage, innate cytokine production, and neutrophil recruitment during murine 109 oropharyngeal candidiasis (OPC). ECE1, a highly expressed, hypha-associated gene 110 encodes a protein (Ece1p) that is processed into eight distinct peptides by the fungal 111 protease Kex2p (15, 16). Genetic, biochemical and functional assays determined that 112 amino acids 62-92 of Ece1p encode a fungal toxin termed Candidalysin, which 113 possesses both lytic and immunostimulatory activity (including MAPK signaling) on oral 114 epithelial cells (16). Importantly, an $ece 1\Delta/\Delta$ null mutant retains the capacity to form 115 hyphae yet is unable to induce an inflammatory response. Moreover, ECE1 expression 116 is largely dependent on the Efg1 transcription factor, likely explaining why efg1 Δ/Δ and 117 $efg1\Delta/\Delta/cph1\Delta/\Delta$ mutants fail to fully activate MAPK signaling and immunopathology in 118 oral or vaginal epithelium (6, 8, 9, 17).

Given these similarities, 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.

126

127 **RESULTS**

128 Differential gene expression and pathway induction in reconstituted human

129 vaginal epithelium following C. albicans challenge. The reconstituted human vaginal 130 epithelium (RVE) model is an excellent in vitro surrogate to study epithelial-specific 131 responses of vaginal candidiasis, as the tissue layer is sufficiently differentiated and 132 supports robust hyphal invasion and infected RVE tissue largely resemble the *in vivo* 133 situation (18, 19). In order to elucidate global host transcriptomic changes in vaginal 134 epithelium in response to challenge with C. albicans as compared to PBS sham control, 135 total epithelial RNA was selectively isolated from RVE at 6 and 24 h post-challenge and 136 subjected to microarray analysis. As with oral epithelium, the intermediate (6 h) time 137 point is associated with initial fungal adherence and detection, while the late (24 h) time 138 point is associated with fungal invasion and cellular damage (8, 20). Approximately 800 139 and nearly 4,000 genes were differentially expressed (P < 0.001) at least 2-fold at 6 and 140 24 h, respectively in response to *C. albicans* (Fig. 1). Comparatively, few genes were regulated in response to PBS-sham treatment at the same time points (Fig. S1). At the 141 142 intermediate stage of infection (6 h post-infection), the majority of differentially 143 expressed genes are up-regulated (Fig 1A) with none showing strong down-regulation. 144 However, by late stages of infection (24 h), there is an increase in the proportion of 145 genes showing down-regulated expression (Fig 1B). Over half of the genes showing up-

146 regulation at 6 h were similarly up-regulated at 24 h (Fig 1C). Surprisingly, relatively few 147 genes were strongly (>4 fold) down-regulated in response to fungal challenge at either 148 time point. Commensurate with these associations, both time points include genes 149 associated with MAPK (phosphatases, accessory proteins and transcription factors), 150 NF- κ B and PI3K signaling. The early time point includes the expression of many genes 151 encoding early/immediate transcription factors (such as c-Fos) and signaling modulators 152 (such as the DUSP phosphatases) that are associated with the immediate signaling 153 response to the presence of C. albicans. However, the late time point contains a far 154 more diverse set of genes, such as downstream effector genes, including cytokines. 155 This pool represents the cumulative responses driven by the continued presence of C. 156 albicans hyphae along with subsequent released host factors. At both time points, the up-regulated expression of a variety of transcription factor genes associated with other 157 158 signaling pathways was observed, as well as genes coding for cytokines and effector 159 molecules.

160 Gene ontology, pathway, and network mapping using the web-based analysis 161 package DAVID revealed profiles from C. albicans infected cells as consistent with 162 MAPK, NF-KB PI3K, ErbB receptor, and TNF signaling pathways (Fig. 2). Pathways 163 involving extracellular matrix remodeling, including proteoglycans in cancer, focal 164 adhesion, adherens junctions, and tight junctions were also significantly enriched during 165 C. albicans infection. Pathways involved in responses to infection by other microbes, 166 including Epstein-Barr virus, *Shigella*, Hepatitis B, Influenza A, Herpes virus, 167 Salmonella, and trypanasome infection were also predicted to be activated, suggesting 168 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 ofindividually expressed genes may be found in Table S1.

171 Genes involved in innate inflammatory signaling were strongly induced by 172 C. albicans, including those encoding cytokines IL-8 (100-fold), IL-1A (18-fold), IL-1B 173 (3.8-fold), CXCL1 (19-fold), CXCL2 (26-fold), GM-CSF (10-fold), and prostaglandin 174 synthase PTGS2 (7.3-fold), many of which play critical roles in recruiting inflammatory 175 cells (particularly neutrophils) to the site of infection. Similar to previous findings, there 176 was clear induction of genes associated with MAPK activity: MAP3K2 (6.8-fold), 177 MAP2K3 (4-fold), MAP3K9 (4-fold), MAP4K4 (2.7-fold). Additionally, C. albicans 178 infection led to epithelial induction of *c-FOS* (32-fold) and *c-JUN* (17.7-fold), which 179 encode members of two families that form the heterodimeric transcription factor AP1, a 180 major effector of MAPK activation. The dual specificity phosphatase 1 (DUSP1) gene, 181 encoding a regulator of MAPK signaling, was also elevated (6.7-fold) in response to C. 182 albicans. 183 A number of other genes were induced that are involved in tissue repair, wound 184 healing, or dampening of active inflammation, including the genes coding for IL-24 (2.3-185 fold) and IL-1RN (4-fold) were increased during C. albicans infection (21, 22). 186 Interestingly, a number of other related genes were also induced, including genes 187 coding for HBEGF (heparin binding EGF-like growth factor, 39.5-fold) and EREG 188 (epiregulin, 6-fold) that are members of the epidermal growth factors (EGFs). They exert 189 their function by binding to their cognate receptors EGFR or v-erb-b2 oncogene 190 homolog (ERBB) to induce cellular proliferation and healing of skin and epidermal 191 tissues (23, 24).

193	Candidalysin damages and activates vaginal epithelial cells. As we observed an
194	up-regulated expression of genes encoding several pro-inflammatory cytokines (e.g. IL-
195	1A, IL-1B, IL-8, GM-CSF) and chemokines during RVE challenge with C. albicans at
196	time points when hyphae invaded the vaginal tissue, we sought to determine whether
197	the hyphae associated peptide toxin Candidalysin similarly elicited these effector and
198	damage responses. Indeed, there was a dose-dependent release of lactate
199	dehydrogenase (LDH) when Candidalysin was applied to A431 cells (Fig. 3A).
200	Significant levels of cellular damage were observed with doses above 15 μM as
201	compared to treatment with the vehicle control.
202	Vaginal epithelial cells respond to <i>C. albicans</i> hyphae by activating the p38-
203	MAPK and ERK1/2-MAPK signalling pathways, resulting in the regulated secretion of
204	proinflammatory cytokines (6). However, the fungal factors governing epithelial
205	activation are unknown. To assess whether Candidalysin is capable of activating p38-
206	MAPK and ERK1/2-MAPK signalling pathways, epithelial cells were exposed to
207	Candidalysin in vitro, and c-Fos production/MKP1 phosphorylation was assessed by
208	Western blotting (Fig. 3B). The c-Fos/p-MKP1 response was induced strongly by 70 and
209	15 μ M Candidalysin, whereas the vehicle was unable to activate signalling. Concomitant
210	with damage, treatment with Candidalysin caused a dose-dependent increase in the
211	release of IL-1 α , IL-1 β , G-CSF, GM-CSF and IL-8 in spent culture supernatants (Fig.
212	3C-H). The lone exception was IL-6, which was only significantly elevated at the highest
213	Candidalysin concentration (70 μ M). With the exception of IL-6, all cytokines assayed
214	were significantly induced at Candidalysin doses above 3 μ M; 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 (16).

218

219 **Candidalysin is required for vaginitis immunopathology.** We next questioned 220 whether Ece1 and/or Candidalysin contribute to immunopathology in an established 221 estrogen-dependent mouse model of vulvovaginal candidiasis (VVC). Therefore, we 222 utilized strains of C. albicans that had been deleted for both copies of ECE1 (ece $1\Delta/\Delta$) 223 and restored with one full-length allele ($ece 1\Delta/\Delta + ECE1$) or one mutant allele lacking the 224 Candidalysin-encoding region of ECE1 (ece1 Δ/Δ +ECE1_{Δ 184-279}), along with the 225 appropriate parental isogenic control (BWP17+Clp30: from here referred to as "WT"). 226 Somewhat surprisingly, recovered fungal burdens from the vaginal lavage fluid were not 227 significantly different between strains at either d 3 (Fig. 4A) or d 7 (Fig. 4B) post-228 inoculation. However, there was a significant reduction in the number of neutrophils 229 recruited into the vaginal lumen during challenge with either $ece 1\Delta/\Delta$ or 230 $ece1\Delta/\Delta + ECE1_{A184-279}$ strains, which was restored to WT levels during infection with the 231 $ece1\Delta/\Delta + ECE1$ re-integrant strain (Fig. 4C,D,G, yellow arrows). Consistent with this 232 phenotype, levels of the damage biomarker LDH were significantly reduced with these 233 same mutants as compared to infection with WT or $ece 1\Delta/\Delta + ECE1$ re-integrant (Fig. 234 4E,F). Given our previous data using hypha deficient strains, a morphogenesis defect 235 may account for this phenotype (9). However, $ece 1\Delta/\Delta$ and $ece 1\Delta/\Delta + ECE1_{\Delta 184-279}$ 236 strains formed hyphae normally at the vaginal mucosa, as did WT and $ece 1\Delta/\Delta + ECE1$ 237 strains (Fig. 4G, green arrows). Thus, these results demonstrate that Ece1 is required

for vaginal immunopathogenesis *in vivo* and that hypha formation alone is insufficient toelicit hallmark immunopathology.

240

241 Candidalysin-dependent innate cytokine expression is conserved between mouse 242 and human. We also wanted to determine whether the Candidalysin-induced innate 243 immune response observed in human vaginal epithelial cells paralleled cytokine 244 expression in the murine vaginal mucosa in vivo. RNA was isolated from whole vaginas 245 of mice challenged with WT, $ece1\Delta/\Delta$, $ece1\Delta/\Delta + ECE1$, $ece1\Delta/\Delta + ECE1_{\Delta 184-279}$ and PBS 246 sham and gene expression assessed by qPCR. Overall, cytokine gene expression 247 patterns were similar between in vitro and in vivo samples, including Candidalysin-248 induced expression of the genes *II-6*, *Cxcl2*, *II-1a*, and *II-1b* (Fig. 5A,C-E). There was a 249 similar trend for expression of the genes Cxcl1 and Gm-csf, although only the ECE1 null 250 mutant (ece $1\Delta/\Delta$) demonstrated a statistically significant reduction in cytokine gene 251 induction (Fig. 5B,F). Unexpectedly, G-csf gene expression was not increased during 252 challenge with any of the fungal strains, unlike that observed with Candidalysin 253 treatment (Fig. 5G). In the oral cavity, C. albicans induces expression of the 254 antimicrobial peptide (AMP) cathelicidin, of which the murine equivalent is the 255 cathelicidin related AMP (CAMP) (25). Interestingly, the gene encoding for CAMP was 256 not induced in the vagina by Candidalysin, and in fact was down-regulated similarly by 257 all strains as compared to sham treatment (Fig. 5H). However, induction of the 258 antimicrobial peptide β -defensin 3 (mBD3) gene was Candidalysin-dependent (Fig. 5I). 259 We also sought to determine if two inflammatory markers previously identified as 260 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) (26, 27). 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 Candidalysindependent fashion (Fig. 5K) (28, 29).

268 Lastly, we validated whether induction of several of these cytokines at the protein 269 level by ELISA at both d 3 and d 7 p.i. was Candidalysin-dependent. Indeed, 270 *C. albicans*-mediated secretion of IL-1 α , IL-1 β , CXCL2, and S100A8 into the vaginal 271 lavage fluid required expression of a functional Candidalysin (Fig. 6A-H). Despite 272 increased expression of the genes encoding for IL-6, CXCL1, and GM-CSF in vaginal 273 tissue, we were unable to demonstrably quantify these cytokines at the protein level in 274 the lavage fluid of mice inoculated with any of the C. albicans strains tested (data not 275 shown).

276

277 **DISCUSSION**

In recent years, vulvovaginal candidiasis has been identified as an
immunopathology, in which the host immune response, orchestrated by a series of proinflammatory cytokines and chemokines, actually exacerbates symptomatic disease. A
landmark live-challenge study conducted by Fidel and colleagues led to this paradigm
shifting hypothesis, as presence of neutrophils in the vaginal lavage fluid of women
intravaginally inoculated with *C. albicans* was tightly correlated to disease

284 symptomatology (e.g. vaginal itching, burning, discomfort) (4). Ultimately, initial 285 activation of innate immune signaling recruit neutrophils to the vaginal mucosa and 286 experimental evidence suggests that these cells then amplify the inflammatory cascade, 287 seemingly without reducing fungal burden (27, 30). Although several data suggest that 288 C. albicans hyphal morphology is associated with immunopathology, the precise 289 virulence attributes that enable *C. albicans* to initiate these early signaling events at the 290 vaginal mucosa have not been well defined. The *in vitro* and *in vivo* data presented in 291 this study identify Candidalysin as the crucial virulence factor that drives both, 292 C. albicans-induced neutrophil recruitment and vaginal immunopathogenesis. 293 The vaginal epithelial response to *C. albicans* infection provides new insight into 294 immunopathological signaling. Microarray data derived from C. albicans-infected 295 human vaginal epithelial cells strongly paralleled what had been observed previously 296 using targeted multiplex cytokine assays to determine host response to vaginal infection 297 (6). Unsurprisingly, many of the genes uncovered by RNA-Seg as differentially 298 regulated during murine vaginitis were not found in the human microarray datasets (11). 299 The first explanation of this is that the human response (microarray data) is not strictly 300 homologous with the murine response. However, given the similarity and linkage of 301 immunopathology with neutrophil influx to the vaginal lumen in both human and murine 302 vaginal infections, this explanation seems less likely. A more plausible explanation is 303 that the microarray data presented here provide an epithelial-specific response that is 304 independent of hormonal modulation or other cell-type intervention. Murine RNA-Seq 305 data were derived from whole vaginal tissue, thus hematopoetic and stromal 306 compartments were similarly represented (11). While each strategy offers its own

307 unique strengths and weaknesses, direct comparison between datasets must be done 308 with caution. However, despite these methodological differences, there was relatively 309 strong conservation between pro-inflammatory responses in both datasets. In both 310 human and murine tissues, expression of phospholipase A2 (PLA2GB4) and 311 prostaglandin synthase 2 (PTGS2) genes were both increased, suggesting activation of 312 eicosanoid signaling at the vaginal mucosa. Indeed, this had been previously (indirectly) 313 confirmed in vivo by measuring prostaglandin E2 metabolites in the vaginal lavage fluid 314 of infected mice and human volunteers (11, 31). Increased expression of *IL-1B* and the 315 IL-1 receptor antagonist (IL1-RN) genes was also identified in both datasets, suggesting 316 that IL-1 circuit is activated in a *C. albicans*-specific manner at the vaginal epithelium. 317 Similarly, previous work conducted by Fidel and colleagues investigating the release of 318 S100A8/A9 alarmins in murine vaginal lavage fluid following *C. albicans* infection and 319 immunohistological staining identified their increased expression in the vaginal 320 epithelium (30). Expression of S100A8/9 genes was not identified in the microarray 321 analysis per se, but a gene encoding the functionally related RAGE-agonist S100P, also 322 implicated in innate inflammation and AP1 activation, was induced following C. albicans 323 challenge (32). In a similar fashion, a gene encoding the neutrophil attractant CXCL2, a 324 downstream effector of Th17 responses, was strongly up-regulated in all datasets, 325 suggesting that this chemokine may play a major role in attracting neutrophils to the site 326 of infection (33).

An interesting observation from the microarray analysis was that a gene encoding the neuronal precursor of cell expressed developmentally down-regulated 9 (*NEDD9*) was increased 5.3-fold post-*C. albicans* challenge. Recently using RNA-Seq,

330 Liu et al. demonstrated that the NEDD9 pathway was activated in oral keratinocytes 331 following C. albicans infection (34). However, further analysis by this group, examining 332 gene expression derived from longitudinal vaginal swabs during healthy and 333 symptomatic vaginitis states revealed that NEDD9 was not activated at the vaginal 334 mucosa, but instead activated pathways were predicted to involve v-erb-b2 335 erythroblastic leukemia viral oncogene homolog 2, (ERBB2) and platelet-derived growth 336 factor (*PDGF-BB*) signaling in the vagina. Given these disparate results, it is unclear if 337 *NEDD9* may play a role in activating gene expression at the vaginal mucosa in 338 response to *C. albicans*. Interestingly, our microarray dataset (human vaginal 339 epithelium) did not directly demonstrate increased expression of genes encoding 340 ERBB2 or PDGF-BB, but several genes coding for downstream targets of these factors 341 were highly induced, including JUNB, FOS, DUSP5, NR4A1, IL-8, IL-1B, TNFAIP3, and 342 EGR1, among others. Many of these same downstream targets were previously found 343 to be induced in oral epithelial cells on the protein level (35). Therefore, *in vitro* infection 344 of A431 cells largely mimics responses observed in both oral epithelial cells and during 345 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 potential 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 (36). Interestingly, treatment of recovered vaginal fluid with heparinase

353 restored the capacity of PMNs to kill C. albicans in vitro, suggesting that heparin sulfate 354 may phenotypically alter or physically inhibit neutrophil-fungus interaction. One potential 355 mechanism was presented whereby heparin sulfate outcompetes the fungal surface 356 antigen Pra1 for its natural ligand Mac1 present on the surface of neutrophils to prevent 357 killing of the fungus (36, 37). Although the precise role of heparin sulfate at the vaginal 358 mucosa remains unclear, it is often up-regulated in other epithelial or epidermal tissues 359 in response to damage, functioning in the tissue repair process (38). Therefore, the 360 capacity of C. albicans hyphae and Candidalysin to damage epithelia and potentially 361 elevate free vaginal heparin sulfate may indirectly contribute to a fungal fitness strategy 362 to defend against PMN-mediated clearance at the mucosal surface. Collectively, these 363 results may help explain why neutrophils are ineffective at reducing fungal burden 364 during vaginitis, despite being robustly recruited to the vaginal lumen.

365 Candidalysin: the key fungal factor driving damage and vulvovaginal 366 **immunopathogenesis.** Previous data generated from our laboratories have 367 demonstrated that both oral and vaginal epithelial cells can differentially sense and 368 respond to yeast and hyphal forms of *C. albicans* (6, 8, 9). However, modestly different 369 signaling mechanisms, cytokine secretion, and hyphal burden thresholds differentiate 370 responses in oral and vaginal epithelial cells. For example, previous studies by Moyes 371 et al. determined that the A431 vaginal cell line responds to *C. albicans* challenge in a 372 similar fashion to TR146 oral cells, albeit at fungal burdens roughly one log greater (6). 373 Moreover, the cytokines CCL20, G-CSF, and IL-6 were robustly secreted by oral 374 epithelia in response to C. albicans infection. However, these responses were 375 comparatively reduced or not observed during in vitro infection of vaginal epithelium or

376 at the murine vaginal mucosa. These differences in responses could be due to the 377 abovementioned apparent elevated signaling threshold in vaginal cells or may represent 378 site specific fine-tuning of mucosal immunity-a hypothesis supported by the observed 379 differences in transcription factors activated in the two cell types (6, 8). Furthermore, 380 some cytokines (e.g. IL-6) were induced by treatment with Candidalysin alone but not 381 during infection with *C. albicans*. It is likely that Candidalysin concentration plays a 382 major factor in its lytic and immunostimulatory function and currently it is unclear what 383 general or microniche concentrations of Candidalysin are present in an ex vivo or in vivo 384 setting or what other host or fungal factors it interacts with during infection.

385 The hypothesis that epithelial surfaces can discriminate between yeast and 386 hyphal forms of *C. albicans* has been established by linking hypha formation with the 387 capacity to damage epithelial surfaces (6, 8). These observations have recently been 388 extended into *in vivo* findings, as a similar phenomenon also appears to be true at the 389 murine vaginal mucosa (9, 39). Indeed, hypha-deficient mutants of *C. albicans* fail to 390 induce pro-inflammatory signaling, despite very high levels of colonization. One key 391 feature explaining these dichotomous responses is lack of elicited epithelial damage 392 and subsequent danger-associated molecular pattern (DAMP) release by hypha-393 deficient strains. This hypothesis is further supported by studies examining the role of 394 the NLRP3 inflammasome, a danger-sensing molecular complex, in mediating 395 inflammation at both oral and vaginal mucosal surfaces (40, 41). The use of NLRP3-/-396 mice during oral and vaginal candidiasis has demonstrated that neutrophil migration and 397 pro-inflammatory signaling is reduced in these animals, presumably due to a defect in 398 the ability to recognize and respond to DAMP signals (11, 25, 42, 43). As for vaginal

399 candidiasis, a population-level genetic study revealed that the 12/9 genotype was 400 significantly associated with high levels of NLRP3 effector cytokines found in the vaginal 401 lavage fluid of women with recurrent VVC (RVVC), suggesting the recognition of DAMP 402 signals likely prime the epithelial pro-inflammatory response (44). This concept of linking 403 fungal pathogenicity to damage was further supported by findings from Schönherr et al. 404 in which the virulence of clinical isolates of C. albicans was directly correlated with their 405 capacity to induce oral mucosal insult (43). Notably, only the expression of 406 ECE1/Candidalysin was strongly correlated with damage and pathogenesis in several 407 (but not all) C. albicans isolates. However, it is likely that simultaneous and combined 408 expression of several attributes (e.g. hyphae and Candidalysin) is required for full 409 virulence.

410 Given that the presence of Candidalysin is sufficient to induce damage to the 411 vaginal mucosa and elicits inflammasome effector responses (i.e. IL-1β), it is possible 412 that Candidalysin serves as a fungal DAMP capable of inflammasome activation. 413 Typically, inflammasome activation requires two independent steps, the first of which is 414 ligation of pattern recognition receptors that prime the response and a second damage 415 step that activates the response (40). Obviously the lytic property of Candidalysin 416 satisfies the second step but activation of the first step would likely depend on other 417 cellular factors of *C. albicans* (e.g. cell surface components). However, results from 418 Moyes et al, in which Candidalysin was mutated to contain a C-terminal alanine-alanine 419 motif (Ece1-III_{62–93AA}) retained immunostimulatory function in the absence of lytic 420 activity, suggesting that structural recognition of this peptide may be sufficient to also

421 prime inflammasome activity or other inflammasome-independent inflammatory

422 responses (16). Investigations are currently underway to address these possibilities.

423 In summary, this study demonstrates that Candidalysin secretion is critical for the 424 induction of immunopathological signaling at the vaginal mucosa, and that these 425 responses are largely conserved at both human and murine epithelial surfaces. 426 Furthermore, our findings decouple hypha formation from disease symptomatology and 427 clearly link vaginitis immunopathogenesis with Candidalysin production and its capacity 428 to directly damage the vaginal mucosa. In light of these findings, studies designed to 429 determine the mechanistic interaction of Candidalysin with the vaginal epithelium are 430 warranted. Therapeutic strategies to either neutralize Candidalysin itself, inhibit its 431 expression, or block downstream host signaling pathways may offer a unique 432 opportunity to more quickly arrest symptomatology of this most prevalent human fungal 433 infection.

434

435 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.

442

443 Cell lines, strains and primers. The A431 human vulvar epidermoid carcinoma cell
444 line was used in this study. All *C. albicans* strains used, including Candidalysin deletion
445 mutants, are those described by Moyes, et al (16, 45). All primers used for quantitative
446 PCR (qPCR) are listed in Table S2.

447

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.

453

454 **Microarray analysis.** Reconstituted human vaginal epithelia (RVE: 5-day) created 455 using the A431 cell line were purchased from SkinEthic Laboratories (France) and used 456 as previously described (6). RNA was isolated from RVE infected with C. albicans 457 SC5314 for 6 and 24 h or an equal volume of PBS using the GenElute total mammalian 458 RNA miniprep kit (Sigma, UK) and trace genomic DNA removed using the Turbo 459 DNase-free kit (Ambion, UK). For microarray analysis, RNA was amplified using the 460 MessageAmp Premier RNA Amplification Kit (Ambion, UK) and hybridized onto U133a 461 2.0 gene chips (Affymetrix, UK) after fragmentation by metal-induced hydrolysis into 35-462 200 nucleotide fragments according to standard protocols. Chips were scanned 463 (Affymetrix GeneChip Scanner 3000) and assessed using Affymetrix Command 464 Console (AGCC) software suite. This data was statistically analyzed using the

Bioconductor R package, PIANO. Gene Ontology and pathway analysis was performed
using both PIANO and DAVID (46, 47).

467

468 Cytokine release. A431 vaginal epithelial cells were cultured in Dulbecco's Modified 469 Eagle Medium Nutrient Mixture + L-glutamine (Life technologies) supplemented with 470 10% (v/v) heat-inactivated fetal bovine serum (Life technologies) and 1% (v/v) penicillin-471 streptomycin (Sigma) at 37°C, 5% CO₂, Candidalysin peptide (SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK) was purchased from Peptide Protein 472 473 Research Ltd (UK). Prior to Candidalysin challenge, confluent A431 epithelial cells were 474 serum-starved overnight and all experiments were carried out in serum-free DMEM 475 medium. Cells were incubated with Candidalysin (prepared as a 10 mg/mL stock in 476 sterile water)) at doses of 1.5, 3, 15 and 70 µM for 2 h at 37°C in 5% CO₂. Sterile water 477 (vehicle only) controls were also included. Culture supernatants were then isolated and 478 human IL-1 α , IL-1 β , IL-6, IL-8, GM-CSF, and G-CSF quantified by Magnetic Luminex 479 Performance Assay (Biotechne) and Bio-Plex 200 System (BioRad) according to the 480 manufacturer's instructions.

481

Epithelial cell damage assay. Damage to epithelial monolayers following a 24 h
challenge with Candidalysin was determined by quantification of lactate
dehydrogenease activity in cell culture supernatants using a CytoTox 96 non-radioactive
cytotoxicity assay (Promega) according to the manufacturer's instructions as previously
described (16). Porcine lactate dehydrogenase (Sigma) was used to create the
standard curve.

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

495

496 **SDS PAGE and Western blotting.** Proteins were resolved by electrophoresis on 12% 497 SDS PAGE gels using a mini-protean tetra cell system (BioRad). Electrophoresed 498 proteins were transferred to nitrocellulose membrane (BioRad) using a mini-transblot 499 electrophoretic transfer cell (BioRad). p-DUSP1/MKP1 (S359) and c-Fos rabbit 500 monoclonal antibodies were purchased from Cell Signaling Technologies. Actin (clone 501 C4) mouse monoclonal antibody was purchased from Millipore. Peroxidase-conjugated 502 Affinipure Goat anti-mouse and anti-rabbit IgG secondary antibodies were purchased 503 from Jackson Immune Research. Membranes were blocked in 1 × TBS (Severn 504 Biotech) containing 0.001% (v/v) tween-20 (Acros Organics) and 5% (w/v) fat free milk 505 powder (Sainsbury's). Primary antibodies diluted (1:1000 or 1:10,000 as suggested by 506 manufacturer) in TBS-tween and 5% milk (c-Fos), or TBS-tween and 5% bovine serum 507 albumin (p-DUSP1/MKP1) were added and membranes incubated overnight at 4°C with 508 gentle shaking. Following incubation, membranes were washed with 1 x TBS containing 509 0.01% (v/v) tween-20, diluted (1:10,000) HRP-conjugated secondary antibody added 510 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.

514

515 Murine model of vaginal candidiasis. A murine model of Candida vaginitis was 516 utilized as described previously (9, 11, 48). Female 6-8 week old C57BL/6 mice were 517 purchased from Charles River laboratories and housed in isolator cages mounted onto 518 ventilated racks. Mice were administered 0.1 mg of estrogen (β -estradiol 17-valerate; 519 Sigma) dissolved in 0.1 mL sesame oil subcutaneously 72 h prior to inoculation with C. 520 albicans. Stationary-phase cultures of C. albicans strains were washed three times in 521 sterile, endotoxin-free phosphate-buffered saline (PBS) and resuspended in a 0.2 x 522 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 523 524 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 525 526 underwent vaginal lavage with 100 µL of PBS. Resultant lavage fluids were spiked with 527 1 µL of 100 × EDTA-free protease inhibitors (Roche) and kept on ice until processing for 528 immunopathological markers. After sacrifice, vaginal tissue was surgically excised and 529 stored for downstream analyses. All animal experiments were conducted with n=4 mice 530 per group, repeated, and data combined unless noted otherwise.

531

532 Assessment of fungal burden and vaginitis immunopathology. All

immunopathological markers were assessed as described previously (9). (i) Lavage

534 fluid was serially diluted 10-fold using the drop-plate method, plated onto YPD agar 535 containing 50 µg/mL chloramphenicol, plates incubated for 24 h at 37°C, and the 536 resulting colonies enumerated. CFU/mL values per group are reported as medians. (ii) 537 Lavage fluid (10 µL) was smeared onto glass slides and stained by the Papanicolaou 538 technique to assess polymorphonuclear leukocyte (PMNs) recruitment (small, blue, 539 cells with multi-lobed nuclei). PMNs were counted in 5 non-adjacent fields by standard 540 light microscopy using a 40X objective and values reported as mean + standard error of 541 the mean (SEM). (iii) Murine IL-1 α , IL-1 β , CXCL2, and S100A8 was assessed in 542 clarified, diluted (1:20-1:100) vaginal lavage fluid using commercial enzyme-linked 543 immunosorbent assays (eBioscience, R&D Systems) according to manufacturer's 544 protocol. Results are reported as the mean + SEM. (iv) Lactate dehydrogenase (LDH) 545 activity was measured in clarified, diluted (1:100) lavage fluid using the commercial 546 available CytoTox 96 nonradioactive cytotoxicity assay (Promega). Results are reported 547 as the mean + SEM.

548

549 **Isolation of RNA from vaginal tissue.** RNA was extracted from whole vaginas as 550 described previously (11). At d 3 p.i., vaginal tissue was surgically excised, immediately 551 placed into RNALater (Thermo Fisher), and incubated at 4°C overnight. The following 552 day, tissues were transferred to TRI Reagent (Sigma), finely minced with scissors, 553 mechanically homogenized (Pro Scientific), and centrifuged at 12,000 x g for 10 min at 554 4°C. RNA was isolated by chloroform-ethanol precipitation and the pellet resuspended 555 in nuclease-free water according to TRI Reagent instructions. RNA concentration was 556 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.

559

560 **gRT-PCR analysis.** RNA from vaginal tissue was isolated as described above. RNA 561 concentrations were equalized amongst samples and 200 ng aliquots were treated with 562 RNase-free DNase according to the manufacturer's instructions (Thermo Scientific). 563 RNA was reverse transcribed using random hexamers and the RevertAid kit according 564 to the manufacturer's protocol (Thermo Scientific). Proprietary primer sets spanning 565 exon-exon junctions were ordered from IDT for murine II-6, Cxcl1, Cxcl2, II-1a, II-1b, 566 Gm-csf, G-csf, Camp, S100a8, Saa3, Defb3, and Act1b (Table S2). All primers were 567 used at the manufacturer's recommended concentrations along with 2 x Maxima Sybr 568 Green mix (Bio-rad) to amplify 20 ng of cDNA. qPCR reactions were monitored and 569 analyzed with the Applied Biosystems 7500 platform and associated software. 570 Expression levels of target genes in infected mice were compared to a reference gene 571 (ACT1B) and naïve controls using the $\Delta\Delta$ Ct method as described previously (49). 572

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- 584 strains. Experimental design was conducted by DLM, JRN, BH and BMP. JPR, HMEW,
- 585 DLM, SS, KSB, SLT, and GEP performed all experimental techniques and data
- analysis. All authors aided in experimental critique and manuscript preparation.

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746

747 **FIGURE LEGENDS**

748 Figure 1. Global differential gene expression induced during *C. albicans* infection 749 of Reconstituted Vaginal Epithelium (RVE). RVE were challenged with C. albicans or 750 mock infected with PBS for 6 or 24 h and differential gene expression was assessed by 751 microarray analysis. Volcano plots depicting log2 fold expression changes of genes 752 between (A) 6 h and (B) 24 h time points following challenge with C. albicans or PBS 753 sham. (C) After adjusting for p-value and false discovery rate (fdr, p< 0.01) gene, Venn-754 diagram plot depicts absolute number of genes expressed between C. albicans at 6 h 755 (red) and 24 h (green).

756

Figure 2. Host signaling pathways predicted to be activated during *C. albicans* infection of RVE. Based on differential gene expression, KEGG pathway analysis using the DAVID web-based package revealed several host pathways predicted to be significantly (p<0.0001) 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.

763

Figure 3. Candidalysin is sufficient to induce cellular damage and
 proinflammatory responses in vaginal epithelial cells. (A) A431 vaginal epithelial

766 cells were exposed to Candidalysin (70, 15, 3 and 1.5 µM) for 24 h and cellular damage 767 quantified by LDH assay. Data are presented as fold change relative to vehicle control. 768 Statistics are applied relative to the vehicle control (n=3 biological repeats). (B) Western 769 blot analysis of the vaginal epithelial response to different concentrations of 770 Candidalysin. Epithelial cell lysates (20 µg total protein) were probed with anti c-Fos and 771 anti p-MKP1 antibodies. One representative blot presented (from n=3 biological 772 repeats). (C-H) Quantification of cytokines (IL-1α, IL-1β, G-CSF, GM-CSF, IL-6, and IL-773 8) secreted from vaginal epithelial cells in response to different concentrations of 774 Candidalysin. Statistics are applied relative to the vehicle control (n=3 biological 775 repeats). Graphs are plotted as the mean + SEM. A and C-H: Statistical significance 776 was calculated using one-way ANOVA and Dunnet's post-test. *** p < 0.001, ** p < 777 0.01, * p < 0.05.

778

779 Figure 4. Candidalysin is required for neutrophil recruitment and mucosal 780 damage in a murine model of vulvovaginal candidiasis. Groups of estrogen-treated 781 C57BL/6 mice (n=4) were intravaginally challenged with WT (black bars), $ece1\Delta/\Delta$ (dark 782 gray bars), $ece 1\Delta/\Delta + ECE 1\Delta_{184-279}$ (white bars), and $ece 1\Delta/\Delta + ECE1$ (light gray bars) 783 strains of *C. albicans* and vaginal lavage fluid assessed longitudinally at d 3 and d 7 for 784 (A, B) fungal burden, median; (C, D) PMNs, mean + SEM; (E, F) the damage biomarker 785 LDH, mean + SEM. (G) Papanicolaou staining was performed on smears made from 786 vaginal lavage fluid to assess PMN influx (yellow arrows) and hypha formation (green 787 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.

791 Figure 5. Candidalysin is required for pro-inflammatory cytokine expression in 792 the murine vagina. Groups of estrogen-treated C57BL/6 mice (n=4) were intravaginally 793 challenged with WT (black bars), ece $1\Delta/\Delta$ (dark gray bars), ece $1\Delta/\Delta + ECE 1\Delta_{184-279}$ 794 (white bars), and ece1 Δ/Δ +ECE1 (light gray bars) strains of C. albicans, whole vaginal 795 tissue excised at d 3 p.i., and extracted RNA processed for qPCR analysis. Genes 796 chosen for qPCR included those previously identified as being induced by candidalysin 797 or C. albicans during in vitro or in vivo challenge, including: (A) II-6, (B) Cxcl1, (C) 798 Cxcl2, (D) II-1a, (E) II-1b, (F) Gm-csf, (G) G-csf, (H) Camp, (I) Defb3, (J) S100A8, and 799 (K) Saa3. All genes were internally compared to the Actb housekeeping gene and to 800 mock-infected controls using the $\Delta\Delta$ Ct method. Graphs are plotted as the mean 801 normalized fold expression + SEM. Statistical significance was calculated using a oneway ANOVA and Tukey's post-test. ** p < 0.01, * p < 0.05. 802

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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), $ece1\Delta/\Delta$ (dark gray bars), $ece1\Delta/\Delta + ECE1\Delta_{184-279}$ (white bars), and $ece1\Delta/\Delta + ECE1$ (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-1, (C, D) IL-1, (E, F) Cxcl2, (G, H) S100a8. All inoculation groups were performed in

811 duplicate and data combined. Graphs are plotted as the mean + SEM. Statistical 812 significance was calculated using a one-way ANOVA and Tukey's post-test. *** p < 813 0.001, ** p < 0.01, * p < 0.05.



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Venn diagram (p-value adjustment: fdr, p<0.001)





hsa04520:Adherens junction

- hsa04010:MAPK signaling pathway
- hsa04064:NF-kappa B signaling pathway
 - hsa04380:Osteoclast differentiation
 - hsa05169:Epstein-Barr virus infection
 - hsa05131:Shigellosis
- hsa04662:B cell receptor signaling pathway
 - hsa04110:Cell cycle
- hsa05120:Epithelial cell signaling in Helicobacter
 - hsa04390:Hippo signaling pathway
 - hsa04510:Focal adhesion
 - hsa04012:ErbB signaling pathway
 - hsa04722:Neurotrophin signaling pathway

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