Published in final edited form as:

J Low Genit Tract Dis. 2018 January ; 22(1): 52–57. doi:10.1097/LGT.0000000000000364.

Toll-like receptor signaling contributes to proinflammatory mediator production in localized provoked vulvodynia

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Abstract

Objectives—Localized provoked vulvodynia (LPV) afflicts ~ 8% of women in the USA and represents a huge financial, physical, and psychological burden. Women with LPV experience intense pain localized to the vulvar vestibule (area immediately surrounding vaginal opening). We have identified mechanisms involved in the development of LPV whereby vulvar fibroblasts respond to proinflammatory stimuli to perpetuate an inflammatory response that causes pain. However, these mechanisms are not fully elucidated. Therefore, we explored the role of Toll-like receptors (TLR), a class of innate immune receptors that rapidly respond to microbial assaults.

Methods—To determine if/which TLRs are expressed by vulvar fibroblasts and whether these contribute to proinflammatory mediator production and pain in LPV, we examined TLR expression and innate immune responses in fibroblasts derived from painful vestibular regions compared to non-painful external vulvar regions.

Results—Human vulvar fibroblasts express functional TLRs that trigger production of inflammatory mediators associated with chronic pain. We focused on the TLR-7-imiquimod proinflammatory interaction, because imiquimod, a ligand of TLR-7, may exacerbate pain in women during treatment of human papilloma virus (HPV) - associated disease.

Conclusions—Human vulvar fibroblasts express a broad spectrum of TLRs (a new finding). A significantly higher TLR-mediated proinflammatory response was observed in LPV case vestibular fibroblasts, and with respect to the imiquimod-TLR7 interaction, development of chronic vestibular pain and inflammation may be a possible sequelae of treatment of vulvar HPV-associated disease. Suppressing enhanced TLR-associated innate immune responses to a spectrum of pathogen-associated molecular patterns (PAMPs) may represent a new/effective therapeutic approach for vulvodynia.

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The authors have no conflict of interest.

All subjects provided informed consent, and the research was approved by the University of Rochester Institutional Review Board (RSRB # 42136).

Keywords

imiquimod; fibroblast; inflammation; interleukin-6; toll-like receptor; vulvodynia

INTRODUCTION

Vulvodynia is a poorly understood, common, chronic, and debilitating condition that afflicts women worldwide^{1–3}. Women with localized provoked vulvodynia (LPV) experience intense pain concentrated within the vulvar vestibule (area immediately surrounding vaginal opening) in response to light touching, clinically known as allodynia¹. Despite experiencing severe and long-lasting pain, LPV patients exhibit no clinically visible signs of disease (no scarring or lesions), making it challenging to identify the underlying cause of disease^{1, 3}. Therefore, currently available therapeutic approaches only manage the outward symptoms of LPV and do not address the underlying origins of pain.

We discovered that fibroblasts isolated from sites of allodynia-type pain in LPV patients are inherently highly sensitive to proinflammatory stimuli (e.g. live yeast, zymosan), producing high levels of proinflammatory mediators ((e.g. interleukin-6 (IL-6) and prostaglandin E₂ (PGE₂)) linked to pain^{4–6}. Furthermore, the levels of fibroblast proinflammatory mediator production accurately predict pain thresholds to mechanical stimuli; the highest amounts of mediators are produced by fibroblasts isolated from the most intense pain threshold-confirmed sites⁵. Although the magnitude of the inflammatory response is greatest in LPV patients, the vestibule appears to have an inherent proclivity to inflammation, even in healthy women^{4, 5}. We have subsequently identified receptors (e.g. Bradykinin Receptors 1 and 2 and Dectin-1) that contribute to this maladaptive inflammation response^{4, 7}. However, inactivating one or more of these receptors does not completely disrupt proinflammatory mediator production, pointing to the existence of other co-existing receptors/mechanisms^{4, 7}.

Toll-like receptors, an important class of pattern recognition receptors (PRRs), are quickly responsive to microbes and play a central role in initiating immune responses to a variety of bacterial, viral, and fungal stimuli^{8–11}. Ten TLRs are expressed in humans, each of which recognizes pathogen-associated molecular patterns (PAMPs) associated with specific harmful stimuli^{8, 9, 12–15}. TLR signaling results in the production of proinflammatory, pain-associated mediators (e.g. IL-6 and PGE₂), among others, that initiate both innate and adaptive immunity and recruit immune cells to sites of infection^{8, 9, 12–15}. However, excessive/inappropriate proinflammatory mediator production can lead to chronic, painful disease states^{9–11}, including rheumatoid arthritis, gout, and systemic lupus erythematosus¹⁰. Because TLRs play a central role in the immune response and may promote chronic pain conditions, we became interested in TLR signaling in the context of LPV^{8–11}. Prior to our work it was not known if vulvar fibroblasts even express TLRs. Here, we present the first evidence that human vulvar fibroblasts do indeed express TLRs, which play a role in vulvar inflammation and pain.

MATERIALS AND METHODS

Patient/Sample selection and fibroblast strains

LPV-afflicted cases (fulfilling Friedrich's Criteria¹⁶) and age/race-matched pain-free controls were recruited by the Division of General Obstetrics and Gynecology at the University of Rochester between December 2012 and February 2014. Selection and sampling procedures were previously published^{5, 6}. All subjects provided written informed consent, and the research was approved by the University of Rochester Institutional Review Board (RSRB # 42136). Primary fibroblast "strains" from individual patients were cultured as previously described⁴⁻⁶.

TLR messenger RNA (mRNA) expression

TLR1-9 mRNA expression was evaluated in three LPV case and two paired control fibroblast strains using reverse transcriptase quantitative PCR (RT-qPCR)^{4, 7}. TLR expression was determined using previously published methods¹⁷⁻²⁰. Messenger RNA expression of TLR-3, TLR-7, and TLR-9 was also evaluated in whole tissue biopsies of 3 LPV cases, collected as previously described^{5, 21}. TLR messenger RNAs levels were compared by anatomic location, within subjects, and by case/control status between subjects.

TLR activity

Paired strains from a prototypical case⁵ were challenged with stimuli that activate the full range of human TLRs using the Human TLR1-9 Agonist Kit (InvivoGen USA, San Diego, CA). IL-6 was analyzed by ELISA^{4, 7}; IL-6 is a measure of TLR pro-inflammatory activity^{8, 9, 12-15}.

TLR-7 protein expression

Protein levels of TLR-7 were measured by Western blotting in three paired case strains^{4, 7} using a primary antibody specific for TLR-7 (GeneTex Inc., Irvine, CA). TLR-7 expression levels were quantified by densitometric analysis and normalized to a control protein (β -tubulin; Cell Signaling Technology, Danvers, MA).

Response of vulvar fibroblasts to live human immunodeficiency virus exposure

Human fibroblasts were treated with live HIV virus (50 or 200 ng of p24), and after 3 and 7 days, cells and supernatants were tested for p24 protein by ELISA to assess HIV-1 particle release. To determine intracellular levels of p24 expression, cells were harvested and analyzed by flow cytometry. TLR-7 mRNA and IL-6 levels were quantified for cells exposed to live HIV.

TLR-7 knockdown

IL-6 levels were compared between untreated (normal) cells and those in which TLR-7 expression was inhibited with a small-interfering RNA (siRNA). A siRNA against human TLR-7 was transfected into fibroblasts using Lipofectamine 2000 (ThermoFisher). Cells were then treated with imiquimod, and IL-6 levels were determined.

Statistical analysis

GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA) was used to conduct the statistical analysis. ANOVA with a Tukey post-test was used to determine statistical significance, set at $P = 0.05$. The data are presented as the mean $\pm$ standard error.

Role of the Funding Source

This work was funded by NIH-NICHD R01 HD069313.

RESULTS

TLRs are expressed in human vestibular and external vulvar fibroblasts

We examined the mRNA expression profiles of TLRs 1–9 in 3 paired case and 2 paired control strains (vestibular versus external vulvar fibroblasts). All nine TLR mRNAs were expressed in all case and control fibroblast strains, irrespective of anatomic location of sampling, but the relative mRNA abundance of TLR 1–9 was consistently higher in cases than in controls (Figure 1). TLRs 1, 2, 4, 7, 8, and 9 were significantly more highly expressed in cases than controls ($P = 0.05$), while TLR-3 and TLR-6 levels were elevated in cases, but these latter differences did not achieve statistical significance ($P = 0.1$). There were no significant differences in TLR expression in the vestibule versus external vulva. However, in many instances, patient vestibular fibroblasts expressed slightly elevated levels of TLRs, specifically TLRs 1, 2, 3, 7, and 9. These findings suggest that TLR expression is up-regulated in cases at sites of pain and may be an important contributor to etiopathogenesis of vulvodynia.

LPV fibroblasts respond to TLR-specific stimuli

We tested the ability of vulvar fibroblasts to respond to a spectrum of TLR-specific stimuli in pain-associated vestibular and pain-free external vulvar fibroblasts obtained from an LPV case. IL-6 production was used as a readout of the TLR-specific proinflammatory response, since IL-6 is made when TLR signaling is turned on^{9–11}. We found that both fibroblast strains responded to all nine TLR stimuli, but vestibular fibroblasts consistently produced higher amounts of IL-6 than external vulvar fibroblasts (Figure 2). Heightened IL-6 production in vestibular fibroblasts likely reflects an inherent sensitivity of the vulvar vestibule to a wide spectrum of proinflammatory stimuli, which has been demonstrated for other activators (e.g. zymosan, a yeast cell wall extract) and reflects LPV case pain profiles^{4–7}. High molecular weight (HMW) Poly(I:C), a viral nucleic acid mimetic, emerged as the most potent inducer of IL-6 production for both vestibular and external vulvar fibroblasts. TLR-3 is one of several TLRs involved in recognizing foreign nucleic acids, including TLR-7 and TLR-9^{8, 15}. TLR-7 and TLR-9 were also more highly expressed in cases at sites of pain (Figure 1). Therefore, we elected to further study these three TLRs and their potential contribution to proinflammatory signaling in LPV.

Endosomal, nucleic acid responsive TLRs are expressed in human vulvar tissue and expression is up-regulated by their cognate ligand

TLR-3, 7, and 9 were expressed in vulvar tissue, albeit in similar levels by anatomic site (Figure 3A). We went on to examine whether exposure to their cognate ligands would alter TLR 3, 7, or 9 mRNA expression. We found that mRNA expression of all three receptors was up-regulated when each was exposed to the appropriate ligand ((Poly(I:C) for TLR-3, imiquimod for TLR-7, and ODN2006 for TLR-9)) (Figure 3B). Furthermore, TLR-7 expression was more highly induced in vestibular versus vulvar fibroblasts (P 0.05), indicating that expression of TLR-7 is highest in areas of pain.

Vulvar fibroblasts express TLR-7 protein

TLR-7 is of particular clinical interest because TLR-7 is well recognized to interact with imiquimod, a common therapeutic used clinically to treat genital warts caused by the human papilloma virus (HPV)^{22, 23}. Vulvar treatment with imiquimod may play a role in eliciting a painful, localized, inflammatory response, particularly with treatment focused on vestibular tissue, potentially promoting a vulvodynia condition. TLR-7 protein expression, examined via Western blotting, was elevated in cases versus controls, with the highest levels of expression occurring in the most painful areas (vestibular fibroblasts taken from cases; Figure 4). This finding agrees with the elevated mRNA expression of TLR-7 in vestibular fibroblasts shown in Figures 1 and 3.

TLR-7 is involved in the response to sexually transmitted pathogens (HIV)

To further study the possible functional roles of TLR-7 in LPV fibroblasts, we examined fibroblast responses to incubation with live human immunodeficiency virus (HIV), which is also recognized by TLR-7²⁴. We first examined whether HIV could infect and replicate within human vulvar fibroblasts. We found no evidence that HIV can invade or replicate within human vulvar fibroblasts (data not shown). However, recent evidence suggests that other cell types not directly infected by HIV (e.g. fibroblasts) can sense and respond to the presence of virus, often enhancing the ability of the virus to infect target cells²⁵. Therefore, we examined the influence of viral exposure on TLR-7 expression; we found that HIV exposure increased TLR-7 mRNA levels in both vestibular and external vulvar fibroblasts after 3 and 7 days of incubation (Figure 5A). To determine if viral exposure induced a proinflammatory response, we monitored IL-6 output and found that levels increased with HIV exposure, especially in vulvodynia-associated vestibular fibroblasts (Figure 5B). Overall, TLR-7 appears to play an important role in the response to pathogenic threats to the vulva.

TLR-7 signaling contributes to IL-6 production

To evaluate whether TLR-7 signaling is directly involved in the production of IL-6, we knocked down TLR-7 mRNA expression using siRNA in fibroblasts from three different LPV cases. Although siRNA knockdown of TLR-7 did not ablate IL-6 production, there was a significant decrease in the amount of IL-6 produced by both vestibular and external vulvar fibroblasts (Figure 6; representative case). Consistent with the relative sensitivity of

fibroblast strains to proinflammatory stimuli, pain-associated vestibular fibroblasts produced greater amounts of IL-6 than their external vulvar counterparts.

DISCUSSION

Although vulvodynia is influenced by one or more underlying etiologies whose origins are incompletely understood¹, recent evidence strongly suggests dysregulated proinflammatory signaling plays a critical role in pain generation^{4-6, 26-28}. Commonly prescribed medical treatments may manage the symptoms of pain and psychological distress but are of unproven efficacy and do not address the underlying causes of disease, which remain unknown^{1, 3, 29, 30}. Therefore, a better understanding of the inflammatory mechanisms that contribute vulvodynia should translate to the development of effective treatments that will target the true causes of disease.

Using IL-6 as a marker of pro-inflammatory pain, we found that human vulvar fibroblasts express all nine human TLRs tested, which are involved in the innate immune response to a wide spectrum of microbiological and environmental PAMPs. Interestingly, the strongest IL-6 response occurred with the TLR-3 ligand, Poly(I:C). This was at first surprising, because Poly(I:C) is a viral nucleic acid mimetic. To date, the only microbial stimulus consistently correlated with LPV onset has been vulvovaginal yeast infection³¹ caused by *Candida albicans*^{8, 32}, a common vulvovaginal yeast pathogen³³⁻³⁵. In addition to patterns expressed directly on the fungi themselves, nucleic acids released by *C. albicans* during infection can additionally interact with TLR-3 to elicit a proinflammatory response³².

At the same time, the presence of numerous active TLRs in LPV fibroblasts suggests that these cells are poised to recognize a wide array of proinflammatory stimuli found in the everyday environment, including bacteria and viruses. A majority of patients (greater than 70%) report the occurrence of chronic or recurrent yeast infections prior to LPV onset³¹. However, because most yeast infections are self-diagnosed and treated with over-the-counter medications³⁶, it is plausible that a significant number of these cases could represent other PAMP-associated inflammatory responses.

TLR-7 was of particular interest because of the TLR-7-imiquimod connection. Our results suggest that imiquimod, although designed to stimulate an inflammatory response that helps to clear viral HPV-associated lesions^{22, 23}, may be accompanied by an increased LPV risk, as it could stimulate or exacerbate an overactive/painful inflammatory response, characterized by the elevated IL-6 levels. Not only is TLR-7 directly involved in the production of IL-6 in response to imiquimod and infectious agents, but it tends to be more highly expressed in cases at sites of pain, suggesting that it plays an important role in LPV. It is unclear at this time whether HPV, per se, may have an influence on TLR-7 or other pattern recognition receptors (PRRs), particularly with respect to vulvodynia risk.

We propose that increased abundance/activity of numerous PRRs renders vestibular fibroblasts inherently sensitive to proinflammatory stimuli of a wide spectrum of fungal, viral, bacterial and other environmental PAMPs. Targeting a single TLR does not completely ablate proinflammatory mediator production, for example knocking down TLR-7 mRNA

with siRNA reduced but did not abrogate IL-6 production. Based on this observation, we suspect that the coordinate activity of many PRRs, including the 9 TLRs examined here, leads to elevated proinflammatory mediator production based on redundancy of a pro-inflammatory, vestibular-focused, innate immune response. Therefore, targeting one, or even a few of these receptors, is unlikely to restore LPV patient proinflammatory mediator levels to a healthy baseline. These findings are in agreement with our previous studies implicating additional receptors (Dectin-1 and BDKRB1/2) in vulvovaginal proinflammatory responses and vulvar pain^{4, 7}. Therefore, additional research is necessary to fully elucidate these pathways and design therapeutics that would target these pathways as a whole, without compromising the vulva's requisite ability to respond to infectious threats and other inflammatory insults.

Acknowledgments

Work funded by NIH-NICHD R01 HD069313.

ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
BDKRB1/2	Bradykinin receptor 1 and 2
<i>C. albicans</i>	<i>Candida albicans</i>
ELISA	Enzyme-linked immunosorbent assay
HIV	Human immunodeficiency virus
HMW	High molecular weight
HPV	Human papilloma virus
IL-6	Interleukin-6
LPV	Localized provoked vulvodynia
mRNA	messenger ribonucleic acid
NFκB	Nuclear factor kappa B
PGE₂	Prostaglandin E ₂
Poly(I:C)	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor
siRNA	Small-interfering ribonucleic acid
TLR	Toll-like receptor

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