Published in final edited form as:

Am J Obstet Gynecol. 2015 July ; 213(1): 38.e1–38.e12. doi:10.1016/j.ajog.2015.02.002.

Identification of novel mechanisms involved in generating localized vulvodynia pain

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Abstract

Objective—Our goal was to gain a better understanding of the inflammatory pathways affected during localized vulvodynia, a poorly understood, common, and debilitating condition characterized by chronic pain of the vulvar vestibule.

Methods—In a control matched study, primary human fibroblast strains were generated from biopsies collected from localized provoked vulvodynia (LPV) cases and age and race-matched controls. We then examined intracellular mechanisms by which these fibroblasts recognize pathogenic *Candida albicans*; >70% of vulvodynia patients report the occurrence of prior chronic *Candida* infections, which is accompanied by localized inflammation and elevated production of pro-inflammatory/pain-associated interleukin 6 (IL-6) and prostaglandin E2 (PGE₂). We focused on examining the signaling pathways involved in recognition of yeast components that are present and abundant during chronic infection.

Results—Dectin-1, a surface receptor that binds *C. albicans* cell wall glucan, was significantly elevated in vestibular versus external vulvar cells (from areas without pain) in both cases and controls, while its abundance was highest in LPV cases. Blocking Dectin-1 signaling significantly reduced pain-associated IL-6 and PGE₂ production during the response to *C. albicans*. Furthermore, LPV patient vestibular cells produced inflammatory mediators in response to low numbers of *C. albicans* cells, while external vulvar fibroblasts were nonresponsive. Inhibition of NFκB (pro-inflammatory transcription factor) nearly abrogated IL-6 and PGE₂ production induced by *C. albicans*, in keeping with observations that Dectin-1 signals through the NFκB pathway.

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No conflict of interest.

Conclusion—These findings implicate that a fibroblast-mediated pro-inflammatory response to *C. albicans* contributes to the induction of pain in LPV cases. Targeting this response may be an ideal strategy for the development of new vulvodynia therapies.

Keywords

fibroblast; vulvodynia; dectin-1; cytokine; inflammation; interleukin 6; prostaglandin

INTRODUCTION

Vulvodynia is a poorly understood and largely understudied pain condition that is common, chronic, and debilitating¹⁻⁶; as high as an estimated 28% of women in the US are afflicted, many of which are of child-bearing age⁷. Vulvodynia is diagnostically categorized into two categories: “generalized” or “localized”⁸. These categories are further differentiated by whether vulvodynia pain can be provoked, unprovoked, or both⁸. Women afflicted with localized provoked vulvodynia (LPV), the most common diagnostic category, experience acute and lasting pain in response to (light) touching of specific areas of the vulva^{1-7, 9}. In contrast, regions of the external vulva (labia minora, labia majora, mons pubis, and perineum) are relatively pain-free to touch^{8, 10, 11}. Pain associated with LPV frequently results in an almost complete disruption of sexual activity, discomfort with tampon insertion, dysuria, and depression, while the current medical therapies do little to permanently alleviate the symptoms of disease^{1-7, 9}. Ultimately, treatments cannot resolve or address the underlying cause(s) of disease, because they are still largely unknown, and only recently has this disease been linked to any preceding immunomodulatory stimulus¹¹⁻¹³. A current report demonstrated that more than 70% of LPV patients report the occurrence of prior, often chronic (> 4/year) vaginal yeast infections⁹. However, limited empirical evidence exists to show a causal link between vulvovaginal yeast infection and the onset of LPV¹³. Very recently, it was demonstrated that repeated yeast infection in mice can lead to an increase in pro-inflammatory mediator production in the vulvar vestibule associated with heightened pain sensitivity, which suggests that prior/chronic infections in human beings are related to the occurrence of LPV¹³. However, we acknowledge that other previously unidentified mechanisms may also exist, as not all patients with LPV have histories of chronic yeast infection⁹.

LPV *pain to light touch*, also known as *allodynia*, is indistinguishable from well-documented experimental and clinical examples of neural pain fiber (nociceptor) sensitization. Allodynia can be induced by intradermal or subcutaneous pro-inflammatory factors such as IL-6 and PGE₂¹⁴⁻¹⁶. IL-6 and PGE₂ are elevated in chronic pain conditions^{17, 18}. IL-6 and PGE₂ induction elicits allodynia^{19, 20}, IL-6 and PGE₂ suppression reduces allodynia^{21, 22}, and in preclinical animal models of pain, IL-6 and PGE₂ factor/receptor knockout mice display reduced allodynia²³. In keeping with these observations, human fibroblasts isolated from LPV patients at sites of allodynia-type pain respond to yeast and yeast-derived products through the heightened production of IL-6 and PGE₂ associated with allodynia-type pain^{10, 11}. Following a live yeast or yeast product challenge, fibroblasts from the vulvar vestibule of LPV patients produce significantly more interleukin 6 (IL-6) and prostaglandin E₂ (PGE₂) than fibroblasts derived from the external

vulva of the same patient and more than fibroblasts derived from the vulvar vestibule and external vulva of pain-free controls. Furthermore, fibroblast-produced IL-6 and PGE₂ were shown to precisely predict mechanical pain thresholds in cases and controls^{10,11}. However, little is known about how human external vulvar and vestibular fibroblasts respond to yeast or yeast products. Even less is known about whether or how the mechanism differs in pain-associated vestibular cells versus external vulvar cells not directly associated with pain.

Vulvar fibroblast strains produce IL-6 and PGE₂ in response to challenge with yeast components, such as zymosan^{10, 11}, a commercially available mixture of yeast cell wall mannoproteins and β -glucan commonly used as an immune stimulus to model responses to yeast infection^{13, 24}. Zymosan is purified from *Saccharomyces cerevisiae*²⁴, which can be found at sites of infection, although it is significantly less pathogenic²⁵⁻²⁷. However, the structure of the *S. cerevisiae* cell wall is highly similar to that of prevalent vaginal yeast pathogens, such as *Candida albicans* and *Candida glabrata*^{26, 28}. With our in-vitro fibroblast model, we have shown that zymosan or live virulent yeast challenges produce comparable pro-inflammatory responses¹⁰. These findings suggest that the recognition of yeast mannoprotein and β -glucan moieties may be critical to the vulvovaginal response to yeast, of which chronic/repeated infection has been associated with the occurrence of LPV.

Mannoprotein is abundant in the outermost layer of the *Candida* cell wall and is a known pathogen-associated molecular pattern (PAMP) that is recognized by cognate pattern recognition receptors (PRRs) of the human immune system²⁹⁻³³. Although β -glucan can be found interior to mannoprotein, it is exposed at bud scars during cell division²⁹⁻³² and is actively secreted into the extracellular milieu³⁴⁻³⁷. In turn, these proteins (also found in zymosan) are presumably abundant during vulvovaginal yeast infection and may serve as key stimuli that contribute to the heightened immune response observed in LPV patients. During infection, *Candida albicans* debrides the epithelial layer through protease secretion and tissue invasion, exposing the underlying tissue (e.g. fibroblasts) to yeast and yeast products^{25, 38-41}. Repeated exposure to yeast or yeast products may result in sensitization, so that even normal (culture-negative) levels of yeast could signal a pro-inflammatory response¹³.

We set out to investigate the underlying mechanisms that may influence pain sensitivity in the vulvar vestibule by evaluating the response to heavy, moderate, and low infectious doses of *C. albicans*, while focusing on the signaling pathways that influence the immune response to yeast PAMPs (β -glucan and mannoprotein). We elected to concentrate on the Dectin-1 receptor, because it is regarded as the paradigm receptor for β -glucan detection, and it triggers a signaling cascade that can activate NF κ B (a protein complex that can regulate pro-inflammatory gene expression), which leads to production of IL-6⁴²⁻⁴⁷. Dectin-1 is expressed on macrophages, monocytes, B-cells, and dendritic cells^{42-44, 46-48}. In addition, it is expressed on gingival fibroblasts and may serve some role in the response to oral candidiasis⁴⁹. In light of these observations, we evaluated Dectin-1 expression on human external vulvar (from the perineum) and vestibular fibroblasts and its role in the production of pro-inflammatory mediators in response to challenge with zymosan or live yeast. We also explored the role of NF κ B signaling in pro-inflammatory mediator production. This work

represents an important step in identifying an underlying intracellular mechanism for LPV; understanding the mechanisms that govern LPV may lead to therapeutic advances.

MATERIALS AND METHODS

Patient/Sample Selection

LPV-afflicted cases (fulfilling Friedrich's Criteria ⁵⁰) and age/race-matched pain-free controls were recruited from the Division of General Obstetrics and Gynecology clinical practice at the University of Rochester between December 2012 and February 2014. All subjects provided informed consent, and the research was approved by the University of Rochester Institutional Review Board (RSRB # 42136). Expanded details on our selection criteria and sampling procedures have been previously published ^{10, 11}. In brief, cases and controls were age- and race-matched with a mean age of 33.5 years. All case and control subjects were Caucasian, non-Hispanic. Furthermore, all subjects denied the use of corticosteroids and non-steroidal anti-inflammatory medications and had no chronic inflammatory illnesses other than LPV. Pain levels (at the vaginal vestibule) using the cotton swab test (CST) ¹⁰ ranged from 7-9 out of a maximal score of 10 for LPV cases and were zero for all pain-free controls. All study subjects had negative yeast cultures at the time of study entry. Tissue was sampled from sites as diagrammed previously ^{10, 11}. A total of 4 paired (vulvar vestibule and external vulva) case and 4 paired control strains (16 total) were used in this study.

Fibroblast strains

Primary fibroblast strains were established from fresh biopsy tissues, which were minced and immobilized on culture dishes and then cultured in RPMI 1640 medium supplemented with 10 mM HEPES, 50 µg/ml, gentamicin, 1 mM sodium pyruvate, 2 mM L-glutamine, antibiotic/antimycotic solution (Gibco/Invitrogen, part of Thermo Fisher Scientific, Waltham, MA), 10% fetal bovine serum (FBS), and 50 µM DMSO (Thermo Fisher) until fibroblasts proliferated onto the culture surface, which was followed by subsequent passages in Minimum Essential Medium (MEM) with 10% FBS, GlutaMAX, gentamicin, and antibiotic/antimycotic solution as previously described ⁵¹. Early passage (4-10) external vulvar and vestibular fibroblast strains were seeded at 5×10^4 cells/well. After achievement of confluence, fibroblasts were serum-reduced for 48 h in fresh media containing 0.05% FBS. Fibroblast cellular identity was confirmed by microscopic inspection and with fibroblast-specific markers (e.g. vimentin, collagen). At the same time, the cells were confirmed to be negative for epithelial cell markers (e.g. cytokeratin), smooth muscle and myofibroblast markers (e.g. α -smooth muscle actin), endothelial cell markers (e.g. CD34), and bone marrow derived cell markers (e.g. CD45) ⁵².

Dose response to live infection with *Candida albicans*

Cultures of fibroblast strains were seeded to 24-well tissue culture plates at roughly half confluence and were allowed to grow until confluent (~3-4 days) at 37°C and 5% CO₂ in Minimal Essential Media (MEM) supplemented with 10% fetal bovine serum (FBS), GlutaMAX, gentamicin, and antibiotic/antimycotic solution (Thermo Fisher). Once confluent, cells were transitioned to serum-reduced media (supplemented with 0.05% FBS)

and incubated for 48 h. The evening prior to infection, *Candida albicans* SC5314 (a virulent wild type strain) yeast cells were inoculated into a 10 ml culture of yeast peptone dextrose broth (YPD; Thermo Fisher Scientific) from a YPD plate culture less than two weeks old. Yeast cultures were incubated overnight at 37°C and 220 rpm. After ~18 h growth, the culture was diluted to OD₆₀₀ = 1.0 in fresh YPD broth. Inoculums were prepared by diluting these yeast cultures to $\sim 1 \times 10^4$ CFU/ml and then serially diluting (10-fold dilutions) to 1×10^1 CFU/ml in antibiotic/antimycotic-free MEM supplemented with 0.05% FBS and GlutaMAX. Confluent fibroblast wells were then infected with 1 ml of each inoculum (1×10^4 , 1×10^3 , 1×10^2 , and 1×10^1 blastoconidia) and incubated for 24 h at 37°C and 5% CO₂. At the same time, additional wells were treated with 100 µg/ml zymosan (Sigma-Aldrich, St. Louis, MO), which was diluted in MEM from a 250X stock dissolved in 100% EtOH. A corresponding vehicle control was also prepared. Standard sandwich ELISAs were performed to measure production of IL-6 (BD Biosciences, Franklin Lakes, NJ) and competitive EIA assays were performed to measure PGE₂ production (Cayman Chemical Company, Ann Arbor, MI). Experiments were performed a minimum of two times in quadruplicate.

Quantitative real-time PCR (qRT-PCR)

Expression profiles of *IL-6* and *CLEC7A* mRNA sequences were evaluated at 30 min, 6, 24, and 72 h following treatment with 100 µg/ml zymosan or vehicle control in fibroblast strains obtained from LPV cases. Cells were propagated and treated in 24-well plates. At each time point, cells were lysed and total mRNA was extracted using the Qiagen RNeasy kit following the manufacturers' instructions (Qiagen Corp., Carlsbad, CA). A NanoDrop ND-1000 (NanoDrop/Thermo Fisher Scientific) was used to quantify the mRNAs, which were used as templates for cDNA synthesis using the iScript cDNA synthesis kit (BioRad, Hercules, CA); 300 ng total RNA template was used in each reaction. Negative reverse transcriptase controls (where no enzyme was added to the reaction) were also prepared to confirm the absence of DNA contamination. cDNA samples were diluted 1:5 in RNase-free molecular grade water (Qiagen) and used as templates for qRT-PCR reactions (5 µl/reaction). A standard curve was constructed for each primer set by preparing 5-fold serial dilutions of a reference set of cDNAs prepared from RNAs purified from cells treated with zymosan. Reactions were prepared in a total of 12 µl using SsoAdvanced Universal SYBR Green Supermix (BioRad). Previously published primer sequences for human CLEC7A were used to quantify Dectin-1 expression⁵³, while primers for human IL-6 (sense: 5'-GTACATCCTCGACGGCATC and anti-sense: 5'-ACCTC AACTCCAAAAGACCAG) were designed using IDT oligo design tools (OligoAnalyzer, <http://www.idtdna.com>). All RT-qPCR values were normalized to the 18S rRNA signal amplified using previously published primer sequences⁵⁴. Each experiment was performed a minimum of two times in quadruplicate.

Dectin-1 protein expression on human external vulvar and vestibular fibroblasts

Fibroblast strains (external vulvar and vestibular strains from both cases and controls) were grown to confluency in 6-well culture dishes (Thermo Fisher Scientific), which were then released from the culture surface using trypsin-EDTA solution and trypsin inhibitor (Gibco/Invitrogen, part of Thermo Fisher Scientific) and then washed with PBS before blocking

non-specific antibody binding with 5% human Fc receptor blocker (Miltenyi Biotech Inc., San Diego, CA) in PBS containing 1% BSA and 0.1% sodium azide. Cells were then either unstained or incubated with phycoerythrin-conjugated anti-human Dectin-1 antibody (GeneTex Inc., Irvine, CA). Unstained and positively stained cells were analyzed on a FACS Canto II flow cytometer running FACSDiva software (BD Biosciences, San Jose, CA) using a 488 nm excitation laser and a 585/42 nm band pass detector and subsequently analyzed using FlowJo software (TreeStar Data Analysis Software, Ashland, OR). A typical result from several flow experiments is depicted.

Additional 6-well cultures of fibroblast strains were prepared as described earlier, then washed with PBS, and lysed in 0.1 M Tris with 2% sodium dodecyl sulfide (SDS) and protease inhibitor cocktail at a 1:10 dilution (Sigma-Aldrich Chemical Co., St. Louis, MO). Protein concentrations were determined using a BioRad DC protein assay, and 10 µg of each protein lysate was run on a 4-20% pre-cast Criterion Tris-HCl gel (BioRad) with Spectra multicolor broad range protein ladder (Thermo Fisher Scientific) and electro-transferred to a 0.45 µm EMD Millipore Immobilon PVDF membrane (Thermo Fisher Scientific). Membranes were stained with Ponceau S (Sigma-Aldrich) for 10 min to visualize total protein on the membrane. Membranes were destained in 5% acetic acid, then washed in western wash buffer (PBS with 0.1% Tween-20) several times before blocking with 2% bovine serum albumin for 30 min (reagents from Sigma-Aldrich). After blocking, membranes were incubated with a mouse monoclonal antibody specific for the Dectin-1 receptor (Genetex) for 1 h at room temperature (RT). Membranes were washed and then incubated with a goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 30 min. Dectin-1 receptor expression was visualized using enhanced chemiluminescent HRP substrate (Thermo Fisher Scientific) and exposure to x-ray film, followed by densitometric analysis using Quantity One 1-D Analysis Software version 4.6.9 (BioRad).

Dectin-1 receptor blockade

Two independent methods were employed to block the function of the Dectin-1 receptor in fibroblast strains derived from women diagnosed with LPV. The impact of blockade was evaluated by quantifying the amount of pro-inflammatory mediators released (IL-6 and PGE₂) in response to challenge with 100 µg/ml zymosan in cells with functional receptor versus those receiving treatment to impair function. IL-6 and PGE₂ were assayed because they are abundantly produced by fibroblast strains from LPV cases^{10, 11} and have been generally associated with the evolution of pain during inflammation^{14-16, 55, 56}. For both methods, cells were grown in 24-well plates in MEM with 10% FBS until confluent, then transitioned to low serum media (0.05% FBS) for 48 h. To physically block receptor function, cells were first incubated for 1 h at 37°C with 1 mg/ml laminarin, a commercially purified soluble β-glucan that binds to Dectin-1, yet fails to signal an inflammatory response (Sigma-Aldrich); cells in control wells with laminarin alone failed to produce IL-6 or PGE₂ as anticipated. Cells were then challenged with vehicle control or 100 µg/ml zymosan by direct addition to the culture media from concentrated stock; after addition, cells were incubated at 37°C for another 24 h, at which point culture supernatants were collected and assayed for IL-6 and PGE₂, as described earlier. A second molecular approach was used to

block the expression of the gene encoding Dectin-1 (CLEC7A). An siRNA against human CLEC7A and Silencer negative control no. 1 siRNA was purchased from Ambion (a division of Thermo Fisher Scientific). The lipofectamine 2000 reagent (Ambion) was used to transfect cells with control and anti-Dectin-1 siRNAs according to the manufacturer's instructions; cells were first washed with PBS, then transfected with a total of 500 ng siRNA/well in MEM with 0.05% FBS. Following the transfection procedure, cells were incubated for 48 h at 37°C. At this time, cells were then challenged with 100 µg/ml zymosan or vehicle control in fresh media for another 24 h, at which point supernatants were collected for detection of IL-6 and PGE₂. The adherent cells were then washed in PBS, and protein was collected for Western blotting against Dectin-1. Western blots confirmed that Dectin-1 protein levels were not affected by the control siRNA, while the levels were dramatically reduced by anti-CLEC7A siRNA. Each experiment was performed a minimum of two times in quadruplicate.

NFκB subunit translocation assays

LPV patient strains were propagated and treated with zymosan or vehicle for a total of 3 h, then the nuclear and cytoplasmic protein fractions were purified separately using a nuclear extract kit per the manufacturer's instructions (Active Motif, Carlsbad, CA). Nuclear extracts were then applied to an NFκB family TransAM assay (Active Motif) to measure NFκB subunit levels in the nucleus. Nuclear and cytoplasmic protein fractions were also analyzed by Western blotting for p65 subunit expression by probing with an anti-p65 monoclonal rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit monoclonal anti-histone H3 in the nucleus, and monoclonal rabbit anti-β-tubulin in the cytoplasm were used as loading controls and to assess the efficiency of nuclear and cytoplasmic separation (antibodies from Cell Signaling Technology, Danvers, MA). Visualization of protein expression was performed as described previously using appropriate secondary antibodies and was performed for more than one set of patient strains.

Activation of NFκB with live yeast challenge

To assess the effect of live yeast infection on the activation of the NFκB pathway, LPV patient fibroblast strains were transfected with an NFκB-firefly luciferase reporter⁵⁷ and pRL-SV40, a commercially available control renilla luciferase reporter (Promega, Madison, WI). Nucleofection of these constructs was performed as previously described⁵⁷. Cells were allowed to reach confluence in 24-well plates and were then serum-starved for 48 h prior to transfection. After transfection, cells were allowed to recover for 24 h before transitioning to fresh media (MEM + 0.05% FBS) containing 1×10^4 *C. albicans* SC5314 cells/ml, 1×10^4 *Saccharomyces cerevisiae* S288C cells/ml, or a matched volume of YPD (vehicle control). Both yeast inoculums were prepared from overnight cultures of each strain diluted to an OD₆₀₀ of 1 in fresh YPD; the overnight culture of *C. albicans* was grown at 37°C, while *S. cerevisiae* was grown at 30°C. After 24 h of infection, reporter activity was determined using the Dual-Glo Luciferase Assay System (Promega, Madison, WI). Luminescence was quantified on a VarioSkan Flash Multimode Reader (Thermo Fisher Scientific). Firefly luciferase activity was then normalized to constitutive renilla activity to adjust for any differences in the final numbers of cells in each well. Assays were performed a minimum of two times in quadruplicate.

Impact of NFκB inhibition on pro-inflammatory mediator release

Patient fibroblast strains were cultured in 24-well plates as described and then simultaneously treated with either zymosan alone or with 100 µg/ml zymosan and 5 µg/ml BAY-11-7082 (NFκB inhibitor; Cayman Chemical) in MEM + 0.05% FBS. Cells were incubated for 24 h at 37°C prior to supernatant collection. The amount of IL-6 and PGE₂ in the supernatant was determined with ELISAs/EIAs as detailed earlier. Assays were performed a minimum of two times in quadruplicate.

Statistical analysis

GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA) was used to conduct the statistical analysis. For most cases, paired t-tests were used to compare between vestibular and external vulvar cells, cases and controls, or treated and vehicle control samples. An ANOVA with a Tukey post-hoc test was used to determine differences in the responses to specific *C. albicans* doses (Figure 1).

RESULTS

Patient vestibular fibroblasts respond strongly to low infectious doses of live *C. albicans* and to isolated yeast components present in zymosan

At the time of diagnosis, LPV cases often show no signs of active yeast infection, yet the cells associated with pain produce pro-inflammatory/pro-pain mediators^{2-7, 9-11, 13, 58}. Therefore, we examined the response of LPV patient fibroblasts to decreasing doses of live yeast to assess whether they may respond to clinically undetectable amounts of yeast in the vulvar vestibule.

In keeping with our hypothesis, we found that vestibular cells from LPV cases respond strongly, through the production of both IL-6 and PGE₂, to as few as 100 *C. albicans* colony-forming units (CFUs), which translates to a multiplicity of infection (MOI; number of yeast cells per each mammalian cell) of ~0.001 (Figure 1A, B), which is lower than the numbers of yeast present during active infection in vivo. There is a statistically significant difference between the vehicle control and a dose of 100 CFUs for both IL-6 and PGE₂ in vestibular strains ($p < 0.05$), while the difference between the vehicle control and a dose of 10 CFUs approaches significance, yet is not statistically different from the vehicle control ($p < 0.1$). External vulvar cells (not typically associated with pain) show a less potent response to even higher doses of *C. albicans* (e.g. 10⁴ CFUs), consistent with previous observations¹⁰. There are no statistically significant differences between vehicle control and any dose of yeast for both IL-6 and PGE₂ in external vulvar cells ($p > 0.05$). Furthermore, with the exception of the vehicle control, the amount of PGE₂ and IL-6 released by vestibular cells is significantly higher than in external vulvar cells ($p < 0.05$). These data support the notion that pain-associated vestibular cells may be able to sense and respond to a very small population of yeast cells normally present in the vagina not actively associated with infection and potentially below the threshold of detection by standard yeast screening techniques, such as DNA probe or culture.

To further investigate the response to specific yeast components, we measured IL-6 mRNA levels before and after treatment with zymosan, which contains the yeast cell wall PAMPs, β -glucan and mannoprotein. We found that *IL-6* mRNA levels were strongly induced in as little as 6 h following zymosan treatment and that *IL-6* was significantly more highly expressed in vestibular versus external vulvar cells and more highly expressed in cases versus controls ($p < 0.05$); the expression of *IL-6* was over 300-fold more highly expressed in vestibular cells from cases compared to those from controls ($p < 0.05$; Figure 1C). These results suggest that human vestibular fibroblasts obtained from LPV patients are exquisitely sensitive to components of the yeast cell wall, namely β -glucan and mannoprotein.

Human vestibular and external vulvar fibroblasts express the yeast β -glucan receptor Dectin-1

Our first step in examining the mechanism by which host fibroblasts recognize yeast components was to test for the expression of Dectin-1, a paradigm receptor for yeast β -glucan, which is a chief component of zymosan and is also present in the cell wall and biofilm matrix of *C. albicans* ^{24, 26, 29, 30, 32, 42-44, 46, 47}. We used published primer sequences for the *CLEC7A* gene encoding Dectin-1 to examine expression in vestibular and vulvar strains obtained from a case demonstrated to respond strongly to challenge with yeast/yeast products ¹⁰. We determined that 1) *CLEC7A* mRNA is expressed in both vestibular and external vulvar cells, 2) zymosan increases *CLEC7A* expression in vestibular cells after 72 h of treatment ($p < 0.05$), and 3) *CLEC7A* is more highly expressed in vestibular versus external vulvar cells following treatment with zymosan, while the expression is slightly higher, but not significantly different between vestibular and external vulvar cells prior to treatment ($p > 0.05$; Figure 2).

We then went on to detect Dectin-1 protein on the surface of vestibular and external vulvar cells from both patient and control strains using flow cytometry analysis with a fluorescently-labeled antibody against Dectin-1. Using this approach, we identified Dectin-1 on the surface of vestibular and external vulvar strains from both case and controls, demonstrating that Dectin-1 is present in these strains in a location that may allow it to readily interact with yeast β -glucan moieties present *in vivo*. However, we did not identify any significant differences in Dectin-1 surface expression between the case and control or between the vestibular and external vulvar strains (Figure 3A). Therefore, we elected to expand our survey to multiple case and control strains using Western blotting to assess Dectin-1 expression in total protein lysates.

Western blotting, loading equivalent amounts of protein and using a second distinct antibody to Dectin-1, showed that a wider survey of fibroblasts strains from both patients and controls express readily detectable levels of Dectin-1 (Figure 3B). Furthermore, densitometry analysis of the Dectin-1 bands revealed that Dectin-1 was slightly more abundant in patient lysates from vestibular cells than from external vulvar cells ($p < 0.05$) and slightly more abundant in cases versus controls ($p < 0.05$; Figure 3C). These data demonstrate that while Dectin-1 is present on and in human vestibular and external vulvar strains (a new finding), its abundance may also be slightly elevated in fibroblast strains obtained from patients at sites where LPV pain is localized.

Functional Dectin-1 receptor plays a role in pro-inflammatory cytokine production

After detecting the Dectin-1 receptor on human fibroblast strains, we sought to investigate its function in pro-inflammatory mediator production by monitoring the release of IL-6 and PGE₂ after impairing the ability of the receptor to recognize zymosan or after inhibiting its transcription/expression. In the first approach, we used laminarin to saturate the binding sites on Dectin-1 prior to challenge with zymosan. Laminarin is a soluble β -glucan moiety that readily binds Dectin-1, while the strength of its interaction with Dectin-1 is not sufficient to elicit an inflammatory response⁵⁹⁻⁶¹. We found that laminarin was highly effective in reducing both IL-6 and PGE₂ release in vestibular and external vulvar fibroblasts; treated values were significantly lower than vehicle control ($p < 0.05$). Furthermore, there was a significant difference in the amount of IL-6 and PGE₂ produced by vestibular versus external vulvar cells for both laminarin-treated and vehicle-treated cells ($p < 0.05$). Even after treatment, vestibular cells continued to produce significantly more pro-inflammatory mediators than their external vulvar counterparts (Figure 4A and B).

In the second approach, we used a small interfering RNA (siRNA) directed against the CLEC7A gene encoding Dectin-1. Before testing its impact on Dectin-1 receptor function, we assessed its ability to reduce the total amount of Dectin-1 protein. Western blotting using the anti-Dectin antibody revealed that siRNA treatment reduced protein expression by greater than 90% (Figure 4C). Therefore, we went on to examine the impact of siRNA treatment on the production of pro-inflammatory mediators. We found that siRNA was highly effective in reducing the production of PGE₂ in both vestibular and external vulvar fibroblasts. The amount PGE₂ was significantly less in siRNA-treated vestibular cells compared to control siRNA-treated cells ($p < 0.05$) and significantly less in siRNA-treated external vulvar cells compared to control ($p < 0.05$; Figure 4E). However, siRNA treatment had no significant effect on the production of IL-6 (Figure 4D). Nonetheless, these results show that functional Dectin-1 is required for maximal pro-inflammatory mediator production, although blocking the function of or reducing the amount of Dectin-1 does not completely abrogate cytokine production.

Recognition of yeast products activates the NF κ B pathway

The NF κ B signaling pathway is a key pathway activated during inflammation, and reports have shown that the Dectin-1 receptor can signal through both the canonical and non-canonical arms of this pathway, which are defined by the subunits involved in activating transcription, which in turn dictates which genes are expressed⁴⁴. Upon activation, NF κ B subunits will migrate to the nucleus to activate the transcription of genes involved in the inflammatory response (e.g. IL-6 and Cox-2, involved in PGE₂ production)^{44, 62-66}. Therefore, to assess whether the response to zymosan might involve the activation of the NF κ B pathway, we used a TransAM assay, which is a modified ELISA/gel shift assay, to gauge the ability of different NF κ B subunits present in the nuclear protein fraction to bind their conserved DNA consensus sequences. Thus, this assay is both a measure of the relative abundance and the binding activity of these transcription factor subunits. While more than one set of patient fibroblast strains was assayed, a typical result from a single set of patient strains is displayed in Figure 5A. All five NF κ B subunits (p50, p65, c-Rel, RelB, and p52) were detected, although the levels of RelB were only marginally above background. Of the

subunits, p50 and p65 (canonical pathway) were especially highly abundant/active, while c-Rel and p52 were in lesser abundance. The levels of nuclear p50 and p65 increased with zymosan treatment, indicating that zymosan can stimulate their activation. The levels of all detected subunits were generally higher in vestibular than external vulvar cells, indicating that activation may be stronger in these cells, consistent with their ability to generate pro-inflammatory mediators.

To confirm these results and assess relative protein abundance, we also analyzed these samples by Western blotting using an antibody specific for p65 (Figure 5B). At the same time, we ran nuclear and cytoplasmic-specific controls to assess the fidelity of our separation; we were unable to detect histone H3 (nuclear-specific) in cytoplasmic fractions and unable to detect β -tubulin (cytoplasmic-specific) in nuclear fractions. In support of our TransAM results, p65 abundance in the nucleus increased with zymosan treatment and was slightly more abundant in vestibular versus external vulvar cells. Densitometry analysis (Figure 5C) estimated the increase in p65 abundance to be roughly 3-fold in zymosan-treated cells (versus vehicle) for both vestibular and external vulvar cells, while there was a ~1.5-fold increase in vestibular versus external vulvar cells. These data indicate that zymosan treatment activates the NF κ B pathway, most likely through the canonical arm of the pathway (associated with expression of IL-6 and Cox-2), and that activation is stronger in vestibular cells.

Infection with live *C. albicans* activates the NF κ B pathway more effectively than non-pathogenic *S. cerevisiae*

Zymosan is a suitable stimulus to model yeast infection, as it contains relevant moieties that interact with PAMP receptors^{29-32, 67, 68}. However, zymosan cannot be used to fully replicate all of the conditions present during infection. Therefore, to assess the impact of live yeast infection on activation of the NF κ B pathway, we transfected primary patient vestibular and external vulvar strains with an NF κ B reporter plasmid that produces luciferase when NF κ B is activated (Figure 6)⁵⁷. Using induced light production as a readout (normalized to a constitutive renilla luciferase control plasmid), we determined that infection with *C. albicans* activates the NF κ B pathway (~3-fold increase over vehicle). *S. cerevisiae* also activates this pathway, but to a lesser extent (~2-fold increased over vehicle); *S. cerevisiae* is a non-pathogen, yet its cell wall structure is highly similar to *C. albicans*²⁶. Infection with *C. albicans* or *S. cerevisiae* activates NF κ B to a greater extent than the vehicle for vestibular and external vulvar strains ($p < 0.05$), while infection with *C. albicans* results in higher activation than *S. cerevisiae* for vestibular and external vulvar strains (~1.3-fold increase with *C. albicans*, $p < 0.05$). Activation may be slightly stronger in vestibular versus external vulvar strains, although this is not statistically significant ($p > 0.05$). Ultimately, live yeast infection activates the NF κ B pathway and may be influenced by the relative pathogenicity and invasiveness of the yeast strain.

Inhibition of the NF κ B pathway nearly abrogates pro-inflammatory mediator production

To assess the relative importance of the NF κ B signaling pathway in the production of pro-inflammatory mediators IL-6 and PGE₂, we treated cells with a highly specific and widely used inhibitor of this pathway, BAY-11-7082, which prevents phosphorylation of I κ B α and

the subsequent translocation of NF κ B subunits to the nucleus⁶⁹. We then measured cytokine output in response to zymosan treatment with and without BAY-11-7082. We found that treatment with BAY-11-7082 reduced pro-inflammatory mediator levels in vestibular and external vulvar strains to near background levels (Figure 7). The amount of IL-6 was significantly lower (~10-fold less) in vestibular cells treated with BAY-11-7082 compared to cells treated with zymosan alone ($p < 0.05$) and ~4-fold less in BAY-11-7082-treated external vulvar cells ($p < 0.05$). A similar trend was observed for PGE₂; there was a ~6-fold reduction in vestibular cells ($p < 0.05$) and a ~2-fold reduction in external vulvar cells ($p < 0.05$). Overall, the NF κ B pathway is a significant contributor to pro-inflammatory mediator production in response to yeast products (zymosan). Impairing its function counteracts the pro-inflammatory influences of zymosan.

DISCUSSION

No currently available therapies for LPV address the underlying pathogenesis of the disease because it is poorly understood. Here, we have identified one possible intracellular mechanism associated with the development of vulvodynia and have pinpointed two possible targets for the resolution of pro-inflammatory signaling and pain: the NF κ B pathway and Dectin-1 mediated signaling. Inhibiting the function of the Dectin-1 receptor or lowering its abundance leads to a significant reduction in the production of pro-pain/pro-inflammatory mediators. However, inhibiting the activation of the NF κ B pathway essentially reduces cytokine production to background levels. Therefore, the NF κ B pathway represents a particularly potent therapeutic target. A number of NF κ B inhibitors are currently used in cancer therapies and may also have a therapeutic role for LPV⁶⁴.

We found that vestibular fibroblasts from LPV cases are intrinsically sensitive to even very low infectious doses of *C. albicans*, cell numbers that fall within the range of what might be present in the normal microbiota of women⁷⁰⁻⁷⁴. Previous studies by our group and others, demonstrate that vestibular cells possess an “immunological memory,” where repeated infections with *C. albicans* predispose these cells to the production of elevated levels of pro-inflammatory mediators, even after the stimulus is removed^{10, 11, 13}. Recent understanding has shown that immunological memory is not limited to the adaptive immune system and that numerous innate immune cell types possess some form of memory⁷⁵. Our results support the concept that vestibular fibroblasts, arising from a distinctly different cell lineage (defined by embryologic origin) than external vulvar cells^{10, 11, 76}, possess or undergo a change that renders them exquisitely sensitive to *C. albicans* and the moieties present in its cell wall. Vestibular fibroblasts produce significantly elevated levels (compared to vehicle) of both IL-6 and PGE₂ in response to as few as 100 yeast cells, while external vulvar cells fail to respond to a dose that is 1000 times greater. This pattern of response does occur in fibroblasts from control patients, but it is greatly accentuated in vestibular fibroblasts associated with LPV pain. We found that upon stimulation with zymosan, vestibular fibroblasts of LPV cases produce as much five times more IL-6 transcript than vestibular fibroblasts from healthy controls. In a preceding study, we demonstrated that numerous strains isolated from patients consistently produce more IL-6 and PGE₂ than healthy volunteers and that IL-6 and PGE₂ production accurately predicts the mechanical pain threshold from that anatomic location¹⁰. In both LPV cases and pain-free controls,

vestibular fibroblasts produce more pro-inflammatory mediators than their external vulvar counterparts, while vestibular fibroblasts from LPV cases produce the highest amount by location and presence or absence of disease. As reported in a number of pain studies of other body sites in both humans and animal models^{14-16, 55, 56}, localized production of pro-inflammatory mediators in the vulvar vestibule may contribute to the profound pain experienced by women with LPV.

One explanation for heightened vestibular sensitivity to yeast and their products would be the presence and/or increased abundance of receptors involved in sensing yeast pathogen associated molecular patterns (PAMPs). In support of this hypothesis, we found that fibroblasts isolated from the vulvar vestibule express at least one pattern recognition receptor (Dectin-1) demonstrated to be involved in the recognition of yeast β -glucan. Our findings demonstrate that vestibular fibroblasts are equipped to sense yeast and their products, which is a significant and new finding. We also show (using two independent methods) that the function of the Dectin-1 receptor is necessary for maximal cytokine production in response to challenge with zymosan. These data verify that Dectin-1 plays a role in the response to yeast in vestibular fibroblasts. In addition to identifying the presence of mechanisms associated with the response to *C. albicans*, we demonstrate that the abundance of the Dectin-1 receptor is comparatively elevated in vestibular versus external vulvar fibroblasts and in cases versus controls. Although further study is necessary to definitively demonstrate that increased receptor abundance accounts for the heightened production of pro-inflammatory mediators, it would stand to reason that the availability of additional binding sites would result in an increase in IL-6 and PGE₂.

Inhibition of Dectin-1 significantly reduces, but does not completely abolish cytokine production. This observation points to the existence of other mechanisms that also contribute to pro-inflammatory mediator release. At the same time, our results clearly show that Dectin-1 signaling feeds into a specific part of the NF κ B pathway that is referred to as the canonical arm and is associated with the production of IL-6 and Cox-2 (rate-limiting enzyme involved in PGE₂ production) (36, 54-58). Inhibition of NF κ B successfully diminishes cytokine production, but the effects of inhibition are broad, considering the relative importance of NF κ B signaling in essential innate immune responses and the initiation of inflammation⁶²⁻⁶⁴. However, topical applications of NF κ B in the context of LPV may pose fewer systemic effects⁷⁷, although this remains to be evaluated. Nonetheless, further examination of the mechanisms contributing to the generation of pro-pain mediators would increase our overall knowledge of a poorly understood disorder and offer a wider selection of therapeutic targets for the design of treatments that will begin to address the underlying causes of disease.

Lastly, the response to *C. albicans* is more involved than the simple recognition of yeast cell proteins. The reason being that NF κ B activation strength is influenced by the infecting yeast species, even when fibroblasts are challenged with species that possess highly similar cell wall structures²⁶. Using an NF κ B reporter, we show that activation is considerably more robust when fibroblasts are challenged with common vaginal pathogen *C. albicans*, versus *S. cerevisiae*, a Brewer's yeast largely regarded as non-pathogenic^{25-28, 78, 79}. In keeping with this observation, our previous work has shown that the response to different yeast strains of

varying abundance and/or pathogenic significance in the vagina is distinct to each strain¹⁰. Infection with common invasive vaginal pathogens, *C. albicans* or *C. glabrata*, results in a high level of IL-6 and PGE₂ production, while infection with less commonly associated strains (e.g. *C. tropicalis*) results in significantly less cytokine production, and *S. cerevisiae* elicits the lowest amount of cytokine production¹⁰. With a high degree of cell wall similarity among *C. albicans*, *C. glabrata*, and *S. cerevisiae*, some other factor(s) must influence the pro-inflammatory response to specific yeast strains. With the goal of finding effective, specific, and selective targets to reduce pro-inflammatory mediator production in the vulvar vestibule, our live infection model presented herein could be used to identify some of these other pertinent factors (e.g. secreted factors or differences in invasive qualities).

In summary, vulvodynia is a serious, yet poorly understood disease^{1-7, 9}. To this end, no available therapies exist that address the underlying cause of disease, pointing to an urgent need to identify targets for the development of new therapeutic agents^{1-7, 9}. Here, we identify two targets that when inhibited result in a decrease in pro-inflammatory/pro-pain mediator production *in vitro*. In addition to identifying Dectin-1 and NFκB as potential therapeutic targets, our data provides clues for isolating other signaling mechanisms or factors that may be involved in the response to *C. albicans* and other yeast species. A clearer understanding of the mechanisms that influence the onset and persistence of vulvodynia is paramount to improving treatment for afflicted women. Due to the existence of approved agents that target certain conserved inflammatory pathways (e.g. NFκB), focusing on how these pathways function in LPV may lead to a faster translation to clinical applications.

Acknowledgments

Work funded by NIH-NICHD R01 HD069313.

REFERENCES

1. BOHM-STARKE N. Medical and physical predictors of localized provoked vulvodynia. Acta Obstet Gynecol Scand. 2010; 89:1504–10. [PubMed: 21080898]
2. Granot M, Friedman M, Yarnitsky D, Tamir A, Zimmer EZ. Primary and secondary vulvar vestibulitis syndrome: systemic pain perception and psychophysical characteristics. Am J Obstet Gynecol. 2004; 191:138–42. [PubMed: 15295354]
3. Kingdon J. Vulvodynia: a comprehensive review. Nurs Womens Health. 2009; 13:48–57. quiz 58. [PubMed: 19207504]
4. Sadownik LA. Etiology, diagnosis, and clinical management of vulvodynia. Int J Womens Health. 2014; 6:437–49. [PubMed: 24833921]
5. Ventolini G. Vulvar pain: Anatomic and recent pathophysiologic considerations. Clin Anat. 2013; 26:130–3. [PubMed: 22951941]
6. Wesselmann U, Bonham A, Foster D. Vulvodynia: Current state of the biological science. Pain. 2014
7. Groyzman V. Vulvodynia: new concepts and review of the literature. Dermatol Clin. 2010; 28:681–96. [PubMed: 20883912]
8. Haefner HK, Collins ME, Davis GD, et al. The vulvodynia guideline. Journal of lower genital tract disease. 2005; 9:40–51. [PubMed: 15870521]
9. Donders G, Bellen G. Characteristics of the pain observed in the focal vulvodynia syndrome (VVS). Med Hypotheses. 2012; 78:11–4. [PubMed: 22041052]

10. Foster D, Falsetta M, Woeller C, et al. Site-specific mesenchymal control of inflammatory pain to yeast challenge in vulvodynia afflicted and pain-free women. *Pain*. 2014
11. Foster DC, Piekarz KH, Murant TI, Lapoint R, Haidaris CG, Phipps RP. Enhanced synthesis of proinflammatory cytokines by vulvar vestibular fibroblasts: implications for vulvar vestibulitis. *Am J Obstet Gynecol*. 2007; 196:346, e1–8. [PubMed: 17403416]
12. Babula O, Linhares IM, Bongiovanni AM, Ledger WJ, Witkin SS. Association between primary vulvar vestibulitis syndrome, defective induction of tumor necrosis factor- α , and carriage of the mannose-binding lectin codon 54 gene polymorphism. *Am J Obstet Gynecol*. 2008; 198:101, e1–4. [PubMed: 18166320]
13. Farmer MA, Taylor AM, Bailey AL, et al. Repeated vulvovaginal fungal infections cause persistent pain in a mouse model of vulvodynia. *Sci Transl Med*. 2011; 3:101ra91.
14. Cunha FQ, Poole S, Lorenzetti BB, Ferreira SH. The pivotal role of tumour necrosis factor α in the development of inflammatory hyperalgesia. *British journal of pharmacology*. 1992; 107:660–4. [PubMed: 1472964]
15. Eliav E, Benoliel R, Herzberg U, Kalladka M, Tal M. The role of IL-6 and IL-1 β in painful perineural inflammatory neuritis. *Brain, behavior, and immunity*. 2009; 23:474–84.
16. Obreja O, Schmelz M, Poole S, Kress M. Interleukin-6 in combination with its soluble IL-6 receptor sensitises rat skin nociceptors to heat, in vivo. *Pain*. 2002; 96:57–62. [PubMed: 11932061]
17. Cavalli F, Mucci MP, Cociancich L, Micheli W, Bacarini L, Cisternino M. [Prostaglandin E2 liberation in the synovial fluid induced by organo-iodinated contrast media. Interrelations with the genesis of post-arthrographic pain]. *La Radiologia medica*. 1987; 74:512–5. [PubMed: 3481097]
18. Firestein GS, Alvaro-Gracia JM, Maki R. Quantitative analysis of cytokine gene expression in rheumatoid arthritis. *Journal of immunology*. 1990; 144:3347–53.
19. Cui JG, Holmin S, Mathiesen T, Meyerson BA, Linderöth B. Possible role of inflammatory mediators in tactile hypersensitivity in rat models of mononeuropathy. *Pain*. 2000; 88:239–48. [PubMed: 11068111]
20. Lin CR, Amaya F, Barrett L, et al. Prostaglandin E2 receptor EP4 contributes to inflammatory pain hypersensitivity. *The Journal of pharmacology and experimental therapeutics*. 2006; 319:1096–103. [PubMed: 16966471]
21. Daymond TJ, Rowell FJ. Reduction of prostaglandin E2 concentrations in synovial fluid of patients suffering from rheumatoid arthritis following tiaprofenic acid or indomethacin treatment. *Drugs*. 1988; 35(Suppl 1):4–8. [PubMed: 3162870]
22. Zanjani TM, Sabetkasaei M, Mosaffa N, Manaheji H, Labibi F, Farokhi B. Suppression of interleukin-6 by minocycline in a rat model of neuropathic pain. *European journal of pharmacology*. 2006; 538:66–72. [PubMed: 16687137]
23. Reinold H, Ahmadi S, Depner UB, et al. Spinal inflammatory hyperalgesia is mediated by prostaglandin E receptors of the EP2 subtype. *The Journal of clinical investigation*. 2005; 115:673–9. [PubMed: 15719070]
24. Ghosh S, Howe N, Volk K, Tati S, Nickerson KW, Petro TM. *Candida albicans* cell wall components and farnesol stimulate the expression of both inflammatory and regulatory cytokines in the murine RAW264.7 macrophage cell line. *FEMS Immunol Med Microbiol*. 2010; 60:63–73. [PubMed: 20618847]
25. Williams DW, Jordan RP, Wei XQ, et al. Interactions of *Candida albicans* with host epithelial surfaces. *J Oral Microbiol*. 2013:5.
26. Klis FM, Boorsma A, De Groot PW. Cell wall construction in *Saccharomyces cerevisiae*. *Yeast*. 2006; 23:185–202. [PubMed: 16498706]
27. Davies S, Johnson E, White D. How to treat persistent vaginal yeast infection due to species other than *Candida albicans*. *Sex Transm Infect*. 2013; 89:165–6. [PubMed: 23180861]
28. Rodrigues CF, Silva S, Henriques M. *Candida glabrata*: a review of its features and resistance. *Eur J Clin Microbiol Infect Dis*. 2014; 33:673–88. [PubMed: 24249283]
29. Perez-Garcia LA, Diaz-Jimenez DF, Lopez-Esparza A, Mora-Montes HM. Role of Cell Wall Polysaccharides during Recognition of *Candida albicans* by the Innate Immune System. *Glycobiology*. 2011; 1:1.

30. Chaffin WL. *Candida albicans* cell wall proteins. *Microbiol Mol Biol Rev.* 2008; 72:495–544. [PubMed: 18772287]
31. Gow NA, van de Veerdonk FL, Brown AJ, Netea MG. *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. *Nat Rev Microbiol.* 2012; 10:112–22. [PubMed: 22158429]
32. Latge JP. Tasting the fungal cell wall. *Cell Microbiol.* 2010; 12:863–72. [PubMed: 20482553]
33. Bourgeois C, Kuchler K. Fungal pathogens-a sweet and sour treat for toll-like receptors. *Front Cell Infect Microbiol.* 2012; 2:142. [PubMed: 23189270]
34. Nett J, Lincoln L, Marchillo K, et al. Putative role of beta-1,3 glucans in *Candida albicans* biofilm resistance. *Antimicrob Agents Chemother.* 2007; 51:510–20. [PubMed: 17130296]
35. Nett JE, Sanchez H, Cain MT, Andes DR. Genetic basis of *Candida* biofilm resistance due to drug-sequestering matrix glucan. *J Infect Dis.* 2010; 202:171–5. [PubMed: 20497051]
36. Nobile CJ, Nett JE, Hernday AD, et al. Biofilm matrix regulation by *Candida albicans* Zap1. *PLoS Biol.* 2009; 7:e1000133. [PubMed: 19529758]
37. Taff HT, Nett JE, Zarnowski R, et al. A *Candida* biofilm-induced pathway for matrix glucan delivery: implications for drug resistance. *PLoS Pathog.* 2012; 8:e1002848. [PubMed: 22876186]
38. Alves CT, Wei XQ, Silva S, Azeredo J, Henriques M, Williams DW. *Candida albicans* promotes invasion and colonisation of *Candida glabrata* in a reconstituted human vaginal epithelium. *J Infect.* 2014
39. Giraldo P, von Nowaskonski A, Gomes FA, Linhares I, Neves NA, Witkin SS. Vaginal colonization by *Candida* in asymptomatic women with and without a history of recurrent vulvovaginal candidiasis. *Obstetrics and gynecology.* 2000; 95:413–6. [PubMed: 10711554]
40. Peters BM, Palmer GE, Nash AK, Lilly EA, Fidel PL Jr, Noverr MC. Fungal morphogenetic pathways are required for the hallmark inflammatory response during *Candida albicans* vaginitis. *Infection and immunity.* 2014; 82:532–43. [PubMed: 24478069]
41. Taylor BN, Staib P, Binder A, et al. Profile of *Candida albicans*-secreted aspartic proteinase elicited during vaginal infection. *Infection and immunity.* 2005; 73:1828–35. [PubMed: 15731084]
42. Dennehy KM, Brown GD. The role of the beta-glucan receptor Dectin-1 in control of fungal infection. *J Leukoc Biol.* 2007; 82:253–8. [PubMed: 17475782]
43. Drummond RA, Brown GD. The role of Dectin-1 in the host defence against fungal infections. *Curr Opin Microbiol.* 2011; 14:392–9. [PubMed: 21803640]
44. Geijtenbeek TB, Gringhuis SI. Signalling through C-type lectin receptors: shaping immune responses. *Nature reviews Immunology.* 2009; 9:465–79.
45. Gringhuis SI, Kaptein TM, Wevers BA, et al. Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1 β via a noncanonical caspase-8 inflammasome. *Nature immunology.* 2012; 13:246–54. [PubMed: 22267217]
46. Huang H, Ostroff GR, Lee CK, Wang JP, Specht CA, Levitz SM. Distinct patterns of dendritic cell cytokine release stimulated by fungal beta-glucans and toll-like receptor agonists. *Infection and immunity.* 2009; 77:1774–81. [PubMed: 19273561]
47. Reid DM, Gow NA, Brown GD. Pattern recognition: recent insights from Dectin-1. *Curr Opin Immunol.* 2009; 21:30–7. [PubMed: 19223162]
48. Taylor PR, Brown GD, Reid DM, et al. The beta-glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages. *Journal of immunology.* 2002; 169:3876–82.
49. Tamai R, Sugamata M, Kiyoura Y. *Candida albicans* enhances invasion of human gingival epithelial cells and gingival fibroblasts by *Porphyromonas gingivalis*. *Microb Pathog.* 2011; 51:250–4. [PubMed: 21742026]
50. Bergeron S, Binik YM, Khalife S, Pagidas K, Glazer HI. Vulvar vestibulitis syndrome: reliability of diagnosis and evaluation of current diagnostic criteria. *Obstetrics and gynecology.* 2001; 98:45–51. [PubMed: 11430955]
51. Baglolle CJ, Reddy SY, Pollock SJ, et al. Isolation and phenotypic characterization of lung fibroblasts. *Methods Mol Med.* 2005; 117:115–27. [PubMed: 16118449]

52. Koumas L, King AE, Critchley HO, Kelly RW, Phipps RP. Fibroblast heterogeneity: existence of functionally distinct Thy 1(+) and Thy 1(-) human female reproductive tract fibroblasts. *Am J Pathol.* 2001; 159:925–35. [PubMed: 11549585]
53. Lefevre L, Lugo-Villarino G, Meunier E, et al. The C-type lectin receptors dectin-1, MR, and SIGNR3 contribute both positively and negatively to the macrophage response to *Leishmania infantum*. *Immunity.* 2013; 38:1038–49. [PubMed: 23684988]
54. Lehmann GM, Woeller CF, Pollock SJ, et al. Novel anti-adipogenic activity produced by human fibroblasts. *Am J Physiol Cell Physiol.* 2010; 299:C672–81. [PubMed: 20554910]
55. Kawabata A. Prostaglandin E2 and pain--an update. *Biological & pharmaceutical bulletin.* 2011; 34:1170–3. [PubMed: 21804201]
56. Sommer C, Kress M. Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia. *Neuroscience letters.* 2004; 361:184–7. [PubMed: 15135924]
57. Baglioni CJ, Maggiorini SB, Gasiewicz TA, Thatcher TH, Phipps RP, Sime PJ. The aryl hydrocarbon receptor attenuates tobacco smoke-induced cyclooxygenase-2 and prostaglandin production in lung fibroblasts through regulation of the NF-kappaB family member RelB. *The Journal of biological chemistry.* 2008; 283:28944–57. [PubMed: 18697742]
58. Ramirez De Knott HM, McCormick TS, Do SO, et al. Cutaneous hypersensitivity to *Candida albicans* in idiopathic vulvodynia. *Contact Dermatitis.* 2005; 53:214–8. [PubMed: 16191018]
59. Brown GD, Taylor PR, Reid DM, et al. Dectin-1 is a major beta-glucan receptor on macrophages. *The Journal of experimental medicine.* 2002; 196:407–12. [PubMed: 12163569]
60. Gantner BN, Simmons RM, Underhill DM. Dectin-1 mediates macrophage recognition of *Candida albicans* yeast but not filaments. *The EMBO journal.* 2005; 24:1277–86. [PubMed: 15729357]
61. Gersuk GM, Underhill DM, Zhu L, Marr KA. Dectin-1 and TLRs permit macrophages to distinguish between different *Aspergillus fumigatus* cellular states. *Journal of immunology.* 2006; 176:3717–24.
62. Hayden MS, Ghosh S. Signaling to NF-kappaB. *Genes & development.* 2004; 18:2195–224. [PubMed: 15371334]
63. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harbor perspectives in biology.* 2009; 1:a001651. [PubMed: 20457564]
64. Luqman S, Pezzuto JM. NF-kappaB: a promising target for natural products in cancer chemoprevention. *Phytotherapy research : PTR.* 2010; 24:949–63. [PubMed: 20577970]
65. Nelson G, Paraoan L, Spiller DG, et al. Multi-parameter analysis of the kinetics of NF-kappaB signalling and transcription in single living cells. *Journal of cell science.* 2002; 115:1137–48. [PubMed: 11884514]
66. Ricciotti E, Fitzgerald GA. Prostaglandins and Inflammation. *Arterioscler Thromb Vasc Biol.* 2011; 31:986–1000. [PubMed: 21508345]
67. Gantner BN, Simmons RM, Canavera SJ, Akira S, Underhill DM. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *The Journal of experimental medicine.* 2003; 197:1107–17. [PubMed: 12719479]
68. Ozinsky A, Underhill DM, Fontenot JD, et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proceedings of the National Academy of Sciences of the United States of America.* 2000; 97:13766–71. [PubMed: 11095740]
69. Pierce JW, Schoenleber R, Jesmok G, et al. Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. *The Journal of biological chemistry.* 1997; 272:21096–103. [PubMed: 9261113]
70. Brown CJ, Wong M, Davis CC, Kanti A, Zhou X, Forney LJ. Preliminary characterization of the normal microbiota of the human vulva using cultivation-independent methods. *Journal of medical microbiology.* 2007; 56:271–6. [PubMed: 17244812]
71. Chaban B, Links MG, Jayaprakash TP, et al. Characterization of the vaginal microbiota of healthy Canadian women through the menstrual cycle. *Microbiome.* 2014; 2:23. [PubMed: 25053998]

72. Danielsson D, Teigen PK, Moi H. The genital econiche: focus on microbiota and bacterial vaginosis. *Annals of the New York Academy of Sciences*. 2011; 1230:48–58. [PubMed: 21824165]
73. Zhou X, Westman R, Hickey R, et al. Vaginal microbiota of women with frequent vulvovaginal candidiasis. *Infection and immunity*. 2009; 77:4130–5. [PubMed: 19528218]
74. van de Wijgert JH, Borgdorff H, Verhelst R, et al. The vaginal microbiota: what have we learned after a decade of molecular characterization? *PloS one*. 2014; 9:e105998. [PubMed: 25148517]
75. Sun JC, Ugolini S, Vivier E. Immunological memory within the innate immune system. *The EMBO journal*. 2014; 33:1295–303. [PubMed: 24674969]
76. Puppo V. Embryology and anatomy of the vulva: the female orgasm and women's sexual health. *European journal of obstetrics, gynecology, and reproductive biology*. 2011; 154:3–8.
77. Jobling P, O'Hara K, Hua S. Female reproductive tract pain: targets, challenges, and outcomes. *Frontiers in pharmacology*. 2014; 5:17. [PubMed: 24592238]
78. Harriott MM, Lilly EA, Rodriguez TE, Fidel PL Jr, Noverr MC. *Candida albicans* forms biofilms on the vaginal mucosa. *Microbiology*. 2010; 156:3635–44. [PubMed: 20705667]
79. Paiva LC, Vidigal PG, Donatti L, Svidzinski TI, Consolaro ME. Assessment of in vitro biofilm formation by *Candida* species isolates from vulvovaginal candidiasis and ultrastructural characteristics. *Micron*. 2012; 43:497–502. [PubMed: 22001373]

Précis

Dectin-1 recognition of *C. albicans* results in heightened pro-inflammatory mediator production in fibroblast strains obtained from vulvodynia patients, which can be mitigated by blocking Dectin-1 or the NFκB pathway.

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Figure 1. *C. albicans* and components of the yeast cell wall are potent inducers of the pro-inflammatory response in vestibular fibroblasts

Panels A (IL-6) and B (PGE₂) depict the amount of pro-inflammatory cytokine released in response to decreasing doses of live *C. albicans*. A single asterisk denotes a difference between the vehicle control and a particular dose of *C. albicans* (for vestibular cells only), while two asterisks denote a significant difference between vestibular and vulvar cells for a particular dose. Significance was determined via ANOVA with a post hoc Tukey test; values less than 0.05 were considered significant. Vestibular cells show a strong response to infection with live yeast and yeast cell wall components (zymosan), with a significant response to as little as 100 CFUs, while vulvar cells show no significant response to a dose up to 1000 times greater. Panel C depicts the transcription of the gene encoding IL-6 in response to treatment with zymosan for 72 h. A single asterisk denotes a difference between paired vestibular and vulvar cells obtained from a patient, while two asterisks denote differences between the normal control and the case for vestibular or vulvar strains. Paired *t*-tests were used to compare between case and control and vestibular and vulvar strains (*n* = 8, *p* < 0.05). After zymosan treatment, expression of *IL-6* was significantly elevated in vestibular versus vulvar cells and in the case versus control, with the highest level of

expression occurring in patient vestibular cells. Data are represented as the mean $\pm$ one standard deviation.

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Figure 2. Dectin-1 mRNA is expressed by patient vestibular strains and its expression is increased with zymosan treatment

This figure depicts expression profiles for CLEC7A, the gene encoding Dectin-1. A single asterisk denotes a difference between the vehicle and zymosan treatment for vestibular cells, while two asterisks denote a difference between vestibular and vulvar cells. Paired *t*-tests comparing vestibular versus vulvar and vehicle versus zymosan were used to determine significance ($n = 9$, $p < 0.05$). Dectin-1 is expressed on human vestibular and vulvar fibroblasts; expression is induced with zymosan treatment and is elevated in vestibular versus vulvar strains. Data is represented as the mean $\pm$ one standard deviation.

Figure 3. Dectin-1 is expressed on the surface of fibroblast strains and is more abundant in vestibular cells and cases

Panel A depicts a typical result from flow cytometry analysis examining surface expression of Dectin-1. Although all cells are strongly positive for Dectin-1, there are no obvious differences between case and control or vestibular and vulvar strains. Panel B depicts a Western blot probing for Dectin-1 examining 4 paired case and 4 paired control strains. The bands alternate between vestibular (vest.) and vulvar (vulv.) strains as noted. All samples were run on a single Western blot with a single exposure to autoradiography film; bands have been cropped and positioned for display purposes. Equivalent loading was confirmed using total protein staining (not shown). Visual assessment suggests that Dectin-1 is more highly expressed in vestibular versus vulvar strains and in cases versus controls, which was confirmed via densitometry analysis, depicted in panel C. A single asterisk denotes a difference between vestibular and vulvar cells for cases, while two asterisks denote a difference between cases and controls for vestibular strains. Paired *t*-tests (case versus control, vestibular versus vulvar) were used to determine significance ($n = 4$, $p < 0.05$). Data is represented as the mean $\pm$ one standard deviation.

Figure 4. Dectin-1 contributes to pro-inflammatory mediator production

Panels A (IL-6) and B (PGE₂) depict the impact of laminarin treatment on pro-inflammatory mediator production in response to zymosan. A single asterisk denotes a significant reduction in pro-inflammatory mediator released between zymosan alone and zymosan with laminarin pre-treatment, while two asterisks denote a difference between vestibular and vulvar strains. Paired *t*-tests (zymosan versus zymosan + laminarin, vestibular versus vulvar) were used to determine significance ($n = 8$, $p < 0.05$). Panel C demonstrates that siRNA against CLEC7A (encoding Dectin-1) is effective in reducing the amount of Dectin-1 protein in total protein lysates compared to treatment control siRNA. Panels D (IL-6) and E (PGE₂) depict the impact of siRNA treatment on pro-inflammatory mediator release; asterisk designations are the same as for panels A and B. Data is represented as the mean $\pm$ one standard deviation. Inhibiting the function of Dectin-1 results in a reduction in the amount of pro-inflammatory mediator released by fibroblasts treated with zymosan.

Figure 5. Zymosan treatment activates the NFκB pathway

This figure depicts a typical result for a representative LPV case (more than one strain was tested). Panel A shows the relative DNA binding activity of NFκB subunits (p65, p50, c-Rel, and p52) detected in the nucleus. RelB was also detected, but the binding activity did not exceed background. Zymosan treatment induces the activity of p65 and p50, which is also higher in vestibular versus vulvar strains, while c-Rel and p52 activity may decline somewhat with zymosan treatment. Panel B depicts Western blotting analysis for p65; zymosan treatment induces translocation of p65 to the nucleus, while translocation may be slightly accentuated in vestibular versus vulvar strains following zymosan treatment. The sensation of zymosan results in the activation of the NFκB pathway, likely through the canonical arm of the pathway, evidenced by specific translocation/activation of the p50 and p65 subunits.

Figure 6. Infection with live yeast activates the NFκB pathway

This figure depicts luciferase activity of an NFκB reporter normalized to constitutive renilla luciferase expression for patient strains infected with *C. albicans* and *S. cerevisiae*. A single asterisk denotes a significant difference between the vehicle control and *C. albicans*-infected cells, while two asterisks denote a significant difference between *C. albicans*- and *S. cerevisiae*-infected cells. Significance was determined via paired *t*-tests (vehicle versus *C. albicans*-infected, *C. albicans*-infected versus *S. cerevisiae*-infected; $n = 8$, $p < 0.05$). Data is represented as the mean $\pm$ one standard deviation. Although not denoted by a symbol, activity was also induced by *S. cerevisiae* compared to vehicle control ($p < 0.05$ for vestibular and for vulvar cells). Clearly, live yeast infection induces the activation of the NFκB pathway, while *C. albicans* infection is particularly potent, and the response is generally greater in pain-associated vestibular cells.

Figure 7. Activation of the NFκB pathway is essential for pro-inflammatory mediator production Panels A (IL-6) and B (PGE₂) show that inhibition of the NFκB pathway with the BAY-11-7082 inhibitor results in a dramatic and significant reduction in the amount of pro-inflammatory mediators released in response to treatment with zymosan. A single asterisk denotes a significant difference between zymosan alone and zymosan with BAY-11-7082. Significance was determined by paired *t*-test (zymosan alone versus zymosan + BAY-11-7082; *n* = 8, *p* < 0.05). Data is represented as the mean ± one standard deviation. Inhibition of the NFκB pathway reduces pro-inflammatory mediators to near background levels.