Transcriptomic Analysis of Vulvovaginal Candidiasis Identifies a Role for the NLRP3 Inflammasome

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ABSTRACT Treatment of vulvovaginal candidiasis (VVC), caused most frequently by Candida albicans, represents a significant unmet clinical need. C. albicans, as both a commensal and a pathogenic organism, has a complex and poorly understood interaction with the vaginal environment. Understanding the complex nature of this relationship is necessary for the development of desperately needed therapies to treat symptomatic infection. Using transcriptome sequencing (RNA-seq), we characterized the early murine vaginal and fungal transcriptomes of the organism during VVC. Network analysis of host genes that were differentially expressed between infected and naive mice predicted the activation or repression of several signaling pathways that have not been previously associated with VVC, including NLRP3 inflammasome activation. Intravaginal challenge of Nlrp3−/− mice with C. albicans demonstrated severely reduced levels of polymorphonuclear leukocytes (PMNs), alarmins, and inflammatory cytokines, including interleukin-1β (IL-1β) (the hallmarks of VVC immunopathogenesis) in vaginal lavage fluid. Intravaginal administration of wild-type (WT) mice with glyburide, a potent inhibitor of the NLRP3 inflammasome, reduced PMN infiltration and IL-1β to levels comparable to those observed in Nlrp3−/− mice. Furthermore, RNA-seq analysis of C. albicans genes indicated robust expression of hypha-associated secreted aspartyl proteinases 4, 5, and 6 (SAP4–6), which are known inflammasome activators. Despite colonization similar to that of the WT strain, ΔSAP4–6 triple and ΔSAP5 single mutants induced significantly less PMN influx and IL-1β during intravaginal challenge. Our findings demonstrate a novel role for the inflammasome in the immunopathogenesis of VVC and implicate the hypha-associated SAPs as major C. albicans virulence determinants during vulvovaginal candidiasis.

IMPORTANCE Vaginitis, most commonly caused by the fungus Candida albicans, results in significant quality-of-life issues for all women of reproductive age. Recent efforts have suggested that vaginitis results from an immunopathological response governed by host innate immunity, although an explanatory mechanism has remained undefined. Using comprehensive genomic, immunological, and pharmacological approaches, we have elucidated the NLRP3 inflammasome as a crucial molecular mechanism contributing to host immunopathology. We have also demonstrated that C. albicans hypha-associated secreted aspartyl proteinases (SAP4–6 and SAP5, more specifically) contribute to disease immunopathology. Ultimately, this study enhances our understanding of the complex interplay between host and fungus at the vaginal mucosa and provides proof-of-principle evidence for therapeutic targeting of inflammasomes for symptomatic vulvovaginal candidiasis.
now linked innate mucosal defenses to disease immunopathology, including robust polymorphonuclear leukocyte (PMN) recruitment and production of inflammatory effectors (e.g., interleukin 1β [IL-1β] and S100 alarmins) (18–21). Despite this paradigm shift in the philosophy of disease pathogenesis, the precise molecular mechanisms responsible for VVC remain poorly understood.

While both OPC and VVC are clinical manifestations of *C. albicans* mucosal infection, significant differences are underscored by differential molecular and epidemiological host-pathogen relationships. For example, HIV-positive women are more susceptible than HIV-negative women to OPC but not VVC (22–27). These findings are supported by the fact that vaginal challenge of SCID mice with *C. albicans* does not result in a greater fungal burden than in WT mice, negating any appreciable role for conventional adaptive-immunity-mediated susceptibility to VVC (15). Furthermore, mice lacking either IL-23α subunit p19 (IL-23p19) or IL-17 receptor A (IL-17RA) develop more severe disease than WT mice during OPC, partly due to decreased neutrophil recruitment (28). In contrast, these deletions do not have a detectable effect on neutrophil recruitment or fungal burden in a murine model of VVC (17). These significant differences highlight the importance of performing discovery-based experiments using specific models to understand VVC instead of drawing solely from previous work on OPC or systemic-disease models.

Several studies have focused on specific vaginal molecular responses to *C. albicans*, using in vitro models (29–31), in vivo models (13, 16, 32–34), or clinical samples (18, 35, 36). However, only one study has examined the host response to *C. albicans* on a global scale in the context of VVC. In that study, Yano et al. used mass spectrometry for *de novo* identification of host proteins that are secreted into vaginal lavage fluid during VVC (20). While that study was instrumental in the identification of the role of the alarmins (S100A8 and S100A9) in PMN recruitment during VVC, the scope of analysis was limited by its targeted proteomic strategy. Transcriptomics, on the other hand, offers a more comprehensive and unbiased systems-level approach to understand host-pathogen responses during infection.

In this study, we used gene expression profiling (transcriptome sequencing [RNA-seq]) to determine the signaling pathways that are activated or repressed during an *in vivo* murine model of VVC. Network analysis of the differentially expressed genes uncovered several signaling proteins that were not previously associated with the vaginal host response to fungal pathogens, as well as those that govern the activation of the inflammasome, a cytoplasmic protein complex that triggers activation of IL-1β and IL-18 to promote inflammatory responses to microbial pathogens (37, 38). Importantly, mice lacking NLRP3 (NLR family, pyrin domain containing 3) and those treated with the potent inflammasome inhibitor glyburide displayed a marked reduction in *C. albicans*-induced PMN infiltration and IL-1β activation, defining a crucial role for inflammasome function in the immunopathogenesis of VVC. RNA-seq analysis of fungal transcripts demonstrated robust expression of hypha-associated secreted aspartyl protease 5 (SAP5). Importantly, intravaginal challenge with a SAP4 to -6 deletion (ΔSAP4–6) mutant led to significantly decreased levels of PMNs and IL-1β in the vaginal lavage fluid. Collectively, this work provides a genome-wide analysis of the complex host and fungal factors that contribute to VVC immunopathology.

**RESULTS**

**RNA-seq analysis of a murine model of VVC.** In an effort to characterize the transcriptomes of both a fungal pathogen and its host during an *in vivo* infection, we performed RNA-seq analysis on vaginal tissues obtained from mice, a well-established model of vulvovaginal candidiasis (39). Groups of C57BL/6 mice were estrogen treated and inoculated with either 5 × 10⁶ *C. albicans* yeast cells (clinical isolate SC5314) or an equal volume of sterile phosphate-buffered saline (PBS). On day 3 postinoculation (p.i.), animals were sacrificed and underwent vaginal lavage for enumeration of fungal burden and PMNs or their vaginal tissues were harvested for extraction of total RNA, which contained a mixture of RNAs expressed by the host as well as *C. albicans*. We chose this time point because our previous work has demonstrated that expression of immunopathological hallmarks (i.e., PMNs, tissue damage, and IL-1β protein levels in the lavage fluid) peaked 3 days p.i. (19). Consistently with our previous observations, infected mice were robustly colonized by *C. albicans* (~1 × 10⁶ CFU/ml), with significant elevation of PMNs in the vaginal lavage fluid, while naive mice were devoid of fungal burden, with little-to-no PMNs present (see Fig. S1 in the supplemental material). Total RNA samples were used to make poly(A)-enriched RNA-seq libraries and subjected to deep sequencing on the Illumina HiSeq2000 platform. All sequencing reads were sequentially aligned to the mouse reference genome (mm10, GRCh38) and the *C. albicans* reference genome (strain SC5314) and then further analyzed as depicted in Fig. 1 (described under Materials and Methods). From each of the 6 sequencing libraries (from 3 infected and 3 naive mice), we obtained an average of 102.6 million reads that mapped to the mouse genome (Table S1). In contrast, for each of the 3 infected samples, we obtained an average of 79,653 reads that matched to the *C. albicans* genome (Table S1).

**Functional enrichment analysis of host gene expression during VVC.** We defined differentially expressed genes as those with a minimum of a 2-fold change in expression (*P* value < 0.05) between the *C. albicans*-infected and naive vaginal tissues. Analysis of host cell gene expression revealed 1,541 genes that were differentially expressed in the *C. albicans*-infected tissue relative to the naive tissues (Table S2). In order to obtain a global picture of the complex biology that takes place in the vaginal mucosa during VVC, we used the Ingenuity Pathway Analysis (IPA; Ingenuity Systems) software package to identify biological functions and activities that are enriched in our list of differentially expressed genes (Table S3). Most notably, many of the differentially expressed genes are involved in activities of the innate immune system, specifically the mobilization of cells (*P* value = 2.68 × 10⁻⁴), accumulation of leukocytes (*P* value = 3.17 × 10⁻⁴), mobilization of myeloid cells (*P* value = 2.14 × 10⁻³), Th17 immune response (*P* value = 3.75 × 10⁻⁴), accumulation of myeloid cells (*P* value = 2.62 × 10⁻⁶), biosynthesis of hydrogen peroxide (*P* value = 4.54 × 10⁻⁶), and infiltration of phagocytes (*P* value = 3.86 × 10⁻⁴). Based on the direction of the gene expression changes, the known relationship between the differentially expressed genes, and the biological functionality of these genes, each of these physiological functions is predicted to be activated in response to *C. albicans* infection (Fig. 2). These results are consistent with those of previous studies that have demonstrated that VVC results in robust infiltration of PMNs, their associated oxidative burst (40), and the
PTGS2 protein activity in the lavage fluid of mice and observed formed a competitive enzyme immunoassay (EIA) to measure coding prostaglandin-endoperoxide synthase 2 (PTGS2). We prepared to the naive tissue, consistent with our RNA-seq results host cells during in vivo C. albicans infection.

To summarize the complex regulatory networks that mediate immunopathology during VVC in vivo, we constructed a diagram of upstream regulators with predicted activation or repression activity (Fig. 4, orange and blue circles, respectively) observed in the murine model. Each of these 11 signaling proteins govern the expression of IL-1β and therefore potentially contribute to an increased levels of prostaglandin E2 (PGE2), the eicosanoid enzymatically generated by PTGS2 induction (Fig. 3B), as previously reported (45). Our RNA-seq results are also validated by previously published experimental data. Specifically, we observed the induction of several genes (IL12B, IL17A, IL17F, IL17C, IL22, CCL2, and S100A9) whose proteins levels are known to increase in vaginal lavage fluid during VVC in rodent models (17, 32, 34, 41, 42, 46–49) as well as the induction of genes (CCL2 and S100A9) whose expression is known to be induced in C. albicans-infected vaginal tissue (20, 48, 49).

Upstream regulator analysis of host gene expression during VVC. We reasoned that gene expression changes in the host may also be used to identify novel signal transduction pathways that are modulated in response to C. albicans infection and that these pathways might be relevant to pathogenesis. We used the upstream Regulator Analysis from the IPA software to identify signaling proteins whose downstream signaling pathways are potentially activated or repressed during the course of infection. This software assesses the overlap between experimentally derived gene lists and an extensively curated database of target genes for each of several hundred known regulatory proteins. It then uses the statistical significance of the overlap and the direction of the differential gene expression to make predictions about activation or repression of these regulatory proteins.

Our upstream regulator analysis of the differentially expressed genes predicted the modulation of a total of 178 signaling proteins, of which 76 are predicted to be activated and 102 are predicted to be repressed (Table S4). The utility of this approach was verified by our identification of multiple signaling molecules whose protein expression or activity is stimulated by or required for the proper response to C. albicans infection. Specifically, tumor necrosis factor (TNF), IL-1α, IL-1β, nuclear factor kappa B (NF-κB), interferon gamma (IFN-γ), Toll-like receptor 4 (TLR4), NLR family CARD domain-containing protein 4 (NLRC4), and myeloid differentiation primary response 88 (MYD88) have all been shown to be activated in host cells in response to C. albicans (21, 29, 30, 41, 46, 47, 49–52) or required for the host defense against C. albicans infection (53–55).

The predicted modulation (activation or repression) of a particular upstream regulatory protein in our analysis may be indicative of a change in biochemical activity due to either (i) post-translational modification, (ii) altered access to a cofactor, or (iii) a simple change in abundance. As further validation of our RNA-seq data, our analysis predicted the activation of several upstream regulatory proteins (colony stimulating factor 2 [CSF2], IFN-γ, IL-12, IL-17C, IL-17A, IL-1α, IL-1β, IL-2, IL-23A, chemokine [C-X-C motif] ligand 2 [CXCL2]) whose protein abundance is known to increase in vaginal lavage fluid in response to infection with C. albicans in either a rodent model or clinical VVC samples (17, 42, 43, 46–48, 56, 57). The analysis also predicted the regulation of several signaling pathways (e.g., microRNA 21 [mir-21] and nuclear receptor subfamily 1 group h [Nrho1h]) that have not previously been reported to play a role in the host response to C. albicans infection (Table S4).

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**FIG 1** Computational pipeline for analysis of RNA-seq data. Schematic illustration of the experimental and computational steps used to identify changes in gene expression that occur during the interaction between C. albicans and host cells during in vivo infection.

secretion of Th17 cytokines, including IL-22, IL17A, IL-17F, and IL-6, into the lavage fluid (17, 34, 41–44).

Validation of differentially expressed host genes. As a validation of our RNA-seq data, we decided to test the increased protein abundance of selected differentially expressed genes in vaginal tissue by performing a multiplex enzyme-linked immunosorbent assay (ELISA) for the following proteins: MIP-1b, IL-1β, IL-12p70, IL-23p19, S100A8, and IL-6. The abundance of each of the proteins tested was elevated in the C. albicans-infected tissue compared to the naive tissue, consistent with our RNA-seq results (Fig. 3A). Our results also indicated induction of the mRNA encoding prostaglandin-endoperoxide synthase 2 (PTGS2). We performed a competitive enzyme immunoassay (EIA) to measure PTGS2 protein activity in the lavage fluid of mice and observed
IL-1β-dependent mechanism of PMN recruitment to the vaginal mucosa during *C. albicans* infection. Four of these proteins (IFN-γ, NLRC4,Nr1h, and mir-21) are known to govern the expression of the NLRP3 inflammasome sensor, whose mRNA is induced 2.2-fold in our data set and known to have a role in IL-1β activation during various diseases (53, 58–60). Thus, we wished to determine the biological relevance of NLRP3 during VVC.

**The role of the NLRP3 inflammasome in the host response during VVC.** The inflammasome is a danger-sensing molecular complex that can respond to pathogenic insult at mucosal surfaces (61). Activation of the inflammasome in response to microbial infection promotes the caspase-dependent cleavage of IL-1β and IL-18 and the subsequent production of proinflammatory cytokines, including those of the Th17 lineage (62, 63). Furthermore, NLRP3 plays an important role in host defense against *C. albicans* during both oral (64) and hematogenously disseminated (65, 66) candidiasis. However, its role in the pathogenesis of VVC has not been previously described.

Three observations from our RNA-seq analysis are consistent with activation of the NLRP3 inflammasome. First, the expression

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**FIG 2** Functional enrichment for innate immune function in our data set. (A) Schematic analysis of select biological functions predicted to be activated in response to *C. albicans* during VVC. Genes colored in red have *C. albicans*-induced expression, and genes labeled in green have *C. albicans*-repressed expression. Gray boxes depict biological functions that are predicted by our analysis to be activated; arrows indicate evidence in the literature that a given gene promotes that biological function. Blunt-ended lines indicate evidence for inhibition. (B) A heatmap is included to demonstrate the relative expression levels of the individual genes included in the functional enrichment schematic. Data are presented as log (base 2)-transformed normalized RPKM. See Table S2 in the supplemental material for a complete list of gene names.
of NLRP3 mRNA is elevated in *C. albicans*-infected mice compared to naive mice. We confirmed the increased expression of NLRP3 with reverse transcription-quantitative PCR (qRT-PCR) analysis of *in vivo*-infected vaginal tissues from an independent experiment (Fig. 5A). We also observed an increase in the levels of NLRP3 protein in *C. albicans*-infected tissue compared to naive tissue (Fig. 5B). Second, our upstream regulator analysis predicted the activation of Nlrc4 (**P value overlap = 1.42E–05**), which is required for robust expression of NLRP3 in a mouse model of oral candidiasis (53). Third, our upstream analysis also predicted the activation of IL-1β, the major inflammasome effector (**P value overlap = 2.04E–06**). We had previously shown that IL-1β protein levels are elevated in vaginal lavage fluid of *C. albicans*-infected mice, but we aimed to determine whether IL-1β was cleaved to its mature form in vaginal tissue (19). Western blot analysis of vaginal tissue extracts using an antibody specific to the mature form of IL-1β confirmed its cleavage (Fig. 5B). Similarly, IL-1β transcript levels were also elevated in vaginal tissue of *C. albicans*-infected mice (Fig. 5A).

Immunohistological analysis of vaginal tissue from naive and infected mice was also performed using an Nlrp3-specific antibody. Both naive and infected mice demonstrated robust staining of the squamous epithelium, while only infected mice showed considerable Nlrp3 expression in the keratinized epithelium and lamina propria (Fig. 5C, yellow arrows). Vaginal-lavage cells, consisting mostly of keratinized epithelium and recruited immune cells, were also analyzed for Nlrp3 staining. *C. albicans*-infected mice demonstrated robust staining of epithelial cells, while naive mice showed no such expression (Fig. 5D). The strong constitutive staining pattern of Nlrp3 in the squamous epithelia of naive and infected mice is similar to that observed in the oral mucosa, esophagus, ectocervix, and vulva (67, 68). Immunostaining with an appropriate isotype control antibody did not exhibit staining in either lavage cells or whole vaginal tissue (data not shown).

In order to directly test the role of NLRP3 in the immunopathogenesis of VVC, we utilized Nlrp3<sup>−/−</sup> knockout mice in our murine model. Despite equivalent colonization rates (Fig. 6A), intravaginal inoculation of *C. albicans* in Nlrp3<sup>−/−</sup> mice led to significantly reduced levels of PMN recruitment (Fig. 6B) in the vaginal lavage fluid compared to those in WT congenic controls. Additionally, multiplex ELISA of the vaginal lavage fluid mice demonstrated a significant reduction in the *C. albicans*-induced expression of IL-12p70, IL-1β, IL-6, MIP-1b, and S100A8 in Nlrp3<sup>−/−</sup> mice compared to that in WT or naive mice (Fig. 6C to G). These results suggest that NLRP3 governs PMN recruitment during VVC, most likely through the induction of alarmins and proinflammatory cytokines, including IL-1β.

In order to address whether the NLRP3 inflammasome could be pharmacologically targeted, we tested the efficacy of glyburide, an FDA-approved K<sup>+</sup> efflux pump antagonist and NLRP3 inhibitor, for effects on PMN infiltration and IL-1β expression. Intravaginal administration of glyburide (2.5 mg/kg of body weight) significantly reduced PMN and IL-1β levels (Fig. 7) compared to those of mice treated with the vehicle (PBS–1% dimethyl sulfoxide [DMSO]). Importantly, *in vitro* studies (Fig. S2A) confirmed that glyburide at this dose did not inhibit *C. albicans* growth or affect morphogenesis; similarly, microscopic analysis of vaginal lavage fluids from both groups of mice confirmed the presence of hyphae (Fig. S2B).

Analysis of *C. albicans* gene expression during VVC. A comprehensive analysis of the *C. albicans* transcriptome during VVC was precluded by our inability to obtain sufficient reads that map to the *C. albicans* reference genome (Table S1). However, unlike microarray data, RNA-seq data (expressed as reads per kilobase per million [RPKM]) can be used to compare the levels of absolute expression between different genes in a sample. Therefore, we examined the list of the most highly expressed genes defined here, conservatively, as the 52 genes that had at least 100 mapped reads in two of the three infected samples. The RPKM value for each “highly expressed” gene in each of the samples is listed in Table S6. We posited that these genes might govern the host-pathogen interaction. One gene on this list, SAP5, encodes a secreted aspartyl proteinase (SAP) with no previously demonstrated role in the immunopathogenesis of VVC. SAP4, SAP5, and SAP6 are part of a subfamily of hypha-associated genes that share 90% codon identity with one another (69). Because of this high homology and functional redundancy, these genes are often deleted and studied together (70). We decided to test a *C. albicans* strain carrying deletions in SAP4, SAP5, and SAP6, as well as SAP5 only, in our murine model of VVC. As a control, we also tested a strain carrying a deletion in the yeast-associated genes SAP1, SAP2, and SAP3. Intravaginal challenge of mice with a triple SAP knockout (ΔSAP4–6) strain and a single SAP5 deletion strain led to significant reductions in PMN recruitment and IL-1β secretion compared to those in a WT strain with no noticeable defect in colonization (Fig. 8). Importantly, use of the SAP1–3 mutant elicited WT levels of these inflammatory mediators. Therefore, hypha-associated SAP5 appears to be an important immunopathogenic factor during the development of disease symptomatology.

Taken together, these data provide evidence that the NLRP3 inflammasome is involved in the immunopathology of VVC and...
that RNA-seq is a valid approach to understand host-pathogen interactions.

DISCUSSION

Decades of experimental and clinical research have demonstrated a protective role for the adaptive immune response against systemic, oral, and gastrointestinal candidiasis (71). Yet, despite mechanistic animal modeling and numerous clinical studies, an attributed role for the adaptive response during vaginitis remains elusive. Instead, recent findings suggest that VVC results from an immunopathology mediated by host innate immunity, including robust recruitment of PMNs and expression of proinflammatory cytokines/chemokines at the vaginal mucosa (72). However, the precise molecular mechanisms governing the symptomatic response remain poorly understood. Importantly, findings from this study have now, for the first time, uncovered a major host innate immunological pathway contributing to the immunopathogenesis of VVC, involving a crucial role for the NLRP3 inflammasome.

Due to the anatomical specificity of the disparate immune responses observed during candidiasis, more-classical targeted methods to understand host responses during VVC would likely be inapplicable. Therefore, we utilized the unbiased systems biology approach of RNA-seq to gain mechanistic insight into the immunopathogenesis of VVC. The initial observation from our RNA-seq and subsequent network analysis is that the murine vaginal infection with C. albicans is presented. Key genes with known functions in host immunity are listed. Orange circles depict upstream regulators that are predicted to activate gene expression, while blue circles depict those that inhibit. Four of the upstream regulators converge upon NLRP3 (solid and dashed black lines). Smaller red and green circles depict individual differentially expressed genes that are up- or downregulated in our data set, respectively. The common signaling pathways largely converge on IL-1β (solid and dashed gray lines), which regulates its own expression (curved black arrow) to drive inflammatory gene expression. See Table S4 in the supplemental material for more information.
inal host response to *C. albicans* is a complex, multifactorial, and functionally redundant set of host proinflammatory mechanisms, consisting of a multitude of cytokines and eicosanoids (see Tables S2 to S4 in the supplemental material). Recognition of pathogens at the mucosal surface is required for initial immune surveillance, and our data set suggests that this process is mediated via a MYD88-dependent mechanism involving multiple signaling cascades through TLR3, -4, -5, and -9. Our previous work had identified a role for surface-expressed TLR4 in the S100 alarmin response in vaginal epithelial cells during *C. albicans* challenge (21). Interestingly, the canonical ligands for TLR3 (double-stranded RNA), TLR5 (flagellin), and TLR9 (unmethylated CpG DNA)
suggest that these TLRs do not play a role in the detection of fungi (73). However, several reports in the literature demonstrate that both fungal RNA and chitin can signal via TLR9 (74, 75). Moreover, genetic polymorphisms in TLR3 can predispose a host to candidiasis (76).

Despite increased gene expression of several c-type lectin receptors (CLRs) during C. albicans challenge, including CLEC4E, CLEC4D, CLEC7A, and CLEC1A, that recognize fungal carbohydrates (e.g., β-glucan), signaling via their common intracellular adaptor protein CARD9 was not predicted to be activated (77). In light of our previous observations that Dectin-1/CLEC7A lacked noticeable immunodetection in murine vaginal epithelial cells, CLR expression in the vagina may suffer from localization issues in the epithelium or be more restricted to the infiltrating immune cells (21). Also surprisingly absent from our data set were genes involved in the mitogen-activated protein kinase (MAPK) pathway, including the terminal signaling proteins c-Fos and c-Jun. These proteins were previously demonstrated to be induced in human oral and vaginal epithelial cells in vitro in response to hyphal forms of C. albicans (29). This discrepancy may be explained by species-specific differences, phosphorylation events as opposed to changes in gene expression, or timing of expression. However, it is important to note that some MAPK pathway proteins (e.g., MAP3K8, activation z score = 1.798) were predicted to be activated but fell below the cutoff threshold.

The main host physiological functions induced by C. albicans included accumulation of myeloid cells, infiltration of phagocytes, and generation of hydrogen peroxide (Fig. 2). These functions are consistent with robust recruitment of neutrophils to the vaginal epithelium during C. albicans challenge (19, 78). However, one surprising result was the strong expression of the Th17 axis of adaptive immunity. Indeed, some of the most highly differentially
strain SC5314 (black symbols). (B and C) Deletion of SAP4–6 or SAP5 – 6 or SAP1–3 does not affect fungal burden during vaginitis. Despite similar levels of colonization at day 3 postinoculation, the SAP4–6 and SAP5 mutants result in less PMN (B) and IL-1β (C) recovered in the vaginal lavage fluid than in that of mice infected with WT C. albicans (light-gray symbols), or IL-1β (white symbols) does not affect fungal burden, infiltration of neutrophils, or S100 expression (17). These findings, coupled with our RNA-seq results, beg the questions of why these cytokines are so highly expressed if they are nonessential and which cell types produce them. Perhaps influences of estrogen or other immunomodulatory regulators at the vaginal mucosa mask potential contributions of Th17-related cytokines or curtail their effectiveness, as observed previously (32, 79, 80).

A major finding of this study identifies the involvement of the NLRP3 inflammasome in governing the immunopathology associated with C. albicans vaginitis. In the context of our and other previously published results, a role for an inflammasome-mediated mechanism of inflammation is strongly supported. For example, Tomalka and colleagues demonstrated that both NLRP3 and NLRC4 play important protective roles in the murine oral epithelium during challenge with C. albicans (53). It was determined that while NLRP3 functions in both epithelial and recruited hematopoietic cells, NLRC4 plays a more prominent role in the stromal compartment to ultimately elicit PMN migration. As with these findings, immunostaining of vaginal tissue demonstrated robust expression of Nlrp3 in the squamous epithelium and increased expression in the keratinized layer that supports fungal growth during C. albicans infection (Fig. 3). NLRP3 inflammasome activation is mediated via a two-step process involving identification of microbes via pattern recognition receptors (PRRs) and surveillance of damage factors (e.g., ATP, K+ efflux, reactive oxygen); basal expression levels are insufficient to generate a response (81, 82). As stated above, C. albicans is likely sensed by epithelial cells via PRR-dependent mechanisms and damage induced via hyphal invasion of vaginal epithelial cells or hypha-associated effectors (19, 83). Interestingly, one of the most highly differentially expressed genes in our data set is the acute-phase protein serum amyloid A3 (SAA3), which is known to be a potent inflammasome activator (84–86). However, it is equally likely that any number of inflammasome-activating endogenous host factors could be released upon hypha-mediated epithelial damage.

Indeed, we have previously shown that hyphal forms of C. albicans are associated with cellular damage and secretion of the major inflammasome effectors IL-1β during VVC (19). Genetic disruption of NLRP3 severely limits PMN infiltration and inflammatory cytokine expression during murine VVC in a fungal-burden-independent manner (Fig. 4). These results now directly link NLRP3 signaling with IL-1β secretion to C. albicans at the vaginal epithelium in vivo. It is important to point out that while genetic disruption of NLRP3 signaling severely reduces the immunopathological response, residual activities of IL-1β and S100 alarmin and PMN recruitment remain (Fig. 4). Notably, ablation of inflammasome signaling predisposes mice to oropharyngeal candidiasis, while genetic blockade of Nlrp3 function during VVC does not seem to enhance fungal burden but actually reduces hallmark immunopathological markers of disease (53). These findings further underscore that VVC results from a nonprotective immune response that contributes to disease symptomatology.

The mouse model of VVC is highly dependent on the administration of the steroid hormone estrogen prior to fungal inoculation. Estrogen has a multitude of systemic and local effects, including immunomodulation, epithelial thickening, and keratinization (72). Similar to this observation is the finding that women are also most clinically susceptible to vaginitis under high...
estrogenic activity (e.g., during the luteal phase of the menstrual cycle and with use of high-estrogen contraceptives) (72). Cholesterol, a sterol precursor to estrogen, is a known activator of Nlrp3 (87). Thus, it is enticing to speculate that estrogen may prime Nlrp3 expression within vaginal epithelial cells. That said, using qPCR, we have not observed increased expression of Nlrp3 in whole vaginal tissue obtained from estrogen- and non-estrogen-treated naive mice (data not shown). However, lack of increased expression does not necessarily correlate with lack of activation. Further detailed experiments using non-estrogen-treated mice will be required to fully elucidate the possible role that estrogen plays in contributing to inflammasome activation during vaginitis.

The essential role of inflammasome signaling in contributing to the immunopathogenesis of VVC has provided a novel target and complementary approach to potentially reduce early disease symptomatology. In support of this, mice intravaginally inoculated with gubride demonstrated significantly fewer PMNs and IL-1β in their vaginal lavage fluid than vehicle-treated controls (Fig. 5). Although its mechanism of action is incompletely defined, gubride acts as a K⁺ efflux inhibitor somewhat downstream of the P2X7 receptor (a known activator of inflammasome signaling, just missing the cutoff threshold as activated in our upstream regulator analysis, with an activation z score equal to 1.963) but upstream of Nlrp3. The efficacies of inflammasome inhibitors as possible novel immunotherapeutics against VVC provide exciting new opportunities for disease management. That said, important considerations remain, including potential off-target effects (e.g., hypoglycemia), cell type specificity, and timing of administration. It is also important to note that treatment with inflammasome inhibitors would likely only reduce symptomatology and that more traditional antifungal therapies would be required in combination to eliminate fungal burden. However, immunomodulatory therapies such as inflammasome inhibition may offer relief, especially to the recurrent-VVC population.

A cursory profiling of C. albicans gene expression during vaginal infection identified SAP5 among the most highly expressed fungal genes. These findings are consistent with those of Taylor et al., who demonstrated SAP5 expression during a mouse model of vaginal infection (88). Our RNA-seq results complement this by demonstrating that SAP5 is one of the most highly expressed genes in the genome during in vivo vaginal infection. Indeed, inoculation of mice intravaginally with C. albicans strains lacking SAP5 and its close homologs SAP4 and SAP6 but not the yeast-associated SAPs (SAP1–3) led to decreases in immunopathological markers (Fig. 8). The same findings were recapitulated using a single SAP5 knockout, suggesting that immunopathogenic activity was mostly attributed to SAP5 expression. SAP5 was previously found to be important for organ invasion in vivo and controlled by the transcriptional regulators Efg1 and, to a lesser extent, Cph1 (89). These observations are consistent with our previous finding that IL-1β secretion during murine VVC is largely Efg1 dependent (19). SAP4 to -6 have optimal activities at a more neutral pH, unlike SAP1 to -3, which are active at a more acidic pH (90). Thus, the relatively neutral pH of the murine vagina may likely explain the preferential expression of SAP4 to -6. Work by Naglik et al. found that SAP5 expression was most highly induced during ex vivo infection and consistently expressed in human oral and vaginal candidiasis samples (91, 92). Furthermore, in the context of inflammasome activation, SAPs (including SAP6) have been shown to directly activate NLRP3, leading to subsequent IL-1β induction (93). However, because SAPs can also induce cell damage, indirect activation of the inflammasome via release of host danger signals (e.g., ion efflux, S100s, ATP) remains an alternative mechanism. Although the SAPs are important for immunopathology, rigorous testing of mutants for other identified known and putative virulence factors will be essential to fully understand the complex interplay between host and pathogen at the vaginal interface.

In conclusion, we have conducted an analysis of the host and fungal transcriptional response during murine vulvovaginal candidiasis. Findings from this study have demonstrated that the physiological functions of innate immunity, leukocyte recruitment, peroxide biogenesis, and Th17 function dominate the vaginal transcriptional response to C. albicans. Furthermore, we have identified the NLRP3 inflammasome as a key signaling pathway mediating the immunopathological response to C. albicans at the vaginal mucosa and have also begun to unravel the strategies used by C. albicans to initiate vaginal inflammation. Now that multiple novel signaling pathways, inflammatory cascades, transcriptional activators, and virulence factors have been uncovered by this analysis, more-traditional detailed immunological and microbiological experiments may be performed to verify their contributions to VVC pathogenesis. Indeed, aside from defining a new and pharmacologically exploitable mechanism of vaginal inflammation, this study features the ability of comprehensive, unbiased genomic approaches to confirm previous research and accurately predict new host-pathogen response pathways that will ultimately contribute to a fuller understanding of vaginal disease.

**MATERIALS AND METHODS**

**Ethics statement.** The animals used in this study were housed in AAALAC-approved facilities located at the LSU Health Sciences Center (LSUHSC) in the School of Dentistry. The LSUHSC Animal Care and Use Committee approved all animals and protocols. Mice were given standard rodent chow and water ad libitum. Mice were monitored daily for signs of distress, including noticeable weight loss and lethargy. All animal experiments were approved by the LSUHSC Division of Animal Care (DAC) under IACUC protocol 3078 (principal investigator, P. L. Fidel). The LSUHSC DAC uses the Public Health Policy on the Humane Care and Use of Laboratory Animals (94) and the Guide for the Care and Use of Laboratory Animals (95) as a basis for establishing and maintaining an institutional program for activities involving animals. To ensure high standards for animal welfare, the LSUHSC DAC remains compliant with all applicable provisions of the Animal Welfare Act (96), guidance from the Office of Laboratory Animal Welfare (OLAW), and the AVMA Guidelines for the Euthanasia of Animals (97).

**Fungal strains and growth.** C. albicans isolate SC5314 and the ΔSAP1–3 (SAP123MS4C), ΔSAP4–6 (SAP456MS4A), and ΔSAP5 (M26) mutants and were maintained as glycerol stocks stored at −80°C (98–100). A small amount of stock was spread onto yeast extract-peptone-dextrose (YPD) agar and incubated at 30°C for 48 h to generate isolated colonies. A single colony was transferred into 10 ml of liquid YPD and incubated at 30°C with shaking at 200 rpm for 18 h prior to intravaginal inoculation.

**Murine model of vulvovaginal candidiasis.** The murine model of vulvovaginal candidiasis has been reported extensively in the literature and was described previously (39). C57BL/6 or Nlrp3<sup>−/−</sup> mice were purchased from Jackson Laboratories and housed in isolator cages mounted onto ventilated racks. Mice were administered 0.1 mg of estrogen (β-estriodol 17-valerate; Sigma) dissolved in 0.1 ml sesame oil subcutaneously 72 h prior to inoculation with C. albicans. Stationary-phase cultures of C. albicans isolates were washed three times in sterile, endotoxin-free...
PBS and resuspended in a 0.2× volume of PBS. Cell suspensions were diluted, counted on a Neubauer hemocytometer, and adjusted to 2.5 × 10⁶ CFU/ml in sterile PBS. Estrogen-treated mice were briefly anesthetized by isoflurane inhalation and then intravaginally inoculated with 20 µl of the standardized blastocandidal cell suspension, generating an inoculum size of 5 × 10⁸ blastocandida. Naïve controls were inoculated with 20 µl of sterile PBS. All animal experiments were conducted in duplicate with 4 to 5 mice per group.

Vaginal lavage. Groups of mice (n = 4 or 5) underwent vaginal lavage immediately after sacrifice with 0.1 ml of sterile PBS containing protease inhibitors (cOmplete, EDTA free; Roche) at day 3 p.i. Recovered lavage fluids were kept on ice during processing. Aliquots were removed to determine fungal burden and for PMN enumeration. The remaining lavage fluid was centrifuged at 3,600 rpm for 3 min at 4°C to remove cellular debris and stored at −80°C until needed.

PMN quantification. Lavage fluid (10 µl) was smeared onto Tissue Path SuperFrost Plus gold slides (Fisher Scientific), allowed to air dry, fixed with CytoPrep spray fixative (Fisher Scientific), and stored at room temperature. Slides were then stained using the Papanicolaou technique (Pap smear). PMNs were identified by their morphology, staining appearance, and characteristic trilobed nuclei. For each smear, PMNs were manually counted in five nonadjacent fields by standard light microscopy using a 40× objective. PMN counts were averaged per field. Values are reported as mean PMN counts per group ± standard error of the means (SEM).

Fungal burden. Lavage fluid was serially diluted 10-fold in sterile PBS and plated onto YPD agar containing 50 µg/ml chloramphenicol to inhibit bacterial overgrowth, using the drop plate method as described previously (101). Plates were allowed to dry and then incubated for 24 h at 37°C, and the resulting colonies were enumerated. Numbers of CFU per milliliter per group are reported as medians.

Enzyme-linked immunosorbent assays for cytokine analysis. Cytokines were measured in both vaginal tissue and lavage samples. To assess cytokine levels in tissue, whole vaginas were isolated 3 days p.i., kept on ice, and immediately minced and mechanically homogenized in tissue extraction reagent I (Invitrogen) supplemented with protease inhibitors (cOmplete, EDTA-free; Roche). Tissue extracts were centrifuged at 4°C for 5 min at 10,000 rpm, the supernatant was collected, and the protein content was measured by the bicinchoninic acid (BCA) assay (Pierce Biotechnology). Tissue extracts were adjusted to 0.8 mg/ml in extraction reagent, and 50 µl was analyzed. Vaginal lavage fluid was also similarly prepared, except that neat lavage fluid was diluted 6-fold in PBS and 50 µl analyzed. All samples were run on the Bio-Plex 200 platform (Bio-Rad) using a custom multiplex cytokine assay (Luminex) according to the manufacturer’s instructions for IL-12p70, IL-23p19, IL-1β, IL-6, MIP-1b, and S100A8. Data were analyzed and concentrations calculated using a 5-parameter logistic curve and best-fit models within the Bio-Plex Manager and MasterPlex software packages. For some experiments (glyburide and SAP mutant studies), a single-plex IL-1β ELISA (R&D Systems) was used according to the manufacturer’s instructions to solely measure this cytokine; lavage fluids were prepared similarly as described above and assayed in a total volume of 100 µl.

Measurement of prostaglandin E₂. Prepared vaginal lavage fluid was analyzed for prostaglandin E₂ production with the prostaglandin E₂ competitive ELISA kit according to the manufacturer’s instructions (Cayman Chemical).

Isolation of RNA from vaginal tissue. At day 3 p.i., vaginal tissue was surgically excised, immediately placed into RNAlater, and incubated at 4°C overnight. The following day, tissues were transferred to TRI Reagent (Sigma), finely minced with scissors, mechanically homogenized (PRO Scientific), and centrifuged at 12,000 × g for 10 min at 4°C. RNA was isolated by chloroform-ethanol precipitation and the pellet resuspended in nuclease-free water according to TRI Reagent instructions. RNA concentration was measured by spectrophotometry at A₂₆₀/A₂₈₀ and its integrity verified by Bioanalyzer (Agilent) analysis.

RNA-seq and gene expression analysis. RNA-seq libraries (non-strand-specific, paired end) were prepared with the TruSeq RNA sample prep kit (Illumina). The total RNA samples were subjected to poly(A) enrichment as part of the TruSeq protocol. One hundred nucleotides of sequence was determined from both ends of each cDNA fragment using the HiSeq platform (Illumina) per the manufacturer’s protocol. Sequencing reads were annotated and aligned to the UCSC mouse reference genome (mm10, GRCh38.75) using TopHat2 (102). Sequencing reads that did not map to the mouse reference genome or whose partner read did not map to the mouse reference genome were subsequently aligned to the C. albicans reference genome (SC5314). For the mouse data, the alignment files from TopHat2 were used to generate read counts for each gene, and a statistical analysis of differential gene expression was performed using the DESeq package from Bioconductor (103). A gene was considered differentially expressed if the P value for differential expression was less than 0.05. For the C. albicans data, RPKM values for each gene in each sample were calculated using in-house scripts.

Identification of signal transduction pathways. We used the upstream regulator analytic of IPA (Ingenuity Systems) to identify signaling proteins that are potentially activated or repressed during the course of infection. This software assesses the overlap between experimentally derived gene lists and an extensively curated database of target genes for each of several hundred known regulatory proteins. It then uses the statistical significance of the overlap and the direction of the differential gene expression to make predictions about activation or repression of these regulatory proteins. This software assesses the overlap between experimentally derived gene lists and an extensively curated database of relationships between genes and biological functions and activities. It then uses the statistical significance of the overlap and the direction of the differential gene expression to make predictions about whether particular biological activities are activated or repressed, in this case during vaginal infection with C. albicans.

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 according to the manufacturer’s instructions (Thermo Scientific). RNA was reverse transcribed using random hexamers and the RevertAid kit according to the manufacturer’s protocol (Thermo Scientific). 6-Carbboxylfluorescein (FAM)-labeled PrimerTime primer/probe sets spanning exon-exon junctions were ordered from IDT for murine IL-1β and nlrp3, while a proprietary VIC-labeled mouse-specific gapdh primer/probe set (Applied Biosystems catalog number 4352399e) was utilized (see Table S5 in the supplemental material for sequences). All primers were used at the recommended concentrations along with SsoFast probe supermix (Bio-Rad) to amplify 20 ng of cDNA. qPCRs were monitored and analyzed with the Bio-Rad iCycler and associated software. Expression levels of target genes in infected mice were compared to those of a reference gene (gapdh) and those in naive controls using the ΔΔCT method (where ΔCT is threshold cycle) as described previously (104).

Western blotting. Vaginal tissue was isolated and protein extracts were prepared as described above. Tissue extracts (40 µg) were subjected to SDS-PAGE and transferred to a nitrocellulose membrane, and the membrane was divided prior to Western blotting. Membranes were blocked in 5% milk–phosphate-buffered saline–Tween 20 (PBST) and incubated overnight at 4°C with the following primary antibodies diluted in 5% milk–PBST according to the manufacturer’s recommendations: monoclonal IgG2A rat anti-mouse Nlrp3 (2 µg/ml; R&D Systems catalog number MBAB7578), polyclonal IgG goat anti-mouse cleaved IL-1β (1:200 dilution; Santa Cruz catalog number sc-23460), and polyclonal IgG goat anti-mouse actin (1:200 dilution; Santa Cruz catalog number sc-1616). The following day, blots were extensively washed in PBST and incubated for 1 h at room temperature with the following horseradish peroxidase (HRP)-coupled secondary antibodies: polyclonal IgG goat anti-rat (1: 1,000; R&D Systems catalog number HAP005) for Nlrp3 blots or polyclonal IgG rabbit anti-goat (1:5,000; Santa Cruz catalog number sc-2768)
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for IL-1β and actin blots. Signals were captured using the Clarity Western enhanced-chemiluminescence (ECL) substrate kit (Bio-Rad) and a ChemiDoc XRS charge-coupled-device (CCD) imager (Bio-Rad). Raw data files were imported and bands quantified using ImageJ software (NIH).

Immunohistochemical staining. Whole vaginas were formalin fixed, paraffin embedded, sliced, and mounted onto slides (LSUHSC Morphology Imaging Core). Additionally, lavage smears containing vaginal epithelial cells were prepared as described above. Both whole tissues and smears were immunostained with a monoclonal IgG2A rat anti-mouse Nlrp3 antibody (R&D Systems) or rat IgG2A isotype control (Bio-X-Cell) at the recommended concentration of 20 μg/ml. All staining steps were performed according to anti-rat cell and tissue staining kits (R&D Systems) utilizing HRP-coupled secondary antibodies. Whole tissues were stained with 3,3’-diaminobenzidine (DAB) to generate a permanent brown stain and counterstained with hematoxylin. Vaginal lavage cells were stained using 3-amino-9-ethylcarbazole (AEC) to produce bright-red staining. All images were captured using standard light microscopy with 4× to 40× objectives.

Pharmacological inhibition of Nlrp3 in vivo. Mice were briefly anesthetized by isoflurane inhalation and intravaginally administered 20 μl vehicle alone (PBS–1% DMSO) or glyburide (InvivoGen) at a dose of 2.5 mg/kg in the vehicle starting 1 day prior to inoculation with C. albicans. The vehicle or glyburide was then administered daily (at least 12 h after the initial colonization) until sacrifice.

Effects of glyburide on C. albicans growth and morphogenesis. Standardized C. albicans cultures were inoculated into YPD medium (at 30°C) or RPMI medium (at 37°C) containing 2.5 mg/ml glyburide–1% DMSO or 1% DMSO alone and observed for effects on growth (YPD, microscopy, and growth at an optical density at 600 nm [OD600]) or the ability to undergo the yeast-to-hypha switch (RPMI medium, microscopy) for up to 16 h.

Statistical analysis. In vivo experiments were performed in biological duplicate using 4 to 5 mice per group as indicated above. Images of Western blots, qPCR gels, and immunohistological stains are representative of at least 3 independent repeats. For CFU, PMN, and cytokine data, statistical comparisons between groups were performed using a Mann-Whitney test; if multiple comparisons were made, then a one-way analysis of variance (ANOVA) and Kruskal-Wallis posttest were used. All statistics for microbiological and immunological assays were analyzed using GraphPad Prism 5.0 software. Transcriptomic data were uploaded to the Ingenuity Pathway Analysis software and analyzed for statistical significance using the IPA software package.

Accession number. All of the processed gene expression data from this study have been submitted to the NCBI Gene Expression Omnibus (GEO) under accession number GSE67688.

SUPPLEMENTAL MATERIAL


Figure S1, TIFF file, 0.1 MB.
Figure S2, TIFF file, 1.4 MB.
Table S1, XLSX file, 0.01 MB.
Table S2, XLS file, 0.02 MB.
Table S3, XLS file, 0.02 MB.
Table S4, XLSX file, 0.1 MB.
Table S5, DOCX file, 0.05 MB.
Table S6, XLSX file, 0.2 MB.

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REFERENCES

18. Fidel PL, Jr, Barousse M, Espinosa T, Ficarra M, Sturtevant J, Martin


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