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FUNGAL AND PARASITIC INFECTIONS

Candidalysin Drives Epithelial Signaling, Neutrophil Recruitment, and Immunopathology at the Vaginal Mucosa

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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 anatomical-site specific, as effective gastrointestinal, oral, and vaginal immunities are 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 proinflammatory cytokine responses (including interleukin 1α [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 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 responsible for the immunopathogenesis of *C. albicans* vaginitis.

KEYWORDS *Candida*, Candidalysin, epithelial cells, immunopathogenesis, mucosal immunity, mucosal pathogens, mycology, vaginitis, vulvovaginal

Vulvovaginal candidiasis (VVC), caused primarily by the polymorphic fungal pathogen *Candida albicans*, remains a serious worldwide health concern leading to significant quality of life issues for immunocompetent women (1). Symptomatic VVC is manifested by itching, burning, and pain sensations at the vaginal and vulvar tissue, often accompanied by odorless vaginal discharge (2). Globally, VVC is estimated to be the most prevalent human fungal infection, with over 75% of women experiencing at least one episode in their lifetime and 5 to 8% suffering from idiopathic recurrent infection (3). In recent years, VVC has been described as an immunopathology in which

Received 7 September 2017 **Returned for modification** 9 October 2017 **Accepted** 3 November 2017

Accepted manuscript posted online 6 November 2017

Citation Richardson JP, Willems HME, Moyes DL, Shoaie S, Barker KS, Tan SL, Palmer GE, Hube B, Naglik JR, Peters BM. 2018. Candidalysin drives epithelial signaling, neutrophil recruitment, and immunopathology at the vaginal mucosa. Infect Immun 86:e00645-17. https://doi.org/10.1128/IAI.00645-17.

Editor George S. Deepe, University of Cincinnati

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the host neutrophil and associated cytokine response actually exacerbates disease symptoms yet fails to adequately control the fungus (4–7). While much effort has been placed on defining host immunological mechanisms contributing to VVC protection, the fungal virulence factors that dictate conversion from asymptomatic colonization to fulminant infection remain poorly understood.

Using model systems, several laboratories have collectively begun to unravel this complex host-pathogen interaction. The studies revealed that vaginal (as well as oral) epithelial cells can discriminate between colonizing yeast and invasive hyphae by activation of the MAPK/c-Fos/MKP1 pathway and that this "sensing" is concomitant with cellular damage (8–10). This was largely recapitulated in the murine model of VVC, where hypha-deficient strains of *C. albicans* (e.g., *efg1*Δ/Δ, *efg1*Δ/Δ *cph1*Δ/Δ, and *NRG1* overexpression) colonized as well as or better than wild-type strains yet failed to induce the hallmark immunopathology (e.g., polymorphonuclear leukocyte [PMN]/neutrophil attraction, S100A8 production, and interleukin 1β [IL-1β] release) or mucosal damage (lactate dehydrogenase [LDH] release). It was fairly unsurprising that fungal burden alone was not sufficient for symptomatic infection, as the vaginal mucosa is often colonized by *C. albicans* without clinical presentation of disease. Similarly, a live-challenge study in women volunteers demonstrated that fungal burden was not solely sufficient to explain VVC susceptibility, as women who were highly colonized did not always develop symptoms, and *vice versa* (4). Therefore, these collective findings suggest that the yeast-to-hypha transition itself or downstream hypha-associated effectors are likely required for tissue damage and subsequent immunopathological inflammation at the vaginal mucosa. However, the precise fungal factors and mechanisms that contribute to neutrophil recruitment, induction of immunopathology, and mucosal damage have remained elusive.

Recently, the *C. albicans ECE1* (extent of cell elongation) gene product was demonstrated to be crucial for cellular damage, innate cytokine production, and neutrophil recruitment during murine oropharyngeal candidiasis (OPC). *ECE1*, a highly expressed, hypha-associated gene, encodes a protein (Ece1p) that is processed into eight distinct peptides by the fungal protease Kex2p (11, 12). Genetic, biochemical, and functional assays determined that amino acids 62 to 92 of Ece1p form a fungal toxin termed Candidalysin, which possesses both lytic and immunostimulatory activities (including MAPK signaling) on oral epithelial cells (12). Importantly, an *ece1*Δ/Δ-null mutant retained the capacity to form hyphae yet was unable to induce an inflammatory response. Given these observations, we hypothesized that Candidalysin may comparably activate vaginal epithelial cells and govern VVC immunopathology *in vivo*. This study demonstrates that a single fungal factor, Candidalysin, is responsible for inducing vaginal cellular damage and proinflammatory responses during *C. albicans* infection *in vitro* and *in vivo*. As such, the identification of a secreted toxin as the factor responsible for driving symptomatic vaginal inflammation may offer novel treatment modalities for arresting symptomatic disease.

RESULTS

Differential gene expression and pathway induction in reconstituted human vaginal epithelium following *C. albicans* challenge. The reconstituted human vaginal epithelium (RVE) model is an excellent *in vitro* surrogate to study epithelium-specific responses of vaginal candidiasis, as the tissue layer is sufficiently differentiated and supports robust hyphal invasion, and infected RVE tissue largely resembles *in vivo* infection dynamics (13, 14). In order to elucidate global host transcriptomic changes in the vaginal epithelium in response to challenge with *C. albicans* (compared to phosphate-buffered saline [PBS] mock control), total epithelial RNA was selectively isolated from three independent RVE at 6 and 24 h postchallenge and subjected to microarray analysis. As with oral epithelium, the intermediate (6-h) time point is associated with initial fungal adherence and microbial recognition, while the late (24-h) time point is associated with fungal invasion and cellular damage (10, 15). Approximately 800 and nearly 4,000 genes were differentially expressed ($P < 0.001$) at 6 and

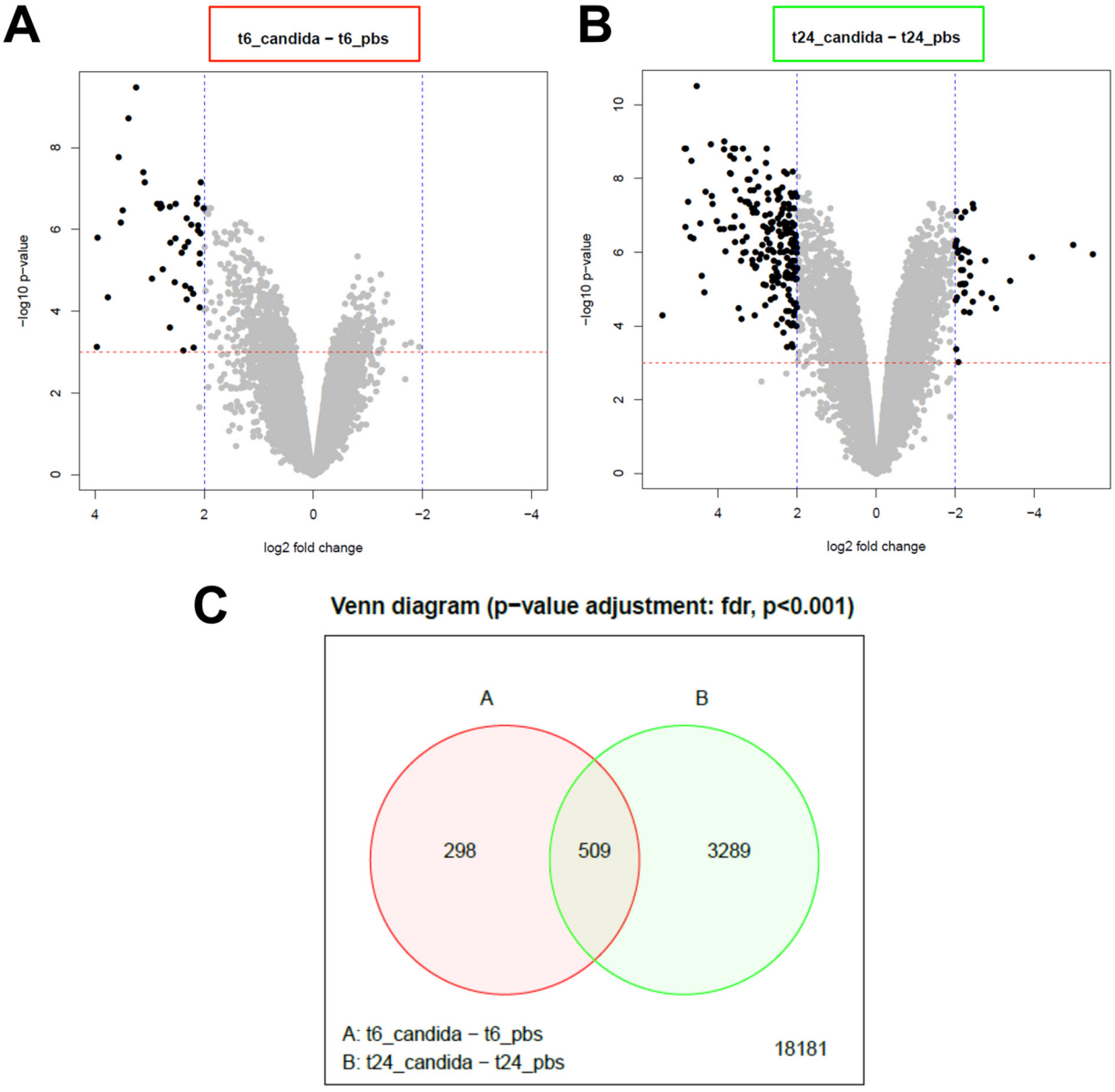

FIG 1 Global differential gene expression induced during *C. albicans* infection of RVE. Three independent RVE were challenged with *C. albicans* or mock infected with PBS for 6 or 24 h, and differential gene expression (≥2-fold change) was assessed by microarray analysis. Shown are volcano plots depicting $\log_2$-fold expression changes (blue dotted lines) at $P < 0.001$ (red dotted lines) of genes between the 6-h (A) and 24-h (B) time points following challenge with *C. albicans* or the PBS mock control. (C) Venn diagram depicting the absolute number of genes expressed between *C. albicans* at 6 h (red) and 24 h (green), after adjusting for *P* value and FDR ($P < 0.01$).

24 h, respectively, in response to *C. albicans* (Fig. 1). Comparatively few genes were regulated in response to PBS mock treatment at the same time points (see Fig. S1 in the supplemental material). At the intermediate stage of infection (6 h postinfection), the majority of differentially expressed genes were upregulated (Fig. 1A), with only 65 genes strongly upregulated (>4-fold) and none showing strong downregulation (>4-fold). However, by late stages of infection (24 h), an increase in the proportion of genes showing downregulated expression was observed (Fig. 1B). Approximately 320 genes were strongly upregulated at 24 h (>4-fold), and over half of the genes showing upregulation at 6 h were also strongly upregulated at the later time point (Fig. 1C). Surprisingly, relatively few genes were strongly (>4-fold) downregulated in response to fungal challenge at either time point.

Gene Ontology, pathway, and network mapping revealed profiles from *C. albicans*-infected cells to be consistent with MAPK, NF-κB, phosphatidylinositol 3-kinase (PI3K),

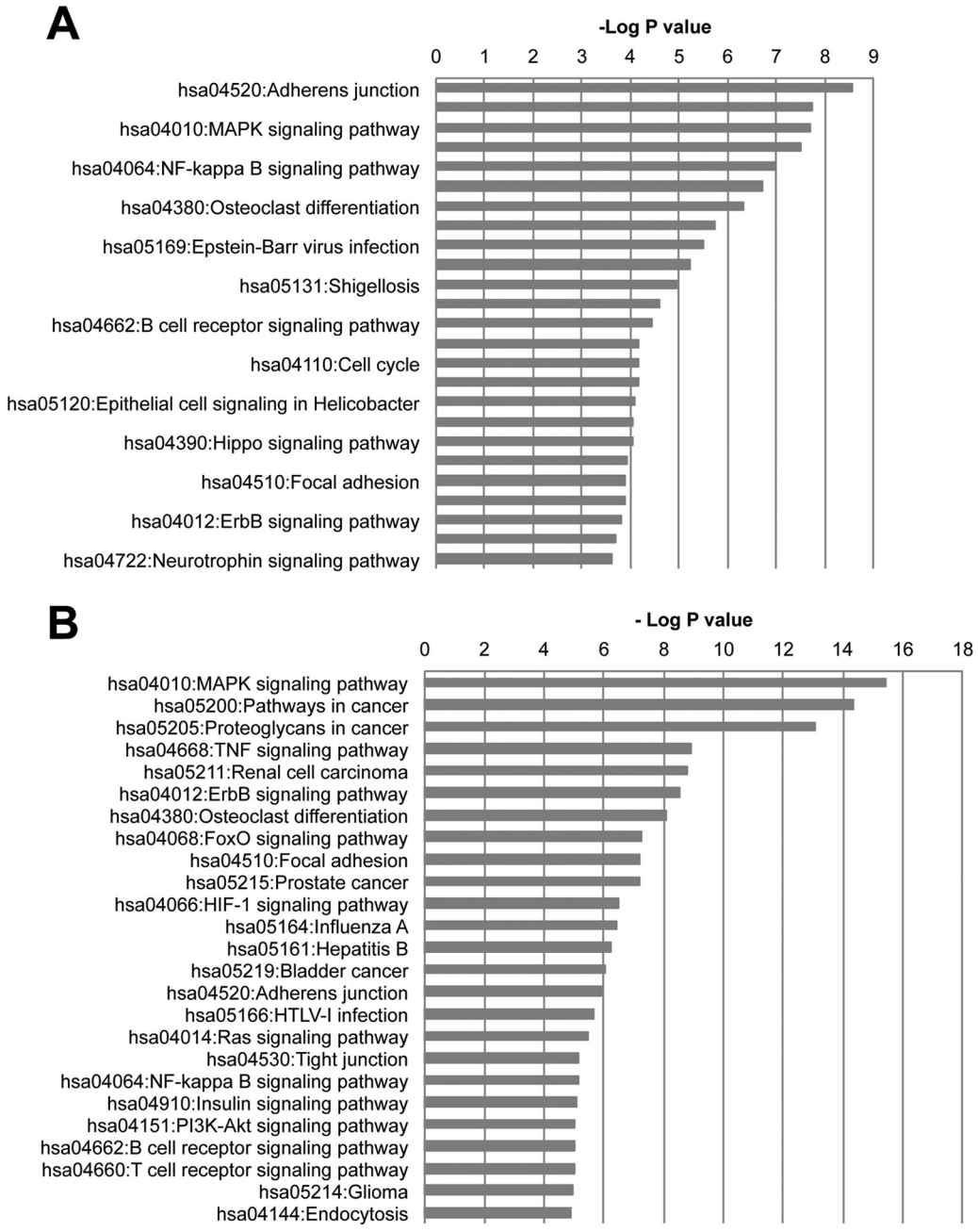

FIG 2 Host signaling pathways predicted to be activated during *C. albicans* infection of RVE. Based on differential gene expression (≥2-fold change), KEGG pathway analysis using the DAVID Web-based package revealed several host pathways predicted to be significantly ($P < 0.001$) activated at 6 h (A) or 24 h (B). Pathways are listed in order of highest probability of activation. Significance was assessed using the DAVID statistical package via analysis of variance (ANOVA).

ErbB receptor, and tumor necrosis factor (TNF) signaling pathways (Fig. 2). Pathways involving extracellular matrix remodeling, including proteoglycans in cancer, focal adhesion, adherens junctions, and tight junctions, were also significantly enriched during *C. albicans* infection. Pathways involved in responses to infection by other microbes, including Epstein-Barr virus, *Shigella*, hepatitis B virus, influenza A virus, herpesvirus, *Salmonella*, and trypanosomes, were also predicted to be activated, suggesting conservation of epithelial responses to a broad array of pathogens. Pathways predicted to be activated were generally conserved at the 6-h and 24-h time points. A list of individually expressed genes may be found in Table S1 in the supplemental material.

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

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

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

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

Candidalysin is required for vaginitis immunopathology. We next questioned whether Ece1p and/or Candidalysin contributes to immunopathology in an established estrogen-dependent mouse model of VVC. Therefore, we utilized strains of *C. albicans* that had had both copies of *ECE1* deleted (*ece1*Δ/Δ) and restored with one full-length allele (*ece1*Δ/Δ + *ECE1*) or one mutant allele lacking the Candidalysin-encoding region of *ECE1* (*ece1*Δ/Δ + $ECE1_{\Delta 184-279}$), along with the appropriate parental isogenic control (BWP17 + CIp30, referred to as here the wild type [WT]). Somewhat surprisingly, recovered fungal burdens from the vaginal lavage fluid were not significantly different between strains at either day 3 (Fig. 4A) or day 7 (Fig. 4B) postinoculation (p.i.). However, there was a significant reduction in the number of neutrophils recruited into the vaginal lumen during challenge with either the *ece1*Δ/Δ or *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$

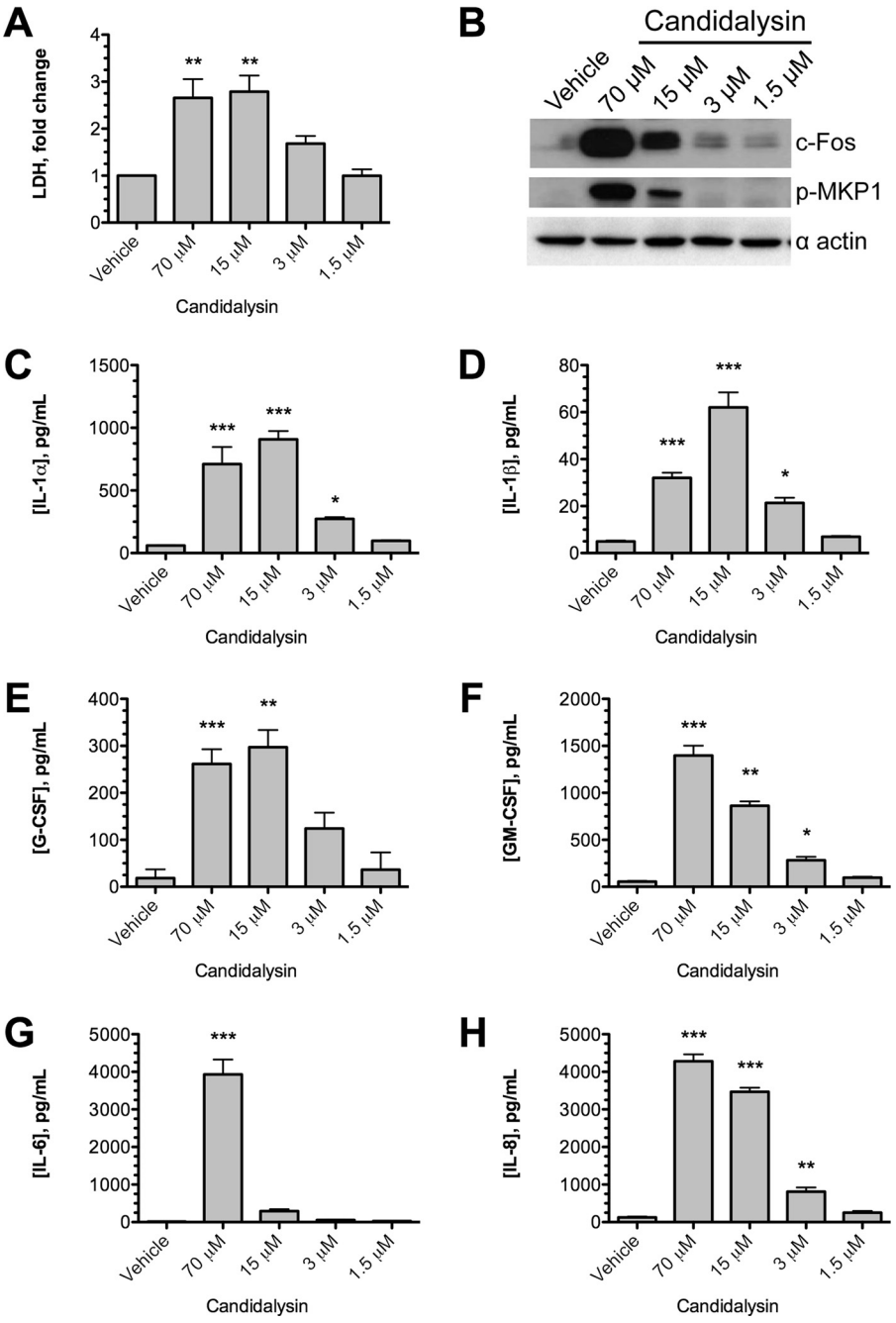

FIG 3 Candidalysin is sufficient to induce cellular damage and proinflammatory responses in vaginal epithelial cells. (A) A431 vaginal epithelial cells were exposed to Candidalysin (1.5, 3, 15, and 70 μM) for 24 h, and cellular damage was quantified by LDH assay. The data are presented as the fold change relative to vehicle control. Statistics were applied relative to the vehicle control ($n = 3$ biological repeats). (B) Western blot analysis of the vaginal epithelial responses to different concentrations of Candidalysin. Epithelial cell lysates (20 μg total protein) were probed with anti c-Fos and anti p-MKP1 antibodies. One representative blot is presented (from 3 biological repeats). (C to H) Quantification of cytokines (IL-1α, IL-1β, G-CSF, GM-CSF, IL-6, and IL-8) secreted from vaginal epithelial cells in response to different concentrations of Candidalysin. Statistics were applied relative to the vehicle control ($n = 3$ biological repeats). The graphs are plotted as means plus SEM. (A and C to H) Statistical significance was calculated using one-way ANOVA and Dunnet's posttest. ***, $P < 0.001$; **, $P < 0.01$, *, $P < 0.05$.

strain, which was restored to WT levels during infection with the *ece1*Δ/Δ + *ECE1* reintegrant strain (Fig. 4C, D, and G, yellow arrows). Consistent with this phenotype, levels of the damage biomarker LDH were significantly reduced with the same mutants compared to infection with the WT or the *ece1*Δ/Δ + *ECE1* reintegrant (Fig. 4E and F).

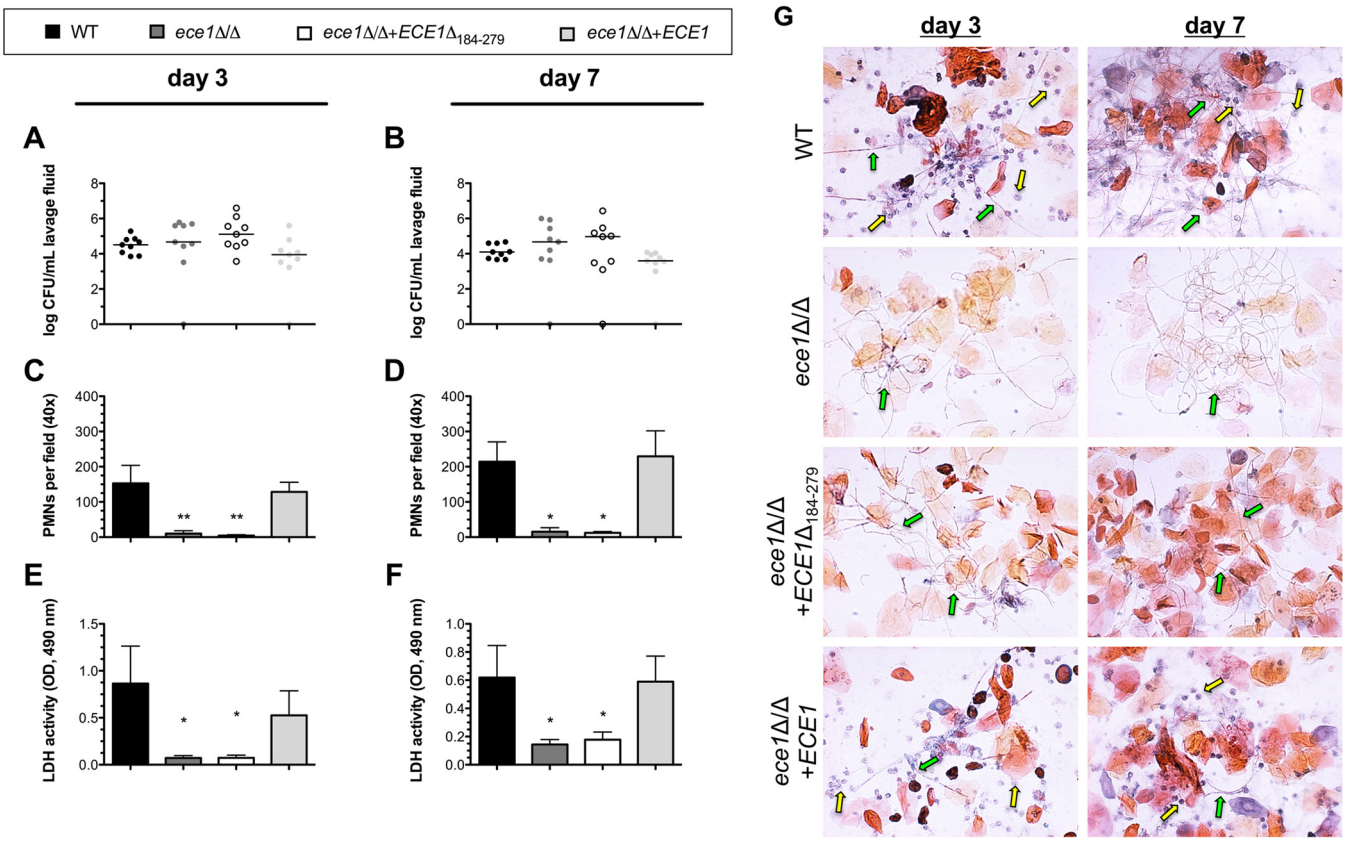

FIG 4 Candidalysin is required for neutrophil recruitment and mucosal damage in a murine model of vulvovaginal candidiasis. (A to F) Groups of estrogen-treated C57BL/6 mice ($n = 4$) were intravaginally challenged with WT, *ece1*Δ/Δ, *ece1*Δ/Δ + *ECE1*$\Delta_{184-279}$, and *ece1*Δ/Δ + *ECE1* strains of *C. albicans*, and vaginal lavage fluid was assessed longitudinally at day 3 and day 7 for fungal burden (horizontal lines, medians) (A and B), PMNs (means plus SEM) (C and D), or the damage biomarker LDH (means plus SEM) (E and F). Statistical significance was calculated using one-way ANOVA and Tukey's posttest. **, $P < 0.01$, *, $P < 0.05$. (G) Papanicolaou staining was performed on smears made from vaginal lavage fluid to assess PMN influx (yellow arrows) and hypha formation (green arrows) at day 3 and day 7 p.i. Representative images are shown. All inoculation groups were performed in duplicate, and the data were combined.

Given our previous data using hypha-deficient strains, a morphogenesis defect may account for this phenotype (6). However, *ece1*Δ/Δ and *ece1*Δ/Δ + *ECE1*$_{\Delta 184-279}$ strains robustly formed hyphae at the vaginal mucosa, as did WT and *ece1*Δ/Δ + *ECE1* strains (Fig. 4G, green arrows). Thus, these results demonstrate that Candidalysin is required for vaginal immunopathogenesis *in vivo* and that hypha formation alone is insufficient to elicit the hallmark immunopathology.

Candidalysin-dependent innate cytokine expression is conserved between mouse and human. We also wanted to determine whether the Candidalysin-induced innate immune response observed in human vaginal epithelial cells paralleled cytokine expression in the murine vaginal mucosa *in vivo*. RNA was isolated from whole vaginas of mice challenged with WT, *ece1*Δ/Δ, *ece1*Δ/Δ + *ECE1*, *ece1*Δ/Δ + *ECE1*$_{\Delta 184-279}$, and PBS, and gene expression was assessed by quantitative PCR (qPCR). Overall, cytokine gene expression patterns were similar between *in vitro* and *in vivo* samples, including Candidalysin-induced expression of the genes *Il-6*, *Cxcl2*, *Il-1a*, and *Il-1b* (Fig. 5A and C to E). There was a similar trend for expression of the genes *Cxcl1* and *Gm-csf*, although only the *ECE1*-null mutant (*ece1*Δ/Δ) demonstrated a statistically significant reduction in cytokine gene induction (Fig. 5B and F). Unexpectedly, *G-csf* gene expression was not increased during challenge with any of the fungal strains, unlike that observed with Candidalysin treatment (Fig. 5G). In the oral cavity, *C. albicans* induces expression of the antimicrobial peptide (AMP) cathelicidin, the murine equivalent of which is the cathelicidin-related AMP (CAMP) (20). Interestingly, the gene encoding CAMP was not induced in the vagina by Candidalysin and in fact was downregulated similarly by all

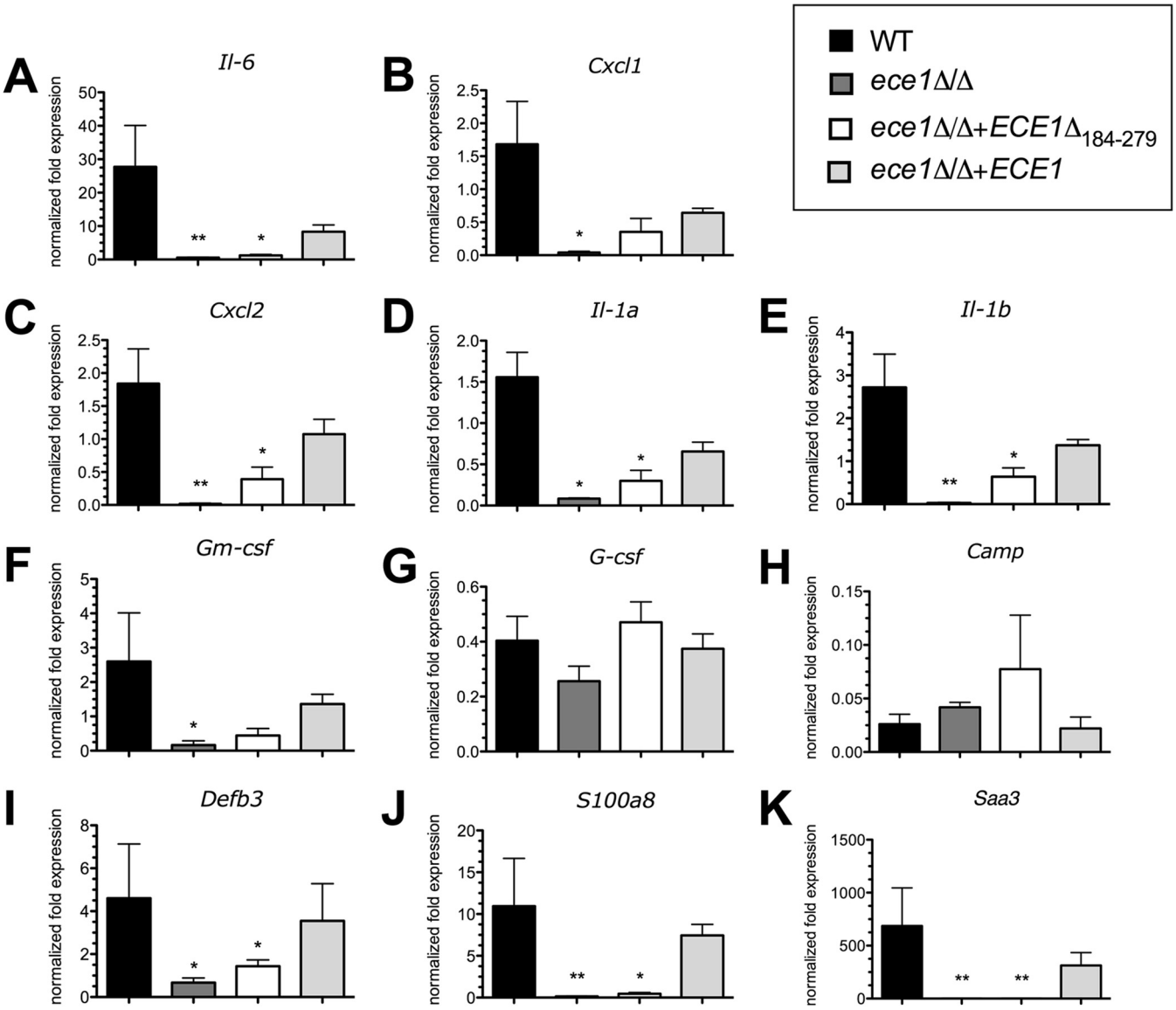

FIG 5 Candidalysin is required for proinflammatory cytokine expression in the murine vagina. Groups of estrogen-treated C57BL/6 mice ($n = 4$) were intravaginally challenged with WT, *ece1*Δ/Δ, *ece1*Δ/Δ + *ECE1*$\Delta_{184-279}$, and *ece1*Δ/Δ + *ECE1* strains of *C. albicans*; whole vaginal tissue was excised at day 3 p.i.; and extracted RNA was processed for qPCR analysis. The genes chosen for qPCR were those previously identified as being induced by Candidalysin or *C. albicans* during *in vitro* or *in vivo* challenge: *Il-6* (A), *Cxcl1* (B), *Cxcl2* (C), *Il-1a* (D), *Il-1b* (E), *Gm-csf* (F), *G-csf* (G), *Camp* (H), *Defb3* (I), *S100A8* (J), and *Saa3* (K). All the genes were internally compared to the *Actb* housekeeping gene and to mock-infected controls using the $\Delta\Delta C_T$ method. The graphs are plotted as the mean normalized fold expression plus SEM. Statistical significance was calculated using one-way ANOVA and Tukey's posttest. **, $P < 0.01$, *, $P < 0.05$.

the strains compared to mock treatment (Fig. 5H). However, induction of the antimicrobial peptide murine β-defensin 3 (mBD3) gene was Candidalysin dependent (Fig. 5I).

We also sought to determine if two inflammatory markers previously identified as associated with VVC immunopathology were regulated in a Candidalysin-dependent manner. Expression of the gene coding for S100A8 (*S100a8*), a calcium-binding protein with important functions in antifungal defense and danger responses and strongly induced during *C. albicans* infection, was almost completely absent during infection with Candidalysin deletion strains (Fig. 5J) (21, 22). Similarly, the gene encoding serum amyloid A3 (*Saa3*), an inducible acute-phase apolipoprotein capable of recruiting immune cells to inflammatory sites, was similarly increased in a Candidalysin-dependent fashion (Fig. 5K) (23, 24).

Finally, we investigated whether production of cytokines at the protein level (at both day 3 and day 7 p.i.) was Candidalysin dependent. Indeed, *C. albicans*-mediated

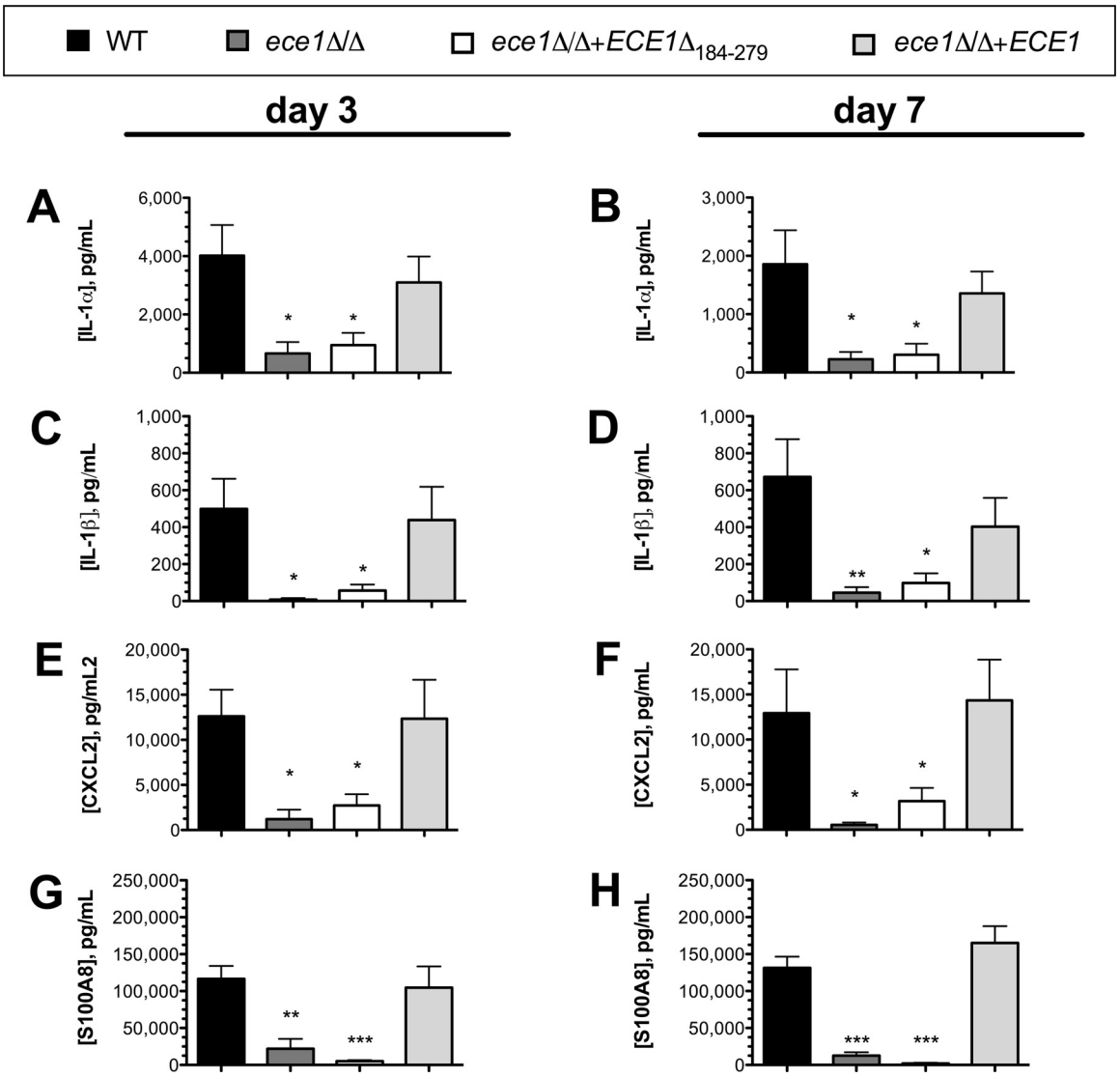

FIG 6 Candidalysin is required for release of hallmark proinflammatory cytokines and chemokines into the vaginal lavage fluid during murine vaginitis. Groups of estrogen-treated C57BL/6 mice ($n = 4$) were intravaginally challenged with WT, *ece1*Δ/Δ, *ece1*Δ/Δ + *ECE1*$\Delta_{184-279}$, and *ece1*Δ/Δ + *ECE1* strains of *C. albicans*, and vaginal lavage fluid was assessed longitudinally by ELISA at day 3 and day 7 p.i. for inflammatory markers: IL-1α (A and B), IL-1β (C and D), Cxcl2 (E and F), and S100A8 (G and H). All inoculation groups were performed in duplicate, and the data were combined. The graphs are plotted as means plus SEM. Statistical significance was calculated using one-way ANOVA and Tukey's posttest. ***, $P < 0.001$; **, $P < 0.01$, *, $P < 0.05$.

secretion of IL-1α, IL-1β, CXCL2, and S100A8 into the vaginal lavage fluid required expression of functional Candidalysin (Fig. 6A to H). Despite increased expression of the genes encoding IL-6, CXCL1, and GM-CSF in vaginal tissue, we were unable to demonstrably quantify these cytokines at the protein level in the lavage fluid of mice inoculated with any of the *C. albicans* strains tested (data not shown).

DISCUSSION

In recent years, vulvovaginal candidiasis has been identified as an immunopathology in which the host immune response, orchestrated by a series of 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 the presence of neutrophils in the vaginal lavage fluid of women intravaginally inoculated with *C. albicans* was tightly correlated with disease symptomatology (e.g., vaginal itching, burning, and discomfort) (4). Activation of innate immune

signaling results in the recruitment of neutrophils to the vaginal mucosa, and experimental evidence suggests that these cells then amplify the inflammatory cascade, seemingly without reducing the fungal burden (22, 25). The *in vitro* and *in vivo* data presented in this study identify Candidalysin as the crucial virulence factor that drives both *C. albicans*-induced neutrophil recruitment and vaginal immunopathogenesis.

The vaginal epithelial response to *C. albicans* infection provides new insight into immunopathological signaling. Microarray data derived from *C. albicans*-infected human vaginal epithelial cells strongly paralleled what had been observed previously using targeted multiplex cytokine assays to determine the host response to vaginal infection (8). Unsurprisingly, many of the genes shown by transcriptomic sequencing (RNA-Seq) to be differentially regulated during murine vaginitis were not found in our human microarray data sets (26). The first explanation of this was that the human response (microarray data) is not strictly homologous with the murine response. However, given the similarity and linkage of immunopathology with neutrophil influx to the vaginal lumen in both human and murine vaginal infections, this explanation seems less likely. A more plausible explanation is that the microarray data presented here provide an epithelium-specific response that is independent of hormonal modulation and the presence of other cell types. Murine RNA-Seq data were derived from whole vaginal tissue, and thus, hematopoietic and stromal compartments were similarly represented (26). While each strategy offers its own unique strengths and weaknesses, direct comparison between data sets must be made with caution. However, despite these methodological differences, there was relatively strong conservation between the proinflammatory responses in the two data sets, including eicosanoid signaling (*PLA2GB4* and *PTGS2*), S100 alarmin expression, and strong CXCL2 chemokine upregulation (25–27). Additionally, increased expression of the *IL-1B* and *IL-1RN* genes was also identified in both data sets, suggesting that the IL-1 circuit is activated in a *C. albicans*-specific manner at the vaginal epithelium.

Recently, RNA-Seq analysis of human cells collected with longitudinal vaginal swabs during healthy and symptomatic vaginitis states revealed that v-*erb-b2* erythroblastic leukemia viral oncogene homolog 2 (*ERBB2*) and platelet-derived growth factor gene (*PDGF-BB*) signaling may have important functions during vaginal candidiasis (28). Interestingly, our microarray data set (human vaginal epithelium) did not directly demonstrate increased expression of *ERBB2* or *PDGF-BB* genes, but several genes coding for downstream targets of these factors were highly induced, including *JUNB*, *FOS*, *DUSP5*, *NR4A1*, *IL-8*, *IL-1B*, *TNFAIP3*, and *EGR1*. Therefore, *in vitro* infection of A431 cells largely mimics responses observed during clinical vaginitis.

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

reducing fungal burden during vaginitis, despite being robustly recruited to the vaginal lumen indirectly by Candidalysin.

Candidalysin is the key fungal factor driving damage and vulvovaginal immunopathogenesis. Previous data generated from our laboratories have demonstrated that both oral and vaginal epithelial cells can differentially sense and respond to yeast and hyphal forms of *C. albicans* with modestly different signaling mechanisms, cytokine secretion, and hyphal burden activation thresholds (6, 8, 10). These differences in responses may represent site-specific fine tuning of mucosal immunity—a hypothesis supported by the observed differences in transcription factors activated in the two cell types (8, 10). Regardless, it is now clear that Candidalysin plays a crucial role in activating epithelial responses at disparate mucosal sites. Interestingly, some cytokines (e.g., IL-6) were induced by treatment with Candidalysin alone, but not during infection with *C. albicans*. Thus, it is likely that the Candidalysin concentration is a major factor in its lytic and immunostimulatory functions, and currently, it is unclear what general or microniche concentrations of Candidalysin are present in an *ex vivo* or *in vivo* setting or what other host or fungal factors (e.g., secreted aspartyl proteinases or cell wall components) it may interact or synergize with during infection.

The hypothesis that epithelial surfaces discriminate between yeast and hyphal forms of *C. albicans* has been established by linking hypha formation with the capacity to damage epithelial surfaces *in vitro* and *in vivo* and the subsequent release of danger-associated molecular patterns (DAMP) to activate the cellular inflammasomes, including NLRP3 (6, 8, 10, 32–34). The use of NLRP3$^{-/-}$ mice during VVC has demonstrated that neutrophil migration and proinflammatory signaling are reduced in these animals, presumably due to a defect in the ability to recognize and respond to DAMP signals (26, 35). Furthermore, a population level genetic study revealed that the 12/9 genotype was significantly associated with high levels of NLRP3 effector cytokines found in the vaginal lavage fluid of women with recurrent VVC (RVVC), suggesting the recognition of DAMP signals is important in disease immunopathogenesis (36). Given that the presence of Candidalysin is sufficient to induce damage at the vaginal mucosa and elicits inflammasome effector responses (i.e., IL-1β), it is possible that Candidalysin serves as a fungal DAMP capable of inflammasome activation. Investigations are under way to address these possibilities.

This concept of linking fungal pathogenicity to damage was further supported by findings by Schönherr et al. in which the virulence of *C. albicans* clinical isolates was directly correlated with their capacity to induce oral mucosal insult (44). Notably, only the expression of *ECE1*/Candidalysin was strongly correlated with damage and pathogenesis in several (but not all) *C. albicans* isolates. However, it is likely that simultaneous and combined expression of several attributes (e.g., hyphae and Candidalysin) is required for full virulence. The interplay of colonization, host response, and damage was elegantly summarized by Casadevall and Pirofski in the "damage-response framework" (DRF), a rubric describing a pathogen's ability to cause disease on a continuum of host immune status and damage capacity (38). Recently, Jabra-Rizk and colleagues revisited the DRF in the context of *C. albicans* pathogenesis specifically, concluding that VVC fits into class 6 of the DRF, in which a pathogen causes damage only in the context of an aggressive immune response (39). Based on these and previous findings, this appears to hold true, given that robust PMN and cytokine levels are strongly associated with disease symptomatology and centrally dependent on the capacity of Candidalysin to cause damage. However, neutrophil-depleted mice (anti-Ly6G) exhibit vaginal LDH levels equivalent to those of isotype-treated controls during experimental VVC, suggesting that mucosal damage still occurs in the absence of robust classical immunopathology (6). Moreover, PMN recruitment in humans is highly associated with disease onset but has not been extensively evaluated as a requirement or worsening criterion for immunopathology. Therefore, it is arguable that VVC may be better categorized in class 5 of the DRF, where the pathogen causes damage across the spectrum of immune responses but the damage may be enhanced by strong immune responses.

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

MATERIALS AND METHODS

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

Cell lines, strains, and primers. The A431 human vulvar epidermoid carcinoma cell line was used in this study. All *C. albicans* strains used, including Candidalysin deletion mutants, were described by Moyes et al. and Gillum et al. (12, 40). All the primers used for qPCR are listed in Table S2 in the supplemental material.

Microorganism growth. *C. albicans* strains were maintained as glycerol stocks stored at −80°C. A small amount of stock was spread onto yeast extract-peptone-dextrose (YPD) agar and incubated at 30°C for 48 h to obtain isolated colonies. A single colony was transferred to 10 ml of YPD liquid medium and incubated at 30°C with shaking at 200 rpm for 16 h prior to vaginal infection.

Microarray analysis. RVE (5 day) created using the A431 cell line were purchased from SkinEthic Laboratories (France) and used as previously described (8). RNA was isolated from three independent RVE infected with *C. albicans* SC5314 for 6 and 24 h or with an equal volume of PBS using the GenElute total mammalian RNA miniprep kit (Sigma, United Kingdom), and trace genomic DNA was removed using a Turbo DNase-free kit (Ambion, United Kingdom). For microarray analysis, RNA was amplified using the MessageAmp Premier RNA amplification kit (Ambion, United Kingdom) and hybridized onto U133a 2.0 gene chips (Affymetrix, United Kingdom) after fragmentation by metal-induced hydrolysis into 35- to 200-nucleotide fragments according to standard protocols. The chips were scanned (Affymetrix GeneChip Scanner 3000) and assessed using the Affymetrix Command Console (AGCC) software suite. The data were statistically analyzed using the Bioconductor R package PIANO. Genes were considered to be differentially up- or downregulated when their expression was changed by at least 2-fold with a false-discovery rate (FDR)-adjusted *P* value of less than 0.01. Gene Ontology and pathway analyses were performed on the generated gene list using both PIANO and DAVID (41, 42).

Cytokine release. A431 vaginal epithelial cells were cultured in Dulbecco's modified Eagle medium (DMEM) nutrient mixture plus L-glutamine (Life Technologies) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Life Technologies) and 1% (vol/vol) penicillin-streptomycin (Sigma) at 37°C and 5% CO_2. Candidalysin peptide (SIIGIIMGILGNIPQVIQIIMSIVKAFKGNK) was purchased from Peptide Protein Research Ltd. (United Kingdom). Prior to Candidalysin challenge, confluent A431 epithelial cells were serum starved overnight, and all experiments were carried out in serum-free DMEM. The cells were incubated with Candidalysin (prepared as a 10-mg/ml stock in sterile water) at doses of 1.5, 3, 15, and 70 μM for 2 h at 37°C in 5% CO_2. Sterile-water (vehicle-only) controls were also included. The culture supernatants were then isolated, and human IL-1α, IL-1β, IL-6, IL-8, GM-CSF, and G-CSF were quantified by magnetic Luminex performance assay (Biotechne) and the Bio-Plex 200 system (Bio-Rad) according to the manufacturers' instructions.

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

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

SDS-PAGE and Western blotting. Proteins were resolved by electrophoresis on SDS-12% PAGE gels using a mini-protean tetra cell system (Bio-Rad). Electrophoresed proteins were transferred to nitrocellulose membranes (Bio-Rad) using a mini-transblot electrophoretic transfer cell (Bio-Rad). p-DUSP1/MKP1 (S359) and c-Fos rabbit monoclonal antibodies were purchased from Cell Signaling Technologies. Actin (clone C4) mouse monoclonal antibody was purchased from Millipore. Peroxidase-conjugated Affinipure goat anti-mouse and anti-rabbit IgG secondary antibodies were purchased from Jackson Immune Research. Membranes were blocked in 1× Tris-buffered saline (TBS) (Severn Biotech) containing 0.001%

(vol/vol) Tween 20 (Acros Organics) and 5% (wt/vol) fat-free milk powder (Sainsbury's). Primary antibodies diluted (1:1,000 or 1:10,000, as suggested by the manufacturer) in TBS-Tween and 5% milk (c-Fos), or TBS-Tween and 5% bovine serum albumin (p-DUSP1/MKP1) were added, and the membranes were incubated overnight at 4°C with gentle shaking. Following incubation, the membranes were washed with 1× TBS containing 0.01% (vol/vol) Tween 20, diluted (1:10,000) horseradish peroxidase (HRP)-conjugated secondary antibody was added, and the membranes were incubated for 1 h at room temperature. The membranes were washed as described above and exposed to Immobilon Western chemiluminescent HRP substrate (Millipore) prior to visualization by exposure to film (GE Healthcare). Alpha-actin was used as a loading control.

Murine model of vaginal candidiasis. A murine model of *Candida* vaginitis was utilized as described previously (6, 26, 43). Female 6- to 8-week-old C57BL/6 mice were purchased from Charles River Laboratories and housed in isolator cages mounted on ventilated racks. The mice were subcutaneously administered 0.1 mg of estrogen (β-estradiol 17-valerate; Sigma) dissolved in 0.1 ml sesame oil 72 h prior to inoculation with *C. albicans*. Stationary-phase cultures of *C. albicans* strains were washed three times in sterile endotoxin-free PBS and resuspended in a 0.2× volume of PBS. The cell suspensions were diluted, counted on a Neubauer hemocytometer, and adjusted to 5×10^8 CFU/ml in sterile PBS. Estrogen-treated mice were intravaginally inoculated with 10 μl of the standardized blastoconidial cell suspension, generating an inoculum size of 5×10^6 blastoconidia. At day 3 and/or day 7 p.i., the mice underwent vaginal lavage with 100 μl of PBS. The resultant lavage fluids were spiked with 1 μl of 100× EDTA-free protease inhibitors (Roche) and kept on ice until they were processed for immunopathological markers. After sacrifice, vaginal tissue was surgically excised and stored for downstream analyses. All animal experiments were conducted with 4 mice per group and repeated, and the data were combined unless otherwise noted.

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

Isolation of RNA from vaginal tissue. RNA was extracted from whole vaginas as described previously (26). At day 3 p.i., vaginal tissue was surgically excised, immediately placed into RNALater (Thermo Fisher), and incubated at 4°C overnight. The following day, the tissues were transferred to TRI Reagent (Sigma), finely minced with scissors, mechanically homogenized (Pro Scientific), and centrifuged at 12,000 × *g* for 10 min at 4°C. RNA was isolated by chloroform-ethanol precipitation, and the pellet was resuspended in nuclease-free water according to the TRI Reagent instructions. The RNA concentration was measured by spectroscopy at A_{260}/A_{280}, and integrity was verified by 3-(*N*-morpholino)propanesulfonic acid (MOPS) gel electrophoresis to visualize intact 18S and 28S rRNA bands.

qRT-PCR analysis. RNA from vaginal tissue was isolated as described above. RNA concentrations were equalized among samples, and 200-ng aliquots were treated with RNase-free DNase (Thermo Scientific) according to the manufacturer's instructions. RNA was reverse transcribed using random hexamers and the RevertAid kit (Thermo Scientific) according to the manufacturer's protocol. Proprietary primer sets spanning exon-exon junctions were ordered from IDT for murine *Il-6*, *Cxcl1*, *Cxcl2*, *Il-1a*, *Il-1b*, *Gm-csf*, *G-csf*, *Camp*, *S100a8*, *Saa3*, *Defb3*, and *Act1b* (see Table S2 in the supplemental material). All the primers were used at the manufacturer's recommended concentrations, along with 2× Maxima Sybr green mix (Bio-Rad) to amplify 20 ng of cDNA. qPCRs were monitored and analyzed with the Applied Biosystems 7500 platform and associated software. Expression levels of target genes in infected mice were compared to that of a reference gene (*ACT1B*) and naive controls using the $\Delta\Delta C_T$ method as described previously (37).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00645-17.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.
SUPPLEMENTAL FILE 2, XLS file, 0.4 MB.
SUPPLEMENTAL FILE 3, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 4, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

We thank Duncan Wilson (University of Aberdeen) for constructing and providing WT (BWP17 + clP30), *ece1*Δ/Δ, *ece1*Δ/Δ + *ECE1*, and *ece1*Δ/Δ + $ECE1_{\Delta 184-279}$ strains.

Experimental design was conducted by D.L.M., J.R.N., B.H., and B.M.P. J.P.R., H.M.E.W., D.L.M., S.S., K.S.B., S.L.T., and G.E.P. performed all experimental techniques and data analysis. We all aided in experimental critique and manuscript preparation.

These studies were supported by National Institute of Allergy and Infectious Disease (NIAID), National Institutes of Health, grant K22AI110541 awarded to B.M.P. and by the Medical Research Council (MR/M011372/1), the Biotechnology and Biological Sciences Research Council (BB/N014677/1), and the National Institute for Health Research at Guys and St Thomas's NHS Foundation Trust and King's College London Biomedical Research Centre (IS-BRC-1215-20006 to J.R.N.).

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