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1 **Title:** Candidalysin drives epithelial signaling, neutrophil recruitment, and

2 immunopathology at the vaginal mucosa.

3

4 **Running title:** Candidalysin and vaginitis

5

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29

ABSTRACT

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 clearly 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 or hyphal burdens as compared to isogenic

54 controls. However, significant decreases in neutrophil recruitment, damage, and pro-
55 inflammatory cytokine expression were observed with these strains. Our findings
56 demonstrate that Candidalysin is a key hypha-associated virulence determinant
57 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.

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

77 MAPK phosphatase MKP1 (6). This MAPK/c-Fos/MKP1 pathway is able to discriminate
78 between *C. albicans* yeast and hyphal cells and is retained between both vaginal and
79 oral epithelia (7, 8). The activation of this signaling pathway is also coupled with lactate
80 dehydrogenase (LDH) release, a marker of epithelial damage (6). Therefore, the ability
81 of *C. albicans* to form hyphae and breach the epithelial barrier governs innate cytokine
82 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*Δ/Δ, *efg1*Δ/Δ/*cph1*Δ/Δ, *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-1β release and PMN recruitment to the
99 vaginal mucosa during *C. albicans* infection, consistent with candidal infection at other

100 anatomical sites (11). However, an important question remained: is hypha formation *per*
101 *se* sufficient for damage-induced inflammation or are distinct fungal factors associated
102 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 *ece1*Δ/Δ 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*Δ/Δ/*cph1*Δ/Δ mutants fail to fully activate MAPK signaling and immunopathology in
118 oral or vaginal epithelium (6, 8, 9, 17).

119 Given these similarities, we hypothesized that Candidalysin may comparably
120 activate vaginal epithelial cells and govern VVC immunopathology *in vivo*. This study
121 demonstrates that a single fungal factor, Candidalysin, is responsible for inducing
122 vaginal cellular damage and pro-inflammatory responses during *C. albicans* infection *in*

123 *vitro* and *in vivo*. As such, the identification of a secreted toxin as the factor responsible
124 for driving symptomatic vaginal inflammation may offer novel treatment modalities for
125 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
141 regulated in response to PBS-sham treatment at the same time points (Fig. S1). At the
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-κB 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
157 up-regulated expression of a variety of transcription factor genes associated with other
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-κB 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

169 predicted to be activated were generally conserved at 6 h and 24 h time points. A list of
170 individually 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).

192

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 *C. albicans* 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 μM 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-1α, IL-1β, 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 μM). With the exception of IL-6, all cytokines assayed
214 were significantly induced at Candidalysin doses above 3 μM; however, this dose was

215 insufficient to cause significant damage (Fig. 3A), suggesting that Candidalysin exhibits
216 dual functionality, serving both immunostimulatory and lytic roles against vaginal
217 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* (*ece1*Δ/Δ)
223 and restored with one full-length allele (*ece1*Δ/Δ+*ECE1*) or one mutant allele lacking the
224 Candidalysin-encoding region of *ECE1* ($ece1\Delta/\Delta+ECE1_{\Delta 184\text{-}279}$), along with the
225 appropriate parental isogenic control (BWP17+CIp30: 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 *ece1*Δ/Δ or
230 $ece1\Delta/\Delta+ECE1_{\Delta 184\text{-}279}$ strains, which was restored to WT levels during infection with the
231 *ece1*Δ/Δ+*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 *ece1*Δ/Δ+*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, *ece1*Δ/Δ and $ece1\Delta/\Delta+ECE1_{\Delta 184\text{-}279}$
236 strains formed hyphae normally at the vaginal mucosa, as did WT and *ece1*Δ/Δ+*ECE1*
237 strains (Fig. 4G, green arrows). Thus, these results demonstrate that Ece1 is required

238 for vaginal immunopathogenesis *in vivo* and that hypha formation alone is insufficient to
239 elicit 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*Δ/Δ, *ece1*Δ/Δ+*ECE1*, *ece1Δ/Δ+ECE1*$_{\Delta 184\text{-}279}$ 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 *Il-6*, *Cxcl2*, *Il-1a*, and *Il-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 (*ece1*Δ/Δ) 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

261 manner. Expression of the gene coding for *S100a8*, a calcium-binding protein with
262 important functions in antifungal defense and danger responses and strongly induced
263 during *C. albicans* infection, was almost completely absent during infection with
264 Candidalysin deletion strains (Fig. 5J) (26, 27). Similarly, the gene encoding serum
265 amyloid A3 (*Saa3*), an inducible acute phase apolipoprotein capable of recruiting
266 immune cells to inflammatory sites, was similarly increased in a Candidalysin-
267 dependent 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-1α, IL-1β, 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**

278 In recent years, vulvovaginal candidiasis has been identified as an
279 immunopathology, in which the host immune response, orchestrated by a series of pro-
280 inflammatory cytokines and chemokines, actually exacerbates symptomatic disease. A
281 landmark live-challenge study conducted by Fidel and colleagues led to this paradigm
282 shifting hypothesis, as presence of neutrophils in the vaginal lavage fluid of women
283 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-Seq 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).
327 An interesting observation from the microarray analysis was that a gene
328 encoding the neuronal precursor of cell expressed developmentally down-regulated 9
329 (*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.

346 Another striking observation was the strong transcriptional up-regulation of the
347 gene encoding EGF ligand heparin-binding EGF-like growth factor (*HB-EGF*, ~40-fold)
348 and the gene coding for the enzyme heparin sulfate 3-O-sulfotransferase 1 (*HS3ST1*),
349 suggesting the potential presence of heparin sulfate at the vaginal mucosa. Indeed,
350 recent work by Yano et al. demonstrated that heparin sulfate can be recovered from the
351 vaginal lavage fluid of mice and that its presence is enhanced by exogenous estrogen
352 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

436 **Ethics statement.** The animals used in this study were housed in AAALAC-approved
437 facilities located at the University of Tennessee Health Sciences Center (UTHSC) in the
438 Regional Biocontainment Laboratory (RBL). The UTHSC Animal Care and Use
439 Committee approved all animals and protocols. Mice were given standard rodent chow
440 and water *ad libitum*. Mice were routinely monitored for signs of distress, including
441 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
448 **Microorganism growth.** *C. albicans* strains were maintained as glycerol stocks stored
449 at -80°C. A small amount of stock was spread onto yeast peptone dextrose (YPD) agar
450 and incubated at 30°C for 48 h to obtain isolated colonies. A single colony was
451 transferred to 10 mL of YPD liquid medium and incubated at 30°C with shaking at 200
452 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

465 Bioconductor R package, PIANO. Gene Ontology and pathway analysis was performed
466 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% CO_2. Candidalysin peptide
472 (SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK) was purchased from Peptide Protein
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% CO_2. Sterile water
477 (vehicle only) controls were also included. Culture supernatants were then isolated and
478 human IL-1α, IL-1β, 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
482 **Epithelial cell damage assay.** Damage to epithelial monolayers following a 24 h
483 challenge with Candidalysin was determined by quantification of lactate
484 dehydrogenease activity in cell culture supernatants using a CytoTox 96 non-radioactive
485 cytotoxicity assay (Promega) according to the manufacturer's instructions as previously
486 described (16). Porcine lactate dehydrogenase (Sigma) was used to create the
487 standard curve.

488

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 × 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

511 described and exposed to Immobilon Western Chemiluminescent HRP substrate
512 (Millipore) prior to visualisation by exposure to film (GE Healthcare). Alpha-actin was
513 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 ×
522 volume of PBS. Cell suspensions were diluted, counted on a Neubauer hemocytometer,
523 and adjusted to 5×10^8 CFU/mL in sterile PBS. Estrogen-treated mice were
524 intravaginally inoculated with 10 µL of the standardized blastoconidial cell suspension,
525 generating an inoculum size of 5×10^6 blastoconidia. At d 3 and/or d 7 p.i. mice
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
533 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-1α, IL-1β, 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 × 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-

557 morpholino)propanesulfonic acid (MOPS) gel electrophoresis to visualize intact 18s and
558 28s rRNA bands.
559
560 **qRT-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 *Il-6, Cxcl1, Cxcl2, Il-1a, Il-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 × 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 ΔΔCt 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

586 analysis. All authors aided in experimental critique and manuscript preparation.

587

588

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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

757 **Figure 2. Host signaling pathways predicted to be activated during *C. albicans***
758 **infection of RVE.** Based on differential gene expression, KEGG pathway analysis
759 using the DAVID web-based package revealed several host pathways predicted to be
760 significantly ($p<0.0001$) activated at **(A)** 6 h or **(B)** 24 h. Pathways are listed in order of
761 highest probability of activation. Significance was assessed using DAVID statistical
762 package via ANOVA analysis.

763

764 **Figure 3. Candidalysin is sufficient to induce cellular damage and**
765 **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*Δ/Δ (dark
782 gray bars), *ece1*Δ/Δ+*ECE1*$\Delta_{184-279}$ (white bars), and *ece1*Δ/Δ+*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

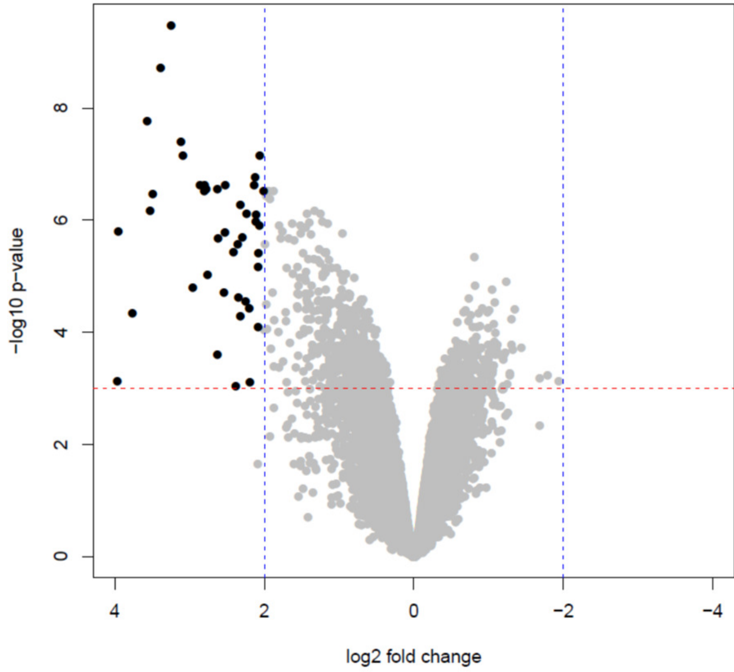
788 performed in duplicate and data combined. A-F: Statistical significance was calculated
789 using a one-way ANOVA and Tukey's post-test. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.
790

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), *ece1*Δ/Δ (dark gray bars), *ece1*Δ/Δ+*ECE1*$\Delta_{184-279}$
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)** *Il-6*, **(B)** *Cxcl1*, **(C)**
798 *Cxcl2*, **(D)** *Il-1a*, **(E)** *Il-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 ΔΔCt method. Graphs are plotted as the mean
801 normalized fold expression + SEM. Statistical significance was calculated using a one-
802 way ANOVA and Tukey's post-test. ** $p < 0.01$, * $p < 0.05$.
803

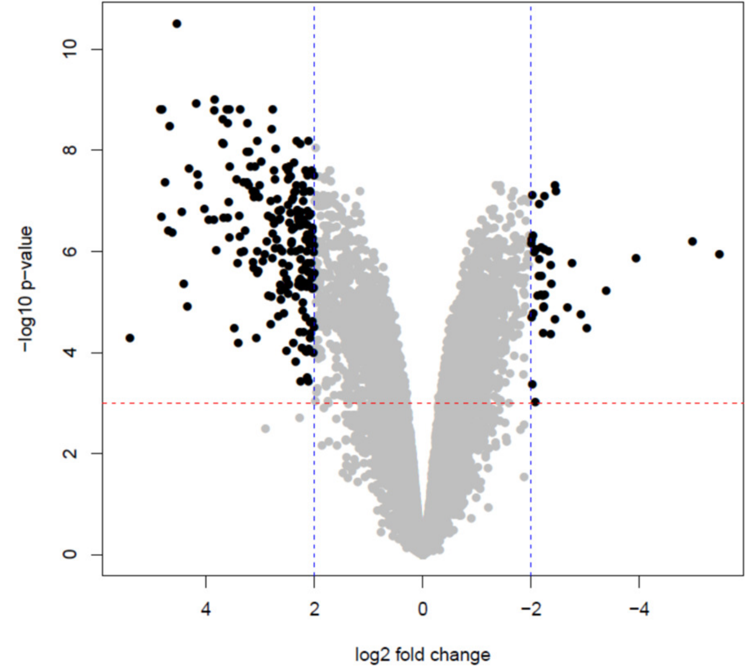
804 **Figure 6. Candidalysin is required for release of hallmark proinflammatory**
805 **cytokines and chemokines into the vaginal lavage fluid during murine vaginitis.**
806 Groups of estrogen-treated C57BL/6 mice (n=4) were intravaginally challenged with WT
807 (black bars), *ece1*Δ/Δ (dark gray bars), *ece1*Δ/Δ+*ECE1*$\Delta_{184-279}$ (white bars), and
808 *ece1*Δ/Δ+*ECE1* (light gray bars) strains of *C. albicans* and vaginal lavage fluid assessed
809 longitudinally by ELISA at d 3 and d 7 p.i. for inflammatory markers, including **(A, B)** IL-
810 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. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.
813
814

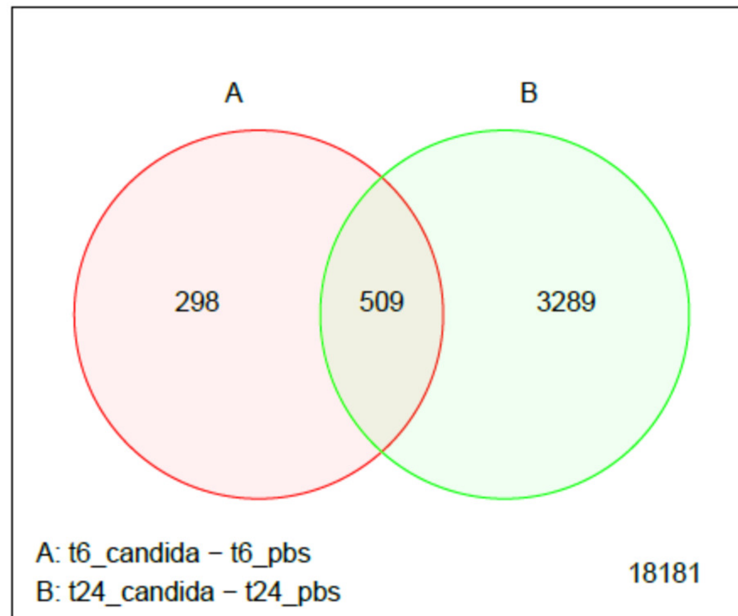
A



B



C



A

-Log P value

0 1 2 3 4 5 6 7 8 9

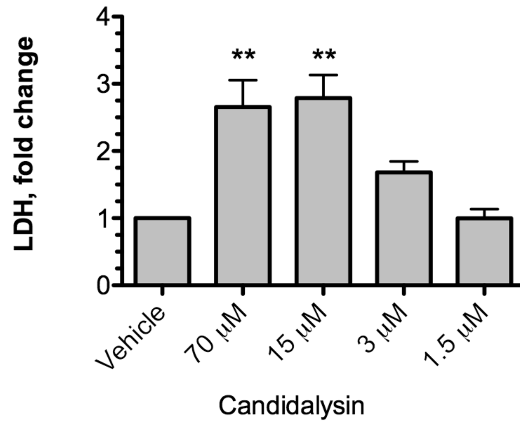
hsa04520:Adherens junction
hsa04010:MAPK signaling pathway
hsa04064:NF-kappa B signaling pathway
hsa04380:Osteoclast differentiation
hsa05169:Epstein-Barr virus infection
hsa05131:Shigellosis
hsa04662:B cell receptor signaling pathway
hsa04110:Cell cycle
hsa05120:Epithelial cell signaling in Helicobacter
hsa04390:Hippo signaling pathway
hsa04510:Focal adhesion
hsa04012:ErbB signaling pathway
hsa04722:Neurotrophin signaling pathway

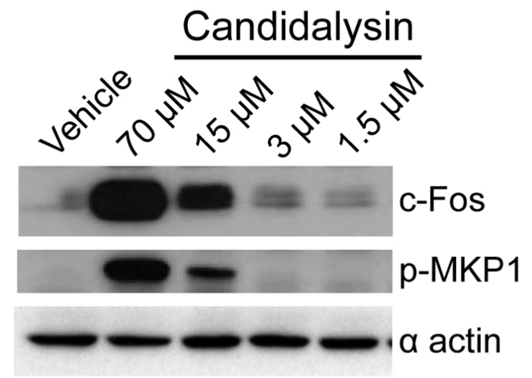
B

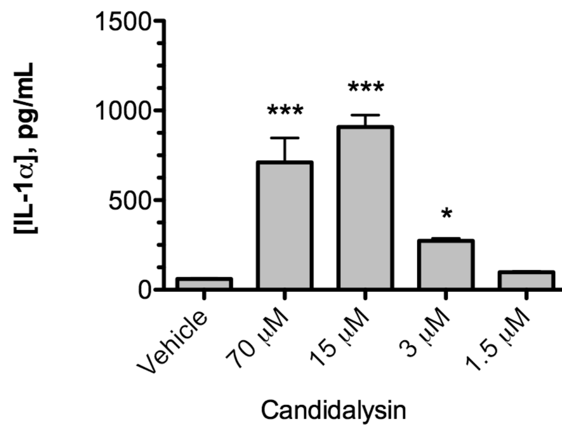
- Log P value

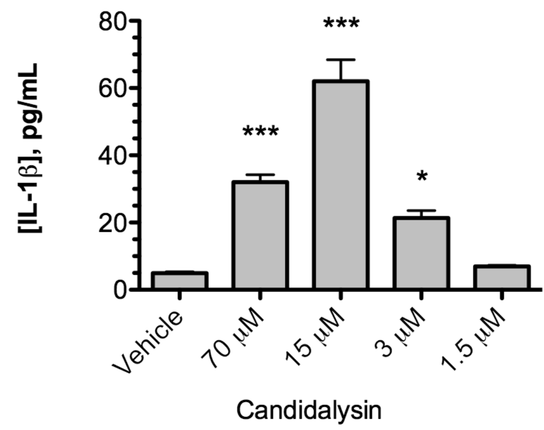
0 2 4 6 8 10 12 14 16 18

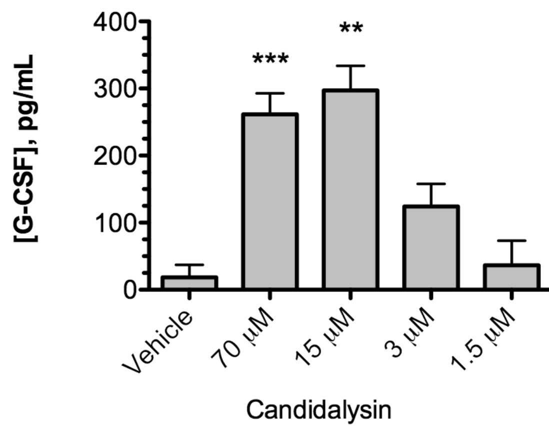
hsa04010:MAPK signaling pathway
hsa05200:Pathways in cancer
hsa05205:Proteoglycans in cancer
hsa04668:TNF signaling pathway
hsa05211:Renal cell carcinoma
hsa04012:ErbB signaling pathway
hsa04380:Osteoclast differentiation
hsa04068:FoxO signaling pathway
hsa04510:Focal adhesion
hsa05215:Prostate cancer
hsa04066:HIF-1 signaling pathway
hsa05164:Influenza A
hsa05161:Hepatitis B
hsa05219:Bladder cancer
hsa04520:Adherens junction
hsa05166:HTLV-I infection
hsa04014:Ras signaling pathway
hsa04530:Tight junction
hsa04064:NF-kappa B signaling pathway
hsa04910:Insulin signaling pathway
hsa04151:PI3K-Akt signaling pathway
hsa04662:B cell receptor signaling pathway
hsa04660:T cell receptor signaling pathway
hsa05214:Glioma
hsa04144:Endocytosis

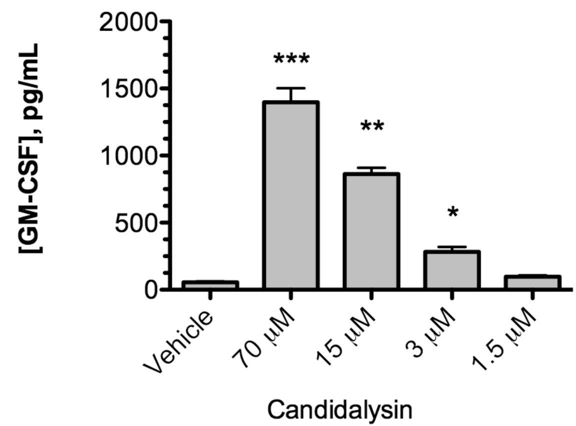

A
LDH, fold change
Vehicle
70 μM
15 μM
3 μM
1.5 μM
Candidalysin

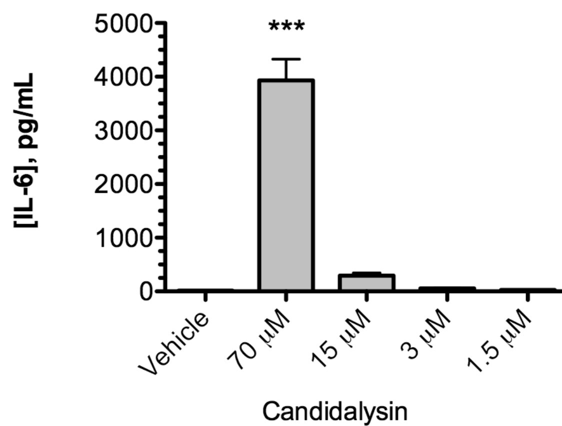

B
Candidalysin
Vehicle
70 μM
15 μM
3 μM
1.5 μM
c-Fos
p-MKP1
α actin

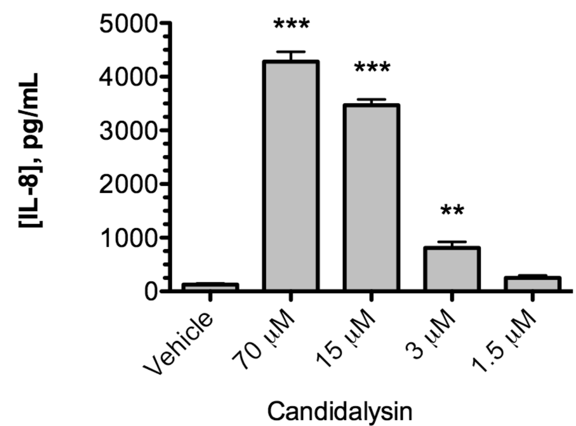

C
[IL-1α], pg/mL
Vehicle
70 μM
15 μM
3 μM
1.5 μM
Candidalysin

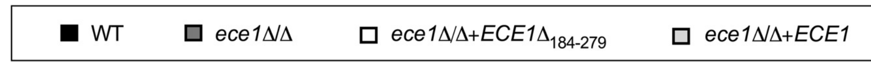

D
[IL-1β], pg/mL
Vehicle
70 μM
15 μM
3 μM
1.5 μM
Candidalysin

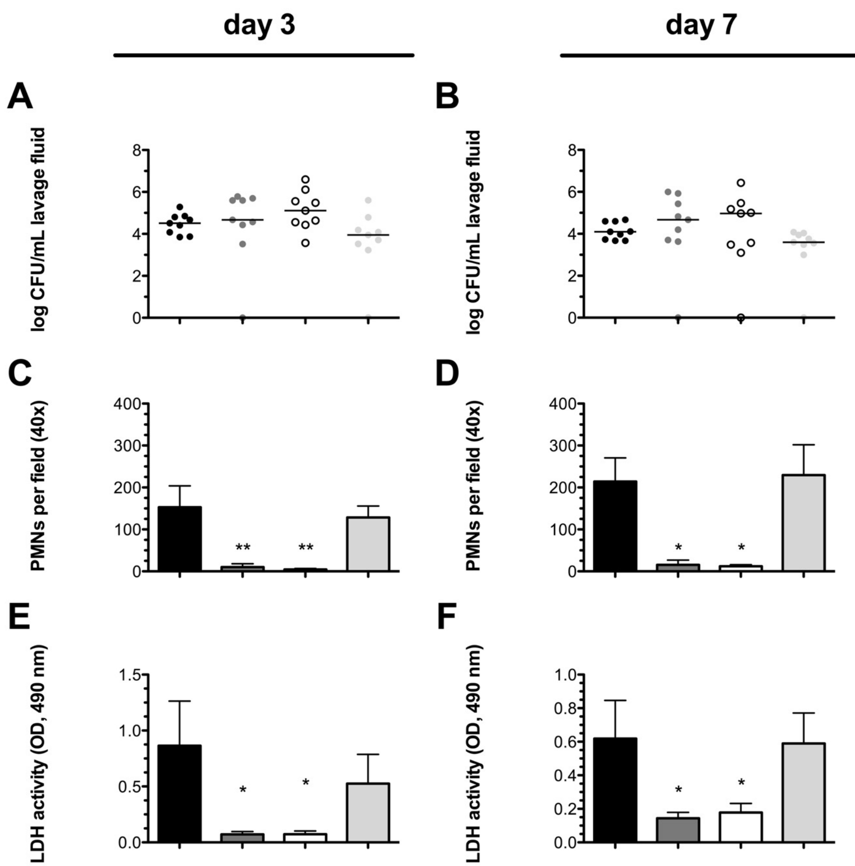

E
[G-CSF], pg/mL
Vehicle
70 μM
15 μM
3 μM
1.5 μM
Candidalysin

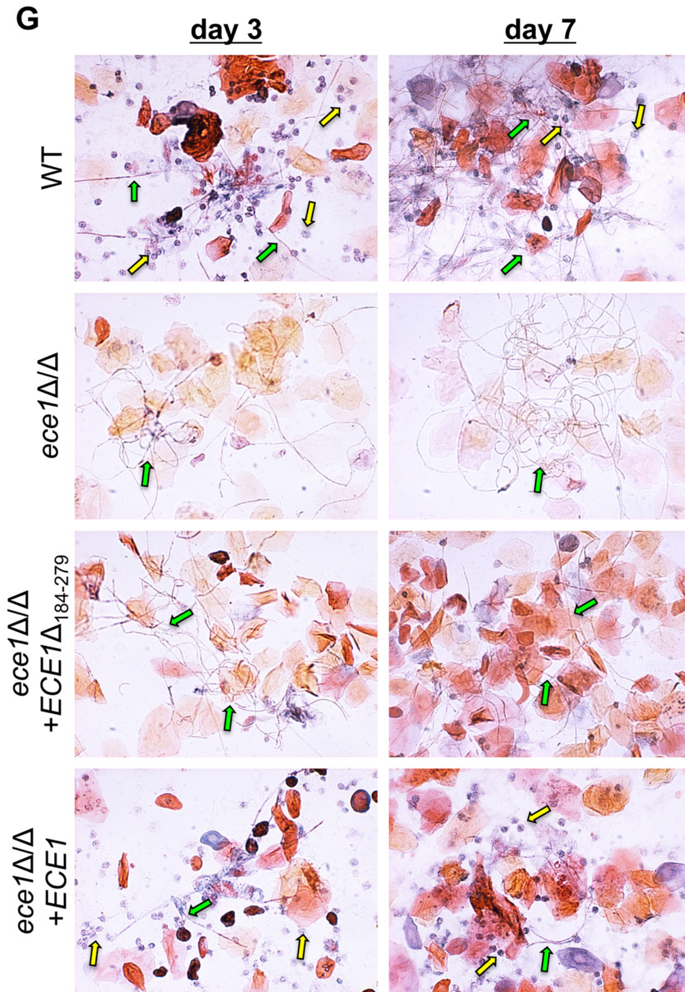

F
[GM-CSF], pg/mL
Vehicle
70 μM
15 μM
3 μM
1.5 μM
Candidalysin

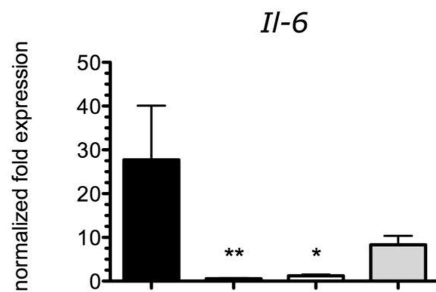

G
[IL-6], pg/mL
Vehicle
70 μM
15 μM
3 μM
1.5 μM
Candidalysin

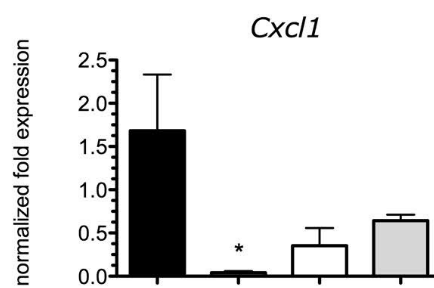

H
[IL-8], pg/mL
Vehicle
70 μM
15 μM
3 μM
1.5 μM
Candidalysin

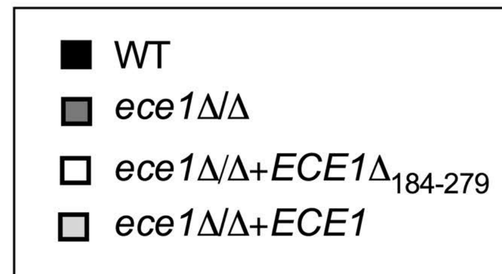

WT
ece1Δ/Δ
ece1Δ/Δ+ECE1Δ184-279
ece1Δ/Δ+ECE1

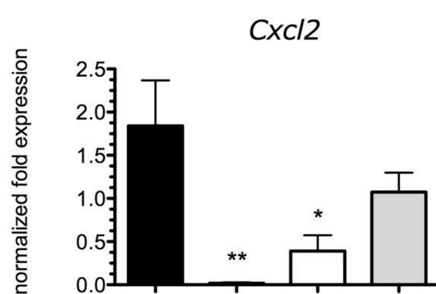

day 3
day 7
A
B
log CFU/mL lavage fluid
C
D
PMNs per field (40x)
E
F
LDH activity (OD, 490 nm)

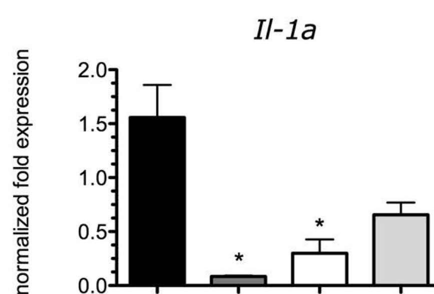

G
day 3
day 7
WT
ece1Δ/Δ
ece1Δ/Δ
+ECE1Δ184-279
ece1Δ/Δ
+ECE1

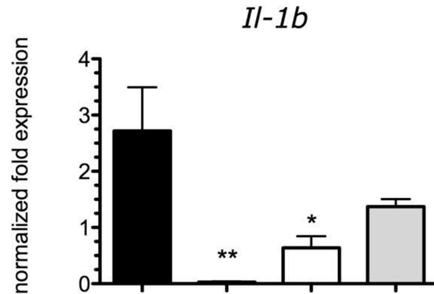

A
Il-6
normalized fold expression
0
10
20
30
40
50
**
*

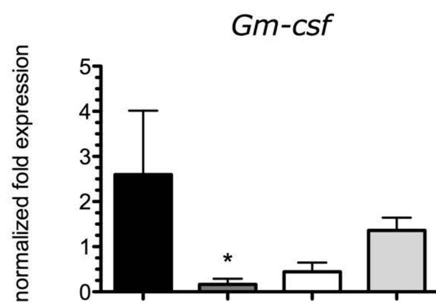

B
Cxcl1
normalized fold expression
0.0
0.5
1.0
1.5
2.0
2.5
*

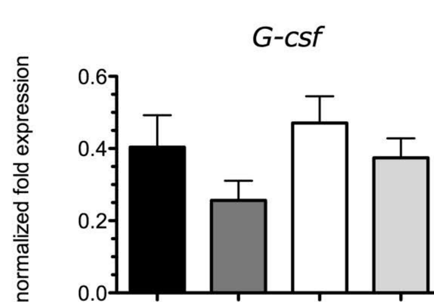

WT
ece1Δ/Δ
ece1Δ/Δ+ECE1Δ184-279
ece1Δ/Δ+ECE1

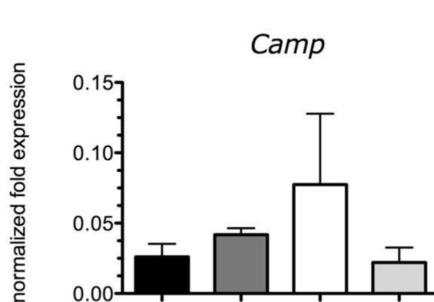

C
Cxcl2
normalized fold expression
0.0
0.5
1.0
1.5
2.0
2.5
**
*

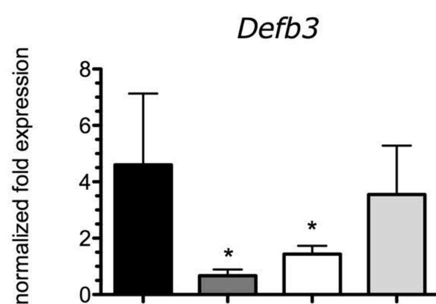

D
Il-1a
normalized fold expression
0.0
0.5
1.0
1.5
2.0
*
*

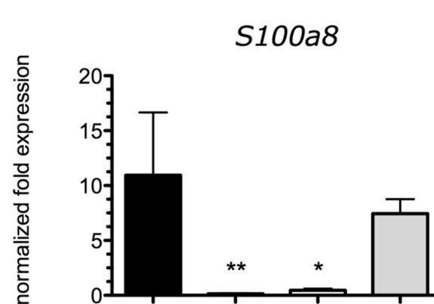

E
Il-1b
normalized fold expression
0
1
2
3
4
**
*

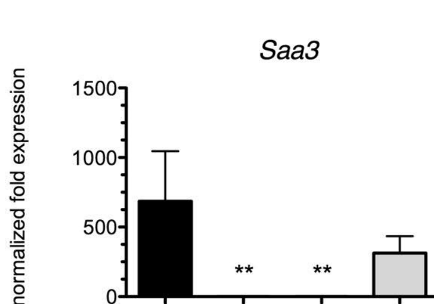

F
Gm-csf
normalized fold expression
0
1
2
3
4
5
*


G
G-csf
normalized fold expression
0.0
0.2
0.4
0.6


H
Camp
normalized fold expression
0.00
0.05
0.10
0.15


I
Defb3
normalized fold expression
0
2
4
6
8
*
*

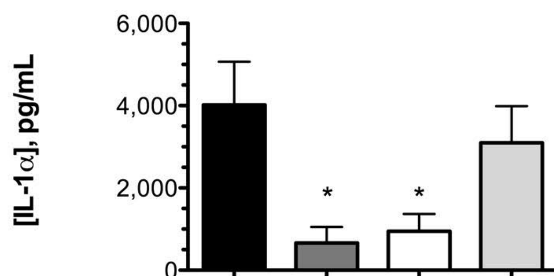

J
S100a8
normalized fold expression
0
5
10
15
20
**
*


K
Saa3
normalized fold expression
0
500
1000
1500
**
**

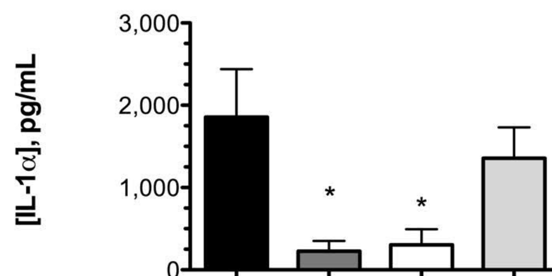
■ WT ■ *ece1*Δ/Δ □ *ece1*Δ/Δ+*ECE1*$\Delta_{184\text{-}279}$ ■ *ece1*Δ/Δ+*ECE1*

day 3 **day 7**

A



B



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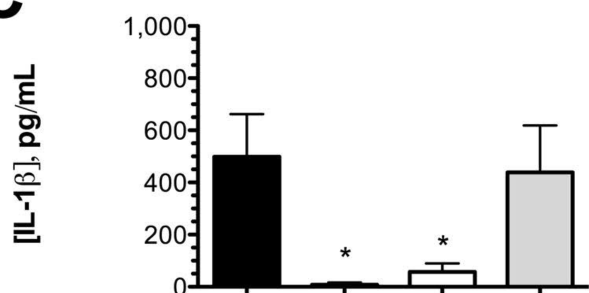

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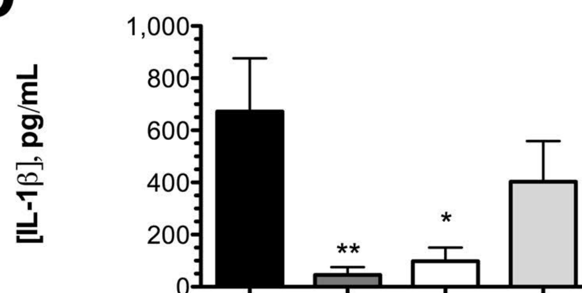
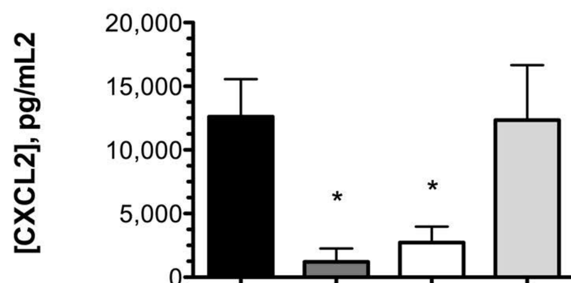


E



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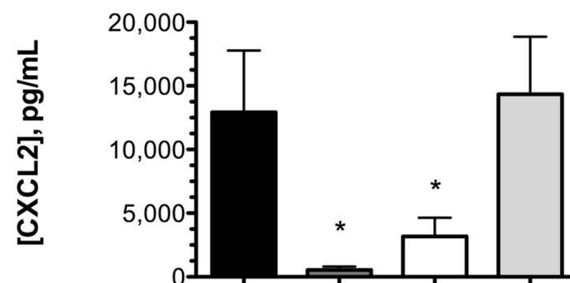
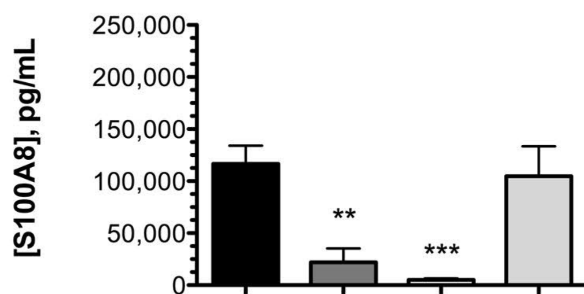


G



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