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Inflammation, lipid dysregulation, and transient receptor potential cation channel subfamily V member 4 signaling perpetuate chronic vulvar pain

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Abstract

Localized provoked vulvodynia is characterized by chronic vulvar pain that disrupts every aspect of the patient's life. Pain is localized to the vulvar vestibule, a specialized ring of tissue immediately surrounding the vaginal opening involved in immune defense. In this article, we show inflammation is the critical first step necessary for the generation of pain signals in the vulva. Inflammatory stimuli alone or combined with the transient receptor potential cation channel subfamily V member 4 (TRPV4) agonist 4α-phorbol 12,13-didecanoate stimulate calcium flux into vulvar fibroblast cells. Activity is blocked by the TRPV4 antagonist HC067047, denoting specificity to TRPV4. Using lipidomics, we found pro-resolving lipids in the vulvar vestibule were dysregulated, characterized by a reduction in pro-resolving mediators and heightened production of inflammatory mediators. We demonstrate specialized pro-resolving mediators represent a potential new therapy for vulvar pain, acting on 2 key parts of the disease mechanism by limiting inflammation and acutely inhibiting TRPV4 signaling.

1. Introduction

Inflammation is an essential process by which the body responds to harmful external stimuli.[18,19,25,27] Yet, there is a delicate balance between inflammation and its resolution; defects in these processes can lead to chronic inflammation and pain.[4,6,21,67,87,88] Pain is one of the most common medical complaints worldwide, affecting ~20% of US adults.[114] Pain therapies are either ineffective or rely on opioids that create dependence and require dose escalation to maintain their effects.[10,26,64,91,105]

In localized provoked vulvodynia (LPV), patients experience intense pain upon touch to the area surrounding the vaginal opening, known as the vulvar vestibule.[30–34,39,41] Sites only a few millimeters away are comparatively pain-free. Pain with non-painful stimuli, or allodynia, is a common form of neuropathic pain.[50] Vulvodynia is a complex pain syndrome that involves other neuropathic pain features, such as central sensitization, although the origins of LPV remain incompletely understood.[47,65,83,86,108] Studying vulvodynia affords the unique opportunity to examine adjacent affected and unaffected sites within the same subject, allowing each patient to serve as their own control. Sadly, LPV destroys the lives of ~8% to 12% of US women.[29,57,80,84,93,111]

Inflammatory stimuli cause heightened inflammation in the vestibule, which is the result of increased expression of nearly a dozen different pattern recognition receptors.[30–34,39] This is an extreme exaggeration of a normal response; the vestibule of control patients is also more sensitive than the adjacent external vulvar tissues, albeit to much less extent.[30–33] In this article, we show a defect in the production of pro-resolving lipids responsible for hastening the return to homeostasis, which may explain why the resolution machinery fails to ablate inflammation.

We found imbalances in pro-resolving lipids and exaggerated responses to inflammatory stimuli in the vulvar vestibule foster activation of the transient receptor potential cation channel subfamily V member 4 (TRPV4). TRPV4 is a member of a family of calcium channels involved in conducting pain signals. TRPVs are ubiquitous, expressed in both neuronal and nonsensory cells, and are implicated in numerous pain conditions, such as peripheral neuropathy, and have been the subject of recent clinical trials to combat pain.[9,63,66,69,82] TRPV4 specifically is implicated in mechanical allodynia (pain with touch)[9,66,69] and

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mechanical hyperalgesia (heightened pain response),[2,3,79] which fits the symptoms of vulvodynia.[30]

Clinical studies have shown the vestibule of patients with LPV can be hyperinnervated, commonly referred to as neuroproliferative vulvodynia.[42,103,112] Patients with neuroproliferative vulvodynia may experience a temporary pain abatement with anesthetic blocks of the pudendal nerve, which innervates the vestibule.[20] Protein gene product 9.5 positive nerve fibers in the vestibule of patients with LPV express elevated levels of vanilloid receptor 1 (VR1), which recognize TRPV1.[102] TRPV1 signaling has been previously implicated in the vulvodynia mechanism, but definitive evidence for targeting TRPVs in vulvodynia has not been demonstrated until now.[7,54,75,102]

By targeting inflammation and TRPV4 signaling simultaneously with the specialized pro-resolving mediator, maresin 1, we can reduce surrogate measures of pain in fibroblasts and quantifiable sensitivity in mice.[34] Maresin 1 is a lipid mediator naturally produced from the dietary polyunsaturated fatty acid docosahexaenoic acid (DHA), which helps to quell inflammation in part by influencing macrophage and neutrophil behavior.[18,19,22,28,89,109] Maresin 1 stops TRPV4 signaling on the order of minutes while compensating for deficits in pro-resolving mediators. Specialized pro-resolving mediators (SPMs) are naturally produced, safe, and will likely lead to faster translation for pain reduction. In this article, we provide evidence that both synthesized drug and natural products represent highly successful and safe treatments for chronic pain that will extend beyond the vulva.

2. Material and methods

2.1. Patient/sample selection

Localized provoked vulvodynia–afflicted cases (fulfilling Friedrich criteria) 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 May 2022. Subjects recruited between December 2012 and November 2015 were labeled as LPV 001-LPV 039, whereas subjects recruited between March 2019 and May 2022 were numbered as LPV 201-LPV 230. 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.[41] In brief, all subjects denied the use of corticosteroids and nonsteroidal anti-inflammatory medications and had no chronic inflammatory illnesses other than LPV. Before biopsy of the vestibular and external vulvar sites, sampling sites underwent Wagner mechanical algometry. We used a method-of-limits technique for vulvodynia mechanical pain threshold initially described by Zolnoun et al.[117] and replicated in our earlier publication.[39] Using the Wagner algometer, an increasing 0.5 N per second force (range 0-5 N) was applied perpendicular to the mucocutaneous surface by a moistened Dacron-tipped swab affixed to the Wagner algometer. Force was terminated at the point of pain development (subject signaled by hand-held clicker) or when the mucocutaneous force reached 5 N. Algometer-site tissue was sampled and used to create fibroblast strains as previously described.[41] A total of 21 cases and 16 controls were consented, including fibroblast stains and tissue samples (see Table S1 and Fig. S1 for demographic information, available as supplemental digital content at http://links.lww.com/PAIN/B937). A mixture of strains and tissues were used for each experiment as appropriate for the experimental design; no fewer than 2 strains were used for each experiment. Any experiments identifying differences between case and control used at minimum 3 case and 3 control strains, whereas most experiments used more.

2.2. Fibroblast culture

Previously established primary fibroblast strains (each obtained from a different patient or healthy control) were cultured in minimal essential medium (MEM) supplemented with 10% FBS, GlutaMAX, gentamicin, and antibiotic/antimycotic solution (Gibco/Invitrogen/Thermo Fisher Scientific, Grand Island, NY). Early passage (4-10) external vulvar and vestibular fibroblast strains were seeded at 2.5×10^4 cells/cm^2. In brief, to establish fibroblast strains, tissues were minced and placed under coverslips and grown in minimal media until fibroblasts emerged. The use of minimal media substantially reduces the chances of epithelial cell contamination, and fibroblast cellular identity was confirmed by microscopic inspection and with fibroblast-specific markers (eg, vimentin and collagen) using flow cytometry. At the same time, the cells were confirmed to be negative for epithelial cell markers (eg, cytokeratin), smooth muscle and myofibroblast markers (eg, α-smooth muscle actin), endothelial cell markers (eg, CD34), and bone marrow–derived cell markers (eg, CD45). These markers remain constant across passages, and the cells consistently respond to stimuli throughout culture.

2.3. RNA extraction, cDNA synthesis, and reverse transcriptase quantitative PCR

RNA was extracted from vulvar tissue and fibroblast cells for qPCR analyses. Tissue samples were processed into 3 pieces, and 1 piece was stabilized in QIAzol Lysis Reagent (Qiagen, Carlsbad, CA) for RNA extraction. See experimental design Fig S2, available as supplemental digital content at http://links.lww.com/PAIN/B937. Total mRNA was extracted using the Qiagen RNeasy kit following the manufacturer's instructions, including the optional QIAshredder step for tissue homogenization and the on-column DNase I digest step (Qiagen Corp). Fibroblast cells were first grown to confluence in 6-well plates in MEM supplemented with 10% FBS, GlutaMAX, gentamicin, and antibiotic/antimycotic solution (Thermo Fisher). Media was then replaced with QIAzol Lysis Reagent, and RNA was extracted from each well individually to generate triplicate RNA samples for each strain. For both patient tissue samples and fibroblast strains, RNA was quantified by spectrophotometric analysis using a NanoDrop One spectrophotometer (Thermo Fisher), and the quality of RNA was checked through gel electrophoresis. RNA that had a 260/280 ratio of around 2.0 and distinct 28S and 18S rRNA bands on the agarose gel were considered pure and were used for cDNA synthesis. RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA). Each reaction contained 300 ng of RNA template diluted in 15 μL of in RNase-free molecular grade water (Qiagen), 1 μL of iScript reverse transcriptase, and 4 μL of 5x iScript reaction mix buffer. Negative reverse transcriptase controls that contained no iScript reverse transcriptase were also prepared to confirm the absence of DNA contamination. After amplification, cDNA samples were diluted 5-fold in RNase-free molecular grade water (Qiagen) and used as templates for qRT-PCR reactions (5 μL/reaction). TRPV mRNA expression was evaluated in 4 LPV case and 6 paired control fibroblast strains using reverse transcriptase quantitative PCR (RT-qPCR). Messenger RNA expression of TPRV4 was also evaluated in whole tissue biopsies of 17 LPV cases and 10 controls, collected as previously described. TRPV4 messenger RNA levels were compared by anatomic location, within subjects,

and by case/control status between subjects. Primer sequences are listed in Table S3, available as supplemental digital content at http://links.lww.com/PAIN/B937.

2.4. Small-interfering RNA knockdown

IL-6 and PGE_2 levels were compared between cells treated with control small-interfering RNA (siRNA) #1 (Silencer Select, Thermo Fisher) and those in which TRPV4 expression was inhibited with siRNA. An siRNA against human TRVP4 (Silencer Select, Thermo Fisher, assay ID s34003) was transfected into fibroblasts using Lipofectamine 2000 (Thermo Fisher). Cells were then treated with IL-1β, and IL-6 and PGE_2 levels were measured. The production of IL-6 was measured using a standard sandwich ELISA (BD Biosciences, Franklin Lakes, NJ), and competitive EIA assays were performed to measure PGE_2 production (Cayman Chemical Company, Ann Arbor, MI). Knockdown exceeded 90% confirmed by RT-qPCR.

2.5. IL-6 and PGE_2 response of vulvar fibroblasts to TRPV4 agonist and antagonist treatment

Cultures of fibroblast strains were seeded to 12-well tissue culture plates at roughly 50% confluence and were allowed to grow until confluent (~3-4 days) at 37°C and 5% CO_2 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-free media and incubated for 24 hours. Confluent fibroblast wells were then treated with various TRPV4 agonists and antagonists as well as vehicle control (see Table S4, available as supplemental digital content at http://links.lww.com/PAIN/B937). Cells incubated with treatments for 48 hours at 37°C and 5% CO_2. Supernatants were collected after the 48 hours incubation and used to measure the production of IL-6 and PGE_2. Standard sandwich ELISAs were used to measure the production of IL-6 (BD Biosciences, Franklin Lakes, NJ), and competitive EIA assays were used to measure the production of PGE_2 (Cayman Chemical Company).

2.6. Calcium imaging assays

Fibroblast cells were seeded at 20% to 40% confluence onto circular 15-mm coverslips that were previously coated with 10-μg/mL poly-d-lysine (Gibco, Thermo Fisher) for 30 minutes and subsequently rinsed with sterile water and allowed to dry. Once cells became confluent, they were pretreated overnight with 50-pg/mL IL-1β or vehicle control, then loaded with 100 μL of 2-μM Fura-2 AM (Molecular Probes, Thermo Fisher) in calcium-containing imaging buffer (137-mM NaCl, 9.6-mM HEPES, 5.5-mM Glucose, 0.56-mM $MgCl_2$, 4.7-mM KCl, 1.0-mM Na_2HPO_4, and 1.3-mM $CaCl_2$, pH 7.4) with 1% BSA for 30 to 45 minutes. Subsequent treatments were applied through superfusion, and calcium flux was monitored using a high temporal/spatial resolution on a TillPhotonics imaging/uncaging system consisting of a high-speed monochromator coupled to a Nikon TE 200 inverted microscope or Olympus IX71 equipped with DIC optics. Images were captured at rates up to 40 frames/sec by a 12-bit cooled digital camera. Analysis was performed using TillVision software. For high-throughput assays, cultures of fibroblast strains were seeded to tissue culture treated black 96-well assay plates with optical clear bottoms and treated for tissue culture at roughly 20% confluence and were allowed to grow until ~80% confluence was reached (~3-4 days) at 37°C and 5% CO_2 in MEM supplemented with 10% fetal bovine serum (FBS), GlutaMAX, gentamicin, and antibiotic/antimycotic solution (Thermo Fisher). Once cells reached optimal percentage of confluence, cells were given an overnight treatment of either vehicle control or an inflammatory stimulus (ie, IL-1β, 5(6)-EET, Poly(I:C), and 4αPDD). After overnight treatment, cells were incubated with a solution containing 2 to 4 μM of calcium indicator Fura-2 AM for 1 hour. Cells were washed 3 times with calcium-containing imaging buffer. Assay plates were then treated with either TRPV4 agonists or TRPV4 antagonists, and calcium activity was measured. Calcium activity was assessed by monitoring fluorescence on a FlexStation 3 microplate reader (Molecular Devices, San Jose, CA) by alternately exciting the Ca^{2+} indicator at 340 and 380 nm and collecting emission fluorescence at 510 nm.

2.7. Lipidomic analysis

Punch biopsies from the vestibule and external vulva of 11 cases and 10 controls were collected and tangentially sectioned into 3 pieces (see Fig. S2 for experimental design, available as supplemental digital content at http://links.lww.com/PAIN/B937). For lipidomic analysis, one piece from each site was flash frozen in an amber glass vial. Samples were then purged with argon gas and stored at −80°C until analysis. Quantitative targeted lipidomic analysis of the tissues was performed by the Wayne State University Lipidomics Core using liquid chromatography–mass spectrometry (LC-MS). Briefly, samples were prepared using C18 reverse phase cartridges (Phenomenex, StrataX SPE cartridge, 30-mg sorbent), and then subjected to reverse phase HPLC on a C18 column (Luna, C18, 3 μm, 2 mm × 150 mm, Phenomenex, CA) as published earlier.[61,62]

2.8. Localized provoked vulvodynia mouse model

All procedures involving mice were approved by the University of Rochester Committee on Animal Resources (UCAR protocol 2016-006). Zymosan, a proinflammatory yeast cell wall preparation (Millipore Sigma, St. Louis, MO), was used to induce sustained vulvar allodynia, measured by pain threshold testing as described previously.[34,36]

During the phase 1 of the experiment, fifty-two 8-week-old female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were acquired and trained to facilitate electronic von Frey determination of vulvar pain thresholds, as previously detailed.[34] Once the mice held relatively still in the testing apparatus upon introduction to the cage, baseline mechanical sensitivity threshold testing began. The mice were shaved, and tails were inked weekly. Four colors were used to distinguish each mouse in a cage; the same 4 colors were used for each cage, so mice from different cages could not be distinguished. A series of 3 individual threshold tests were conducted to determine the baseline threshold before zymosan injections commenced.

During threshold evaluation using the MouseMet electronic von Frey (TopCat Metrology, Ltd., Cambridge, United Kingdom), a cage of 4 mice was tested at once, alternating the mice stimulated, working from left to right and repeating the series until 5 values were collected for each mouse. The electronic von Frey device was gently rotated upwards at approximately 1 g/s, increasing the pressure, until the mouse stepped or jumped off the hair, at which point the peak force was automatically recorded (mechanical sensitivity threshold). The investigator was blinded to cage and mouse identity. Blinding was maintained by reading

cage barcodes into custom LabVIEW software for use with the MouseMet electronic von Frey device.

To be a valid test, all the following must be true: (1) The force increased ~1 g/s across within an acceptable range of 0.5 to 4 g/s, (2) the hair contacted the injection site, (3) the hair did not leave the injection site before mouse response, and (4) the hair and device arm were not depressed by anything other than contact with the mouse. With our custom software, the device automatically records the peak value when pressure on the hair is relieved (eg, the mouse steps off the hair), eliminating the need for the tester to make a determination of if/when the mouse reacts to the stimulus.

During the induction phase, the mice received 4-weekly injections to the midline posterior vulva under isoflurane anesthesia and underwent weekly threshold testing. Mice were determined to have allodynia if we observed a ≥33% drop in threshold (vs baseline threshold) for 2 consecutive weeks. Mechanical sensitivity threshold testing and injections occurred at the same day and time each week; thresholds were determined the day before injection each week. Mice were shaved weekly the day before testing. A final threshold was determined after the injection series had concluded, at which point mice that had at least 2 consecutive pain tests with a greater than 33% reduction in threshold were eligible for drug testing. Any mouse not developing allodynia, as defined by these criteria, remained in the analysis to serve as sentinels for any ill effects of treatment and to reduce testing bias.

For Lipinova testing, we tested a high and a low dose compared with mock (saline-moistened swab) and vehicle treatment. Each treatment group contained a total of 12 mice with allodynia. Mice were randomized into 4 groups: vehicle, mock, low, and high doses of Lipinova. Mice were held by the tail, and a pea-sized amount of cream was applied to the entire shaved area by rolling a sterile cotton–tipped swab over the area twice daily (morning and evening) Monday through Friday and once daily Saturday and Sunday. The treatments were gently rolled onto the entire shaved area for ~15 seconds each application; a small amount of treatment entered the vaginal opening during application. For mock treatment, a saline-moistened swab was used. Threshold testing was performed blinded each week as described.

A 1.9% (high) and 0.7% (low) topical formulation (cream) of Lipinova was prepared containing the following: 3% glyceryl monostearate, 3% cetyl alcohol, 2.5% polyoxyl-40, 3.5% isopropyl myristate, 4% white petrolatum, 1% benzyl alcohol, 0.5% vitamin E acetate, 15% neosorb 70/20 B, 65.5% purified water, and highly purified fish oil enriched for DHA and SPM precursors 18-HEPE, 17-HDHA, and 14-HDHA (Lipinova, Solutex GC, SL, Madrid, Spain; ~70% DHA by volume). To prepare the cream, the oil and water phases were heated to 70°C and combined by high-speed homogenization for 5 minutes and then mixed while cooling. Active phase ingredients were premixed with antioxidant vitamin E and preservative benzyl alcohol and added to the emulsion at 45°C. A vehicle cream containing all the elements, save Lipinova, was also prepared. Creams were prepared in a small batch under non-GMP conditions by the Ferndale Pharma Group (Detroit, MI).

2.9. Statistical analysis

Mixed-effect models were fit for each experiment. The technical replicates for each subject were random effects, whereas the treatment, location, and case differences were fixed effects. We tested for differences in the fixed effects and reported significant differences if the *P*-value for the difference was less than 0.05. The weekly threshold values for mice were compared between the 4 treatments in each week, separately. This was repeated for the percent change in threshold values from week 8. There were 15 to 20 mice measured repeatedly each week, and these values were displayed as boxplots against time within each treatment group. No outliers were removed, and all data points are displayed or presented wherever possible.

3. Results

3.1. TRPV4 is involved in inflammatory signaling in vulvodynia

Targeted lipidomic analysis was performed using mass spectrometry to detect metabolites of arachidonic acid (AA), eicosapentaenoic acid (EPA), and DHA involved in inflammation and resolution. Tissue biopsies were collected from vestibular (site of pain in patients) and nonpainful external vulvar sites in 11 LPV cases and 10 controls to identify alterations in lipid profiles. We identified several arachidonic acid–derived lipids, namely epoxyeicosatrienoic acids (EETs), that were deficient in painful areas (case vestibule), corresponding to reduced resolution capacity and ongoing inflammation and pain (**Fig. 1A**).[60,96] The full lipidomic data set may be found in Table S5, available as supplemental digital content at http://links.lww.com/PAIN/B937.

In an attempt to parse out whether reduced abundance was due to reduced production or enhanced degradation of EETs, we compared ratios of each active EET over its respective inactive dihydroxyeicosatrienoic acid (DHET) product. Unexpectedly, the only significant difference we found was an increase in the active pool of 5(6)-EET in the painful vestibule compared with the other 3 non-painful sites (**Fig. 1B**). Although 5(6)-EET is presumed to play a role in the resolution of inflammation, there is no empiric evidence to support this role.[60,96] Rather, 5(6)-EET has an established role in initiating TRPV4 signaling, which is associated with mechanical allodynia.[16,23,76,95,104,106,116,118] Mechanical allodynia is the key feature of LPV, which prompted further investigation of the TRPV4 pathway.[30]

We next examined the expression of TRPV4 mRNA in fibroblasts cultured from painful and nonpainful areas (**Fig. 1C**) and in whole tissue from the same sites (**Fig. 1D**). We found that expression of TRPV4 was significantly elevated in the painful vestibule of cases compared with nonpainful sites from the exterior vulva and the vestibule of controls. This was consistent across a survey of 21 cases and 16 controls (see Table S1 for demographic information, available as supplemental digital content at http://links.lww.com/PAIN/B937), and it mirrored the expression of pattern recognition receptors involved in inflammatory signaling from previous studies.[31–33] We also examined expression profiles for the remaining TRPVs; TRPV4 was the only one that showed enhanced expression in the case vestibule (Fig. S3, available as supplemental digital content at http://links.lww.com/PAIN/B937). Overall, the painful vestibule seems to express receptors involved in inflammation and mechanical pain signaling more robustly than nonpainful areas.[30–33]

To determine whether TRPV4 contributes to the inflammatory signaling associated with LPV,[30–34,39,41] we knocked down expression of TRPV4 using siRNA and then measured the amount of IL-6 and PGE_2 produced, which serve as surrogate measures of pain.[39] Knockdown of TRPV4 (confirmed through qRT-PCR) significantly decreased both PGE_2 and IL-6 levels (**Fig. 1E**). Reducing the levels of these mediators should at least partially ameliorate pain signaling,[30–34,39] suggesting TRPV4 represents another target for LPV pain therapy. Of advantage, targeting TRPV4

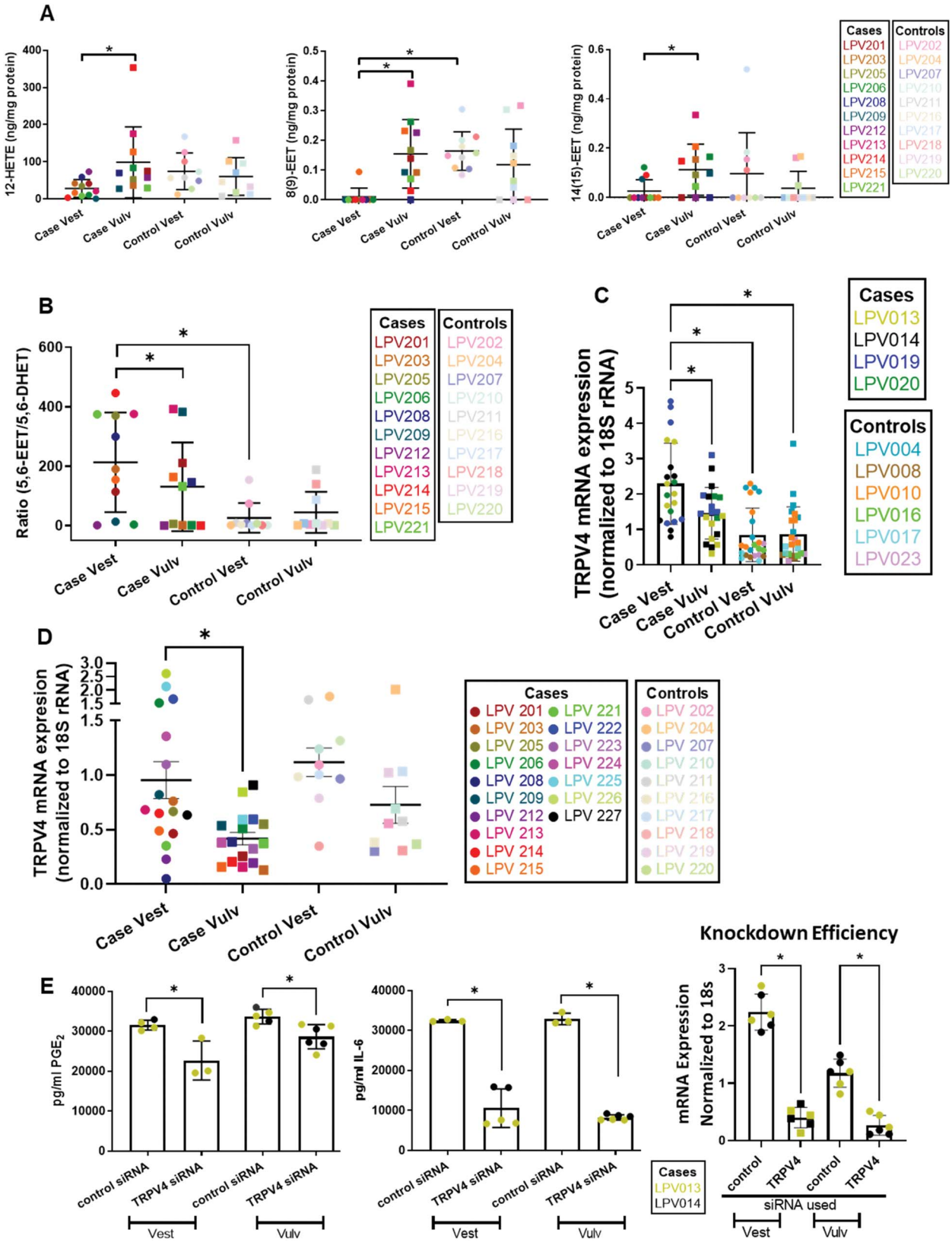

Figure 1. TRPV4 is connected to inflammatory signaling in LPV. Dots denote vestibular (vest) samples, squares denote external vulvar (vulv) samples, and each patient is numbered and color-coded for transparency and to illustrate technical replication. (A) Lipids found to be significantly reduced in painful vestibular tissue, all of which are derived from AA metabolism, n = 21, $^*P < 0.05$. (B) Lipid ratio of 5(6)-EET/5,6-DHET is elevated in the painful vestibule, n = 21, $^*P < 0.05$. (C) mRNA profiles of TRPV4 in fibroblast strains normalized to 18S rRNA show enhanced expression in the painful vestibule, n = 10, $^*P < 0.05$. (D) TRPV4 expression is also enhanced in mRNA from whole tissue biopsies, n = 27, $^*P < 0.05$. (E) siRNA knockdown with anti-TRPV4 siRNA (confirmed through quantitative reverse transcriptase polymerase chain reaction-PCR) vs control siRNA in fibroblasts shows decreases in prostaglandin E_2 (PGE_2) and interleukin-6 (IL-6) levels in culture supernatants in cells challenged with IL-1β, n ≥ 3, $^*P < 0.05$. AA, arachidonic acid; DHET, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; LPV, localized provoked vulvodynia; siRNA, small-interfering RNA.

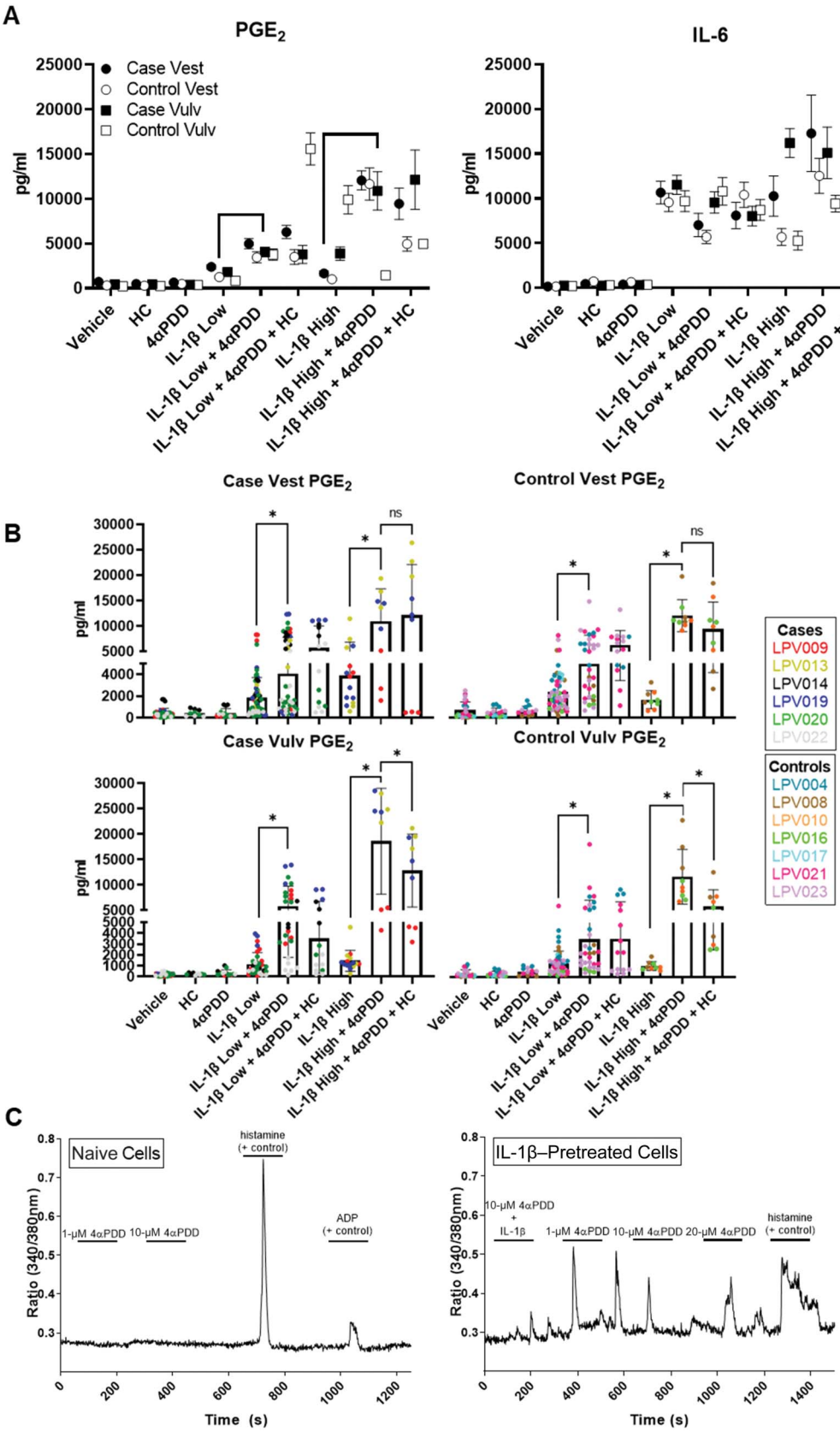

Figure 2. TRPV4 exacerbates inflammatory signaling. (A) Summary of all ELISA data (significance stars not displayed), showing 4alpha-phorbol 12,13-didecanoate (4αPDD) alone does not induce an inflammatory response. Treatment of 4αPDD in combination with interleukin-1beta (IL-1β) augments prostaglandin E_2 (PGE_2), but not interleukin-6 (IL-6) production; brackets denote areas where significant differences were suspected, which were further examined in panel (B). (B) enzyme-linked immunosorbent (ELISA) data showing significant inductions in PGE_2 production when 1-μM 4αPDD is added to IL-1β (50 pg/mL low; 100 pg/mL high), which is at least partially attenuated by treatment with TRPV4 antagonist 10-μM HC067047 (HC). Strains are color-coded; dots represent replicates, n = 13, $^*P < 0.05$. (C) Calcium imaging showing that IL-1β–pretreated cells respond to 4αPDD, whereas naive cells do not; increases in the 340/380 ratio reflect calcium influx into the cell indicative of TRPV4 activity. Drugs were applied by superfusion during the period indicated by the application bars.

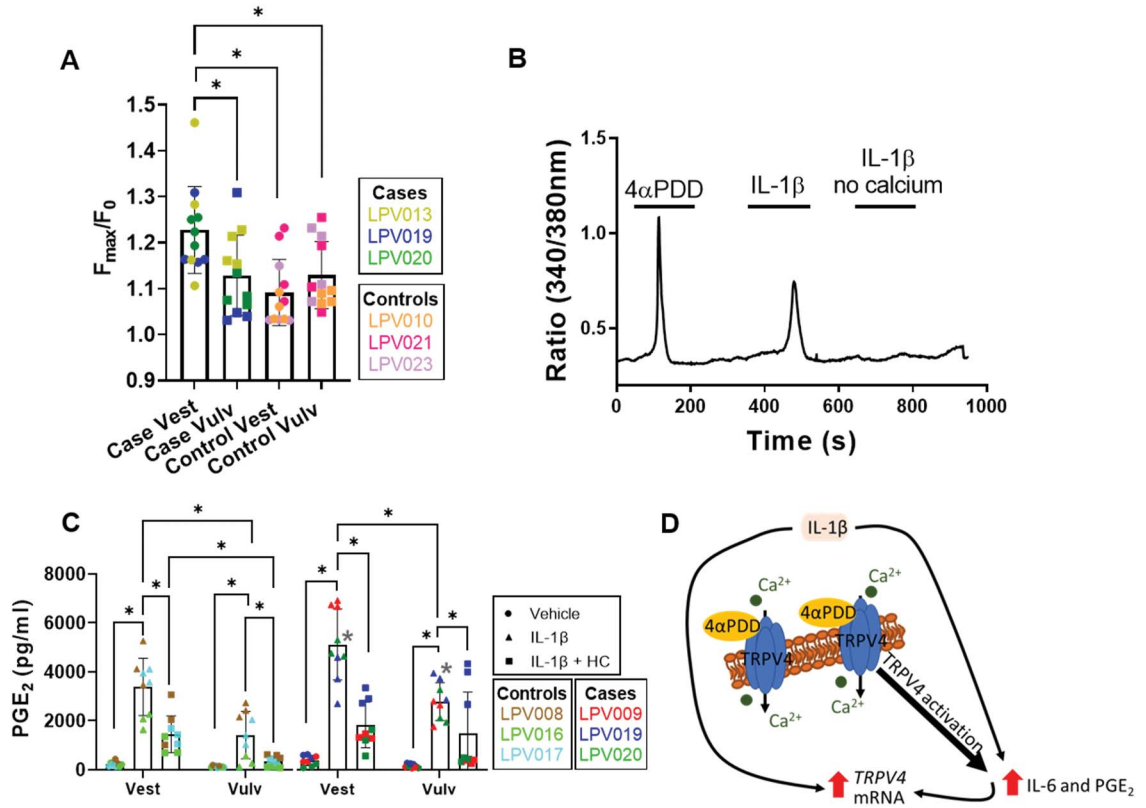

Figure 3. Inflammation is necessary and sufficient for TRPV4 activation. (A) TRPV4 activity in cells challenged with 1-μM 4alpha-phorbol 12,13-didecanoate (4αPDD) after overnight pretreatment with 50-pg/mL interleukin-1beta (IL-1β) shows highest activity measured by 340/380 ratio in the painful vestibule (vest), n = 6, $P < 0.05$. (B) Acute 50-pg/mL IL-1β treatment after overnight 50-pg/mL IL-1β pretreatment induces calcium flux, only in the presence of calcium. Drugs were applied by superfusion during the period indicated by the application bars. (C) 10-μM HC067047 attenuates the inflammatory response, n = 6, $^*P < 0.05$. Case vs control differences are denoted by a gray asterisk. (D) Feed-forward loop showing how inflammation and TRPV4 activation perpetuate one another.

poses minimal risk,[23,24,66,69,70] has no abuse potential, and may in fact reduce the potential for substance abuse.[51]

3.2. Activation of TRPV4 exacerbates inflammatory signaling in the context of ongoing inflammation

To better understand the relationship between inflammation and TRPV4 signaling, we treated cells with the TRPV4 selective agonist 4alpha-phorbol 12,13-didecanoate (4αPDD) and measured the production of pronociceptive proinflammatory mediators IL-6 and PGE_2 (**Fig. 2A**). Based on siRNA knockdown results (**Fig. 1E**), we hypothesized that activation of TRPV4 would raise IL-6 and PGE_2 levels. However, when we treated cells with 4αPDD alone, we saw no increase in IL-6 or PGE_2 over vehicle (**Figs. 2A and B**).

Therefore, we tested whether TRPV4 is active in human vulvar fibroblasts. We used calcium flux assays as a readout of TRPV4 activity. TRPV4 is a nonspecific cation channel that is permeable to calcium.[23,106] Measuring intracellular calcium is a convenient and sensitive measurement of calcium flux through the channel and thus its activity. Consistent with the IL-6 and PGE_2 results, 4αPDD treatment alone did not activate calcium flux (**Fig. 2C**). However, positive controls histamine and adenosine diphosphate (ADP) increased intracellular calcium, demonstrating these calcium channels are functional in vulvar fibroblasts.

Based on the current vulvodynia literature, inflammation is believed to play a key role in the disease mechanism and is present throughout the disease course; exogenous stimuli such as conserved pathogen-associated molecular patterns (PAMPs) or endogenous stimuli such as interleukin-1β (IL-1β) can stimulate this response.[1,8,13,30–34,39,40,58,59,78,97,101,115] Therefore, we evaluated the effect of 4αPDD on cells either pretreated or concurrently treated with the inflammatory stimulator IL-1β. In cells pretreated overnight with IL-1β, calcium signaling activity was apparent with all doses of 4αPDD, with or without an additional IL-1β treatment (**Fig. 2C**). TRPV4 mRNA expression more than triples in the presence of IL-1β, which may explain the enhancement in TRPV4 signaling (Fig. S4A, available as supplemental digital content at http://links.lww.com/PAIN/B937). Likewise, concurrent treatment with 4αPDD and IL-1β augmented the production of PGE_2 compared with cells treated with IL-1β alone (**Figs. 2A and B**). PGE_2 levels increased with increasing 4αPDD doses, provided IL-1β was also present (Fig. S4B, available as supplemental digital content at http://links.lww.com/PAIN/B937). Effects on IL-6 production were nominal (**Fig. 2A**). The vulvodynia mechanism seems to involve lipid dysbiosis, namely alterations in arachidonic acid (AA) metabolism. Fittingly, PGE_2 is AA-derived,[56,110] whereas IL-6 is gene-encoded.[44] In many cases, the TRPV4 selective antagonist HC067047 was able to reduce PGE_2 production (**Fig. 2B**), suggesting TRPV4 inhibition could be a useful strategy for reducing inflammation.

3.3. TRPV4 activity is enhanced in the painful vestibule, and inflammation is both necessary and sufficient to activate TRPV4 signaling

We used a FlexStation3 to examine TRPV4 signaling simultaneously in several case and control strains from the vestibule and external vulva. We found that TRPV4 activity, initiated by treating IL-1β–primed cells with 4αPDD, was significantly higher in the

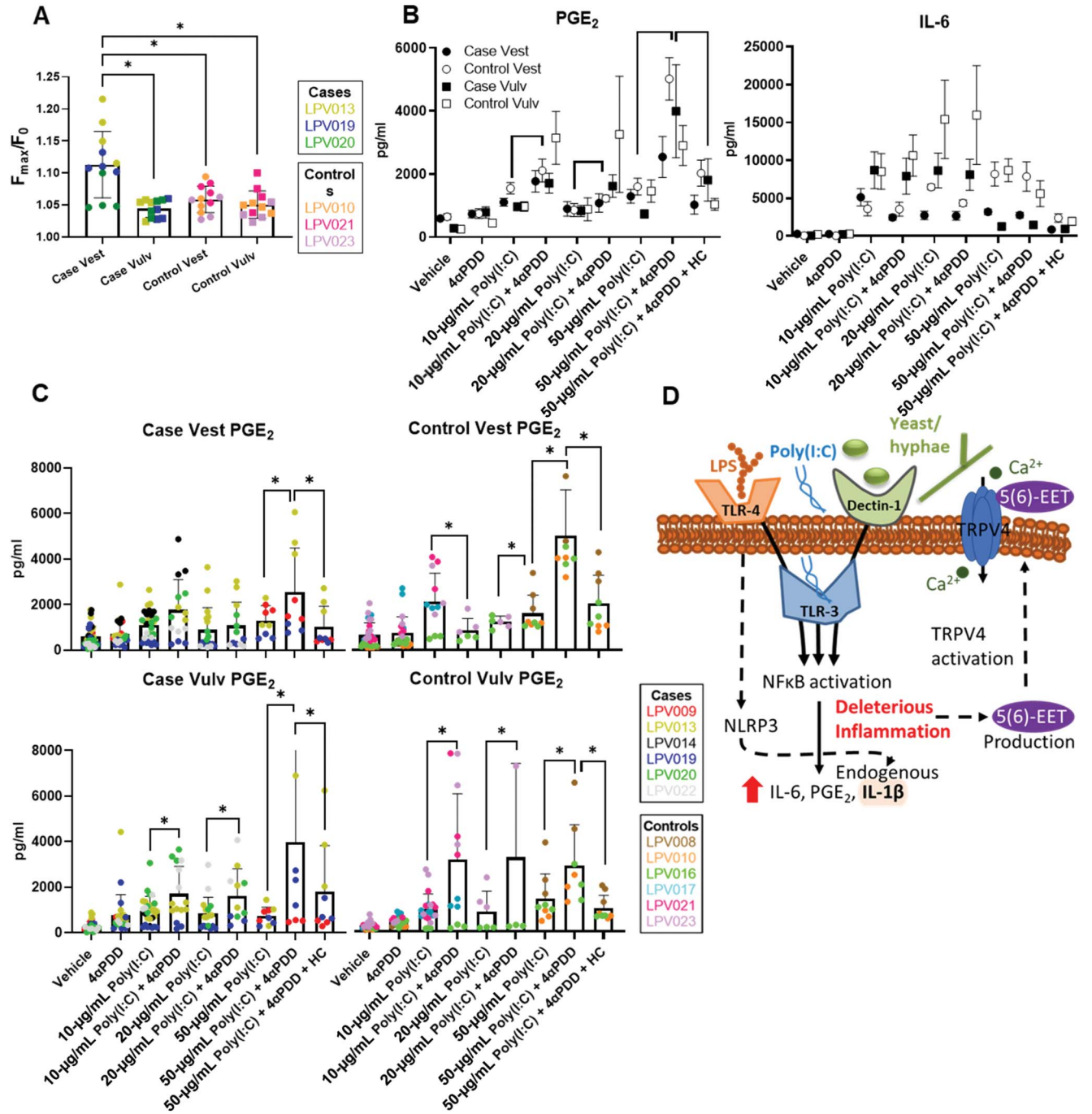

Figure 4. Exogenous stimuli initiate TRPV4 signaling. (A) Calcium flux is activated by 50-μg/mL Poly(I:C), and activity is highest in the painful vestibule, n = 6, $^*P < 0.05$. (B) Summary of all enzyme-linked immunosorbent assay (ELISA) data (significance stars not displayed), showing 1-μM 4alpha-phorbol 12,13-didecanoate (4αPDD) alone does not induce an inflammatory response, yet augments PGE_2, but not IL-6 production in combination with Poly(I:C), whereas addition of 10-μM HC067047 inhibits both interleukin-6 (IL-6) and prostaglandin E_2 (PGE_2) production; brackets denote areas where significant differences were suspected, which were further examined in panel (C). (C) PGE_2 ELISA data showing significant inductions in PGE_2 production when 1-μM 4αPDD is added to Poly(I:C), which is attenuated by treatment with TRPV4 antagonist 10-μM HC067047 (HC). Strains are color-coded; dots represent replicates, n = 12, $^*P < 0.05$ (D) Published[30-32] data show inflammatory stimuli elicit production of pronociceptive IL-6 and PGE_2 through NFκB activation. PGE_2 and IL-6 induce TRPV4 channel expression to increase activity when TRPV4 ligands are applied (eg, 4αPDD) or produced (eg, 5(6)-EET). Dashed arrows indicate tentative relationships. EET, epoxyeicosatrienoic acid.

painful vestibule compared with all 3 nonpainful areas from cases and controls (**Fig. 3A**). This increase in activity may account for enhanced nociception and contribute to the elevated levels of inflammatory mediators in these painful areas by exacerbating PGE_2 production as observed in **Figures 2A and B**.

Next, we investigated whether inflammation on its own might be sufficient to initiate TRPV4 signaling using calcium flux assays. 4αPDD induced a strong spike in calcium flux in IL-1β–primed cells as anticipated (**Fig. 3B**). However, we also observed a strong peak with a second acute IL-1β treatment that was not present when calcium was depleted from the media. IL-1β alone was clearly sufficient to initiate calcium flux and demonstrates that inflammation has a direct link to initiation of calcium flux. In cells treated with IL-1β, HC067047 reduces PGE_2 production

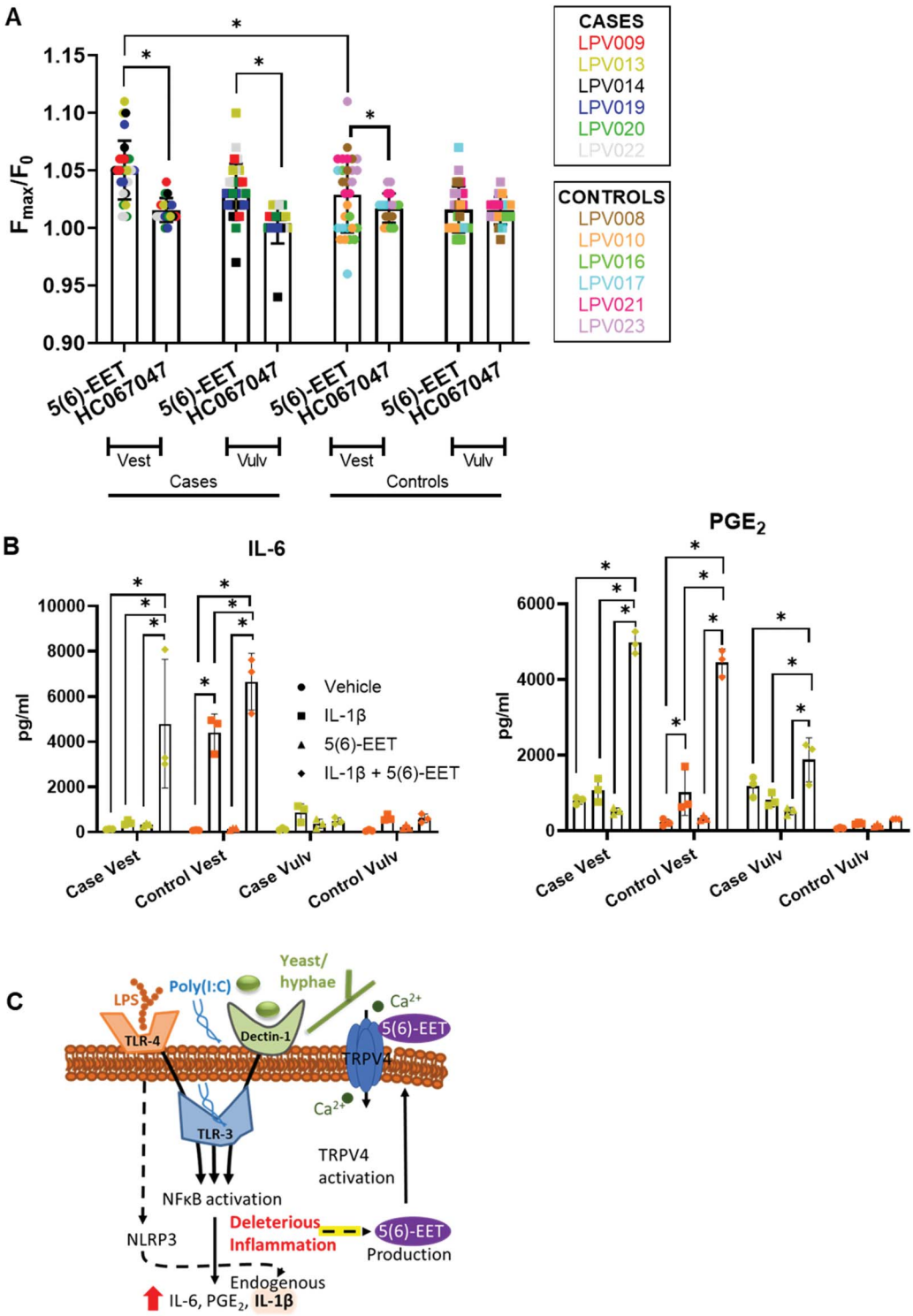

Figure 5. 5(6)-EET initiates TRPV4 signaling and exacerbates inflammation. (A) Calcium flux is activated by 1.12-μM 5,(6)-EET, and activity is highest in the painful vestibule. Blocking TRPV4 with 10-μM HC067047 (HC) attenuates calcium flux, indicative of 5(6)-EET specificity for TRPV4, n = 12, $^*P < 0.05$. (B) ELISA data show 1.12-μM 5(6)-EET alone does not instigate proinflammatory signaling, but significantly exacerbates it in the presence of ongoing inflammation, similar to 4alpha-phorbol 12,13-didecanoate (4αPDD), n = 4, $^*P < 0.05$. Patient samples are color-coded according to the key in panel (A). (C) Updated mechanism model showing TRPV4 is activated by 5(6)-EET and highlighting possible link between deleterious inflammation and 5,(6)-EET levels. EET, epoxyeicosatrienoic acid; PGE_2, prostaglandin E_2; IL-1β, interleukin-1β.

(**Fig. 3C**). Altogether, these results suggest that inflammation itself, represented by IL-1β treatment, initiates TRPV4 signaling, whereas antagonizing TRPV4 reduces inflammation. This points to a feed-forward loop where TRPV4 activation enhances inflammation, whereas inflammation increases TRPV4 expression and activity (**Fig. 3D**). These observations provide further rationale that TRPV4 inhibition would have therapeutic value for LPV.

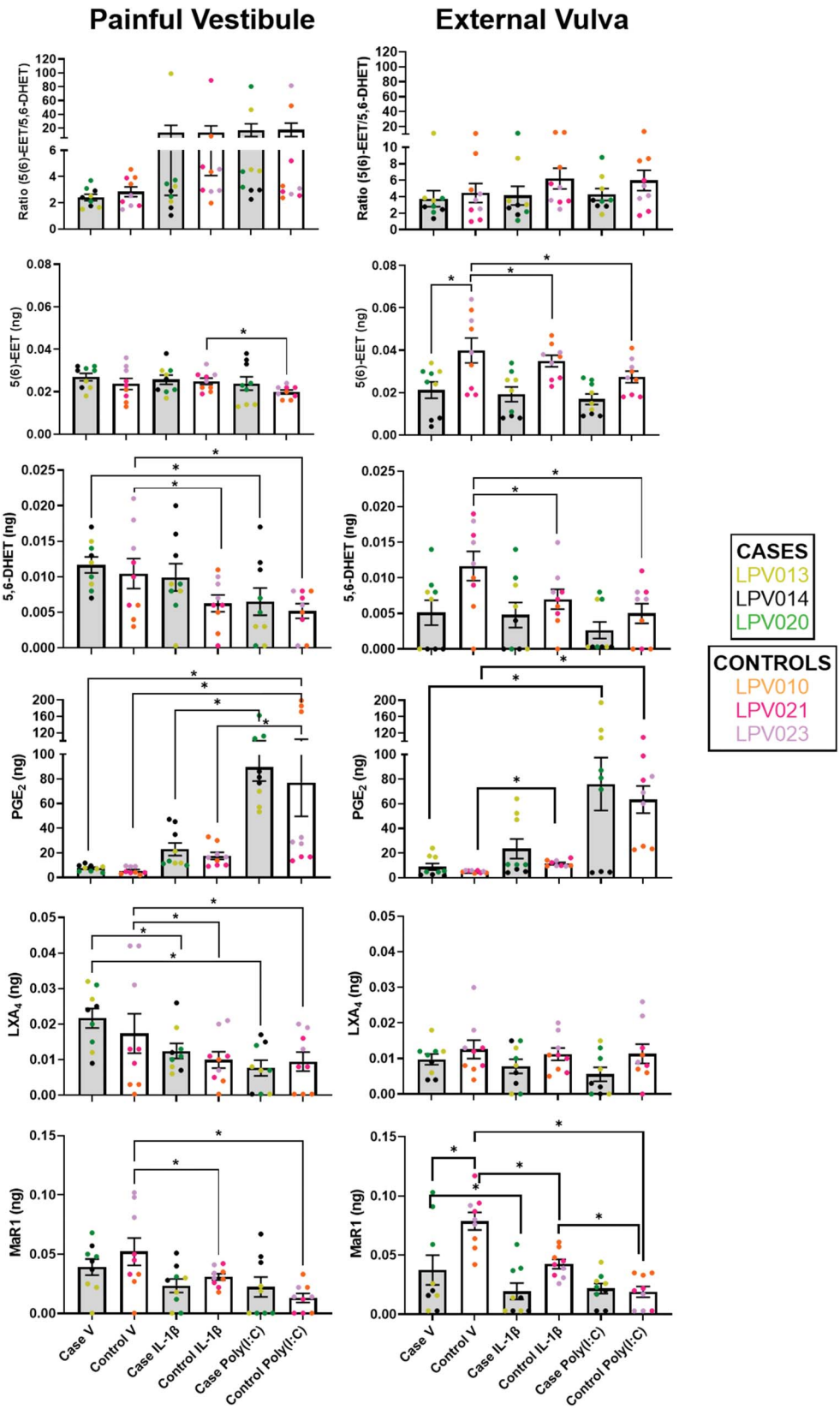

Figure 6. Inflammatory stimuli alter lipid profiles, favoring inflammation, reduced resolution, and enhanced TRPV4 signaling. Fibroblast cultures were treated with vehicle (V), 50-pg/mL IL-1β, or 50-μg/mL Poly(I:C) for 48 hours before cells, and supernatants were homogenized through probe sonication and preserved with 15% methanol containing butylated hydroxytoluene before flash freezing at −80°C. Lipids were quantified through mass spectroscopy. Gray bars = case, white bars = control, n = 6, replicates color-coded by strain, $^*P < 0.05$, 3 technical replicates were processed for each strain. EET, epoxyeicosatrienoic acid; LXA_4, lipoxin A_4, MaR1, MaR1(n-3DPA); PGE_2, prostaglandin E_2; IL-1β, interleukin-1β.

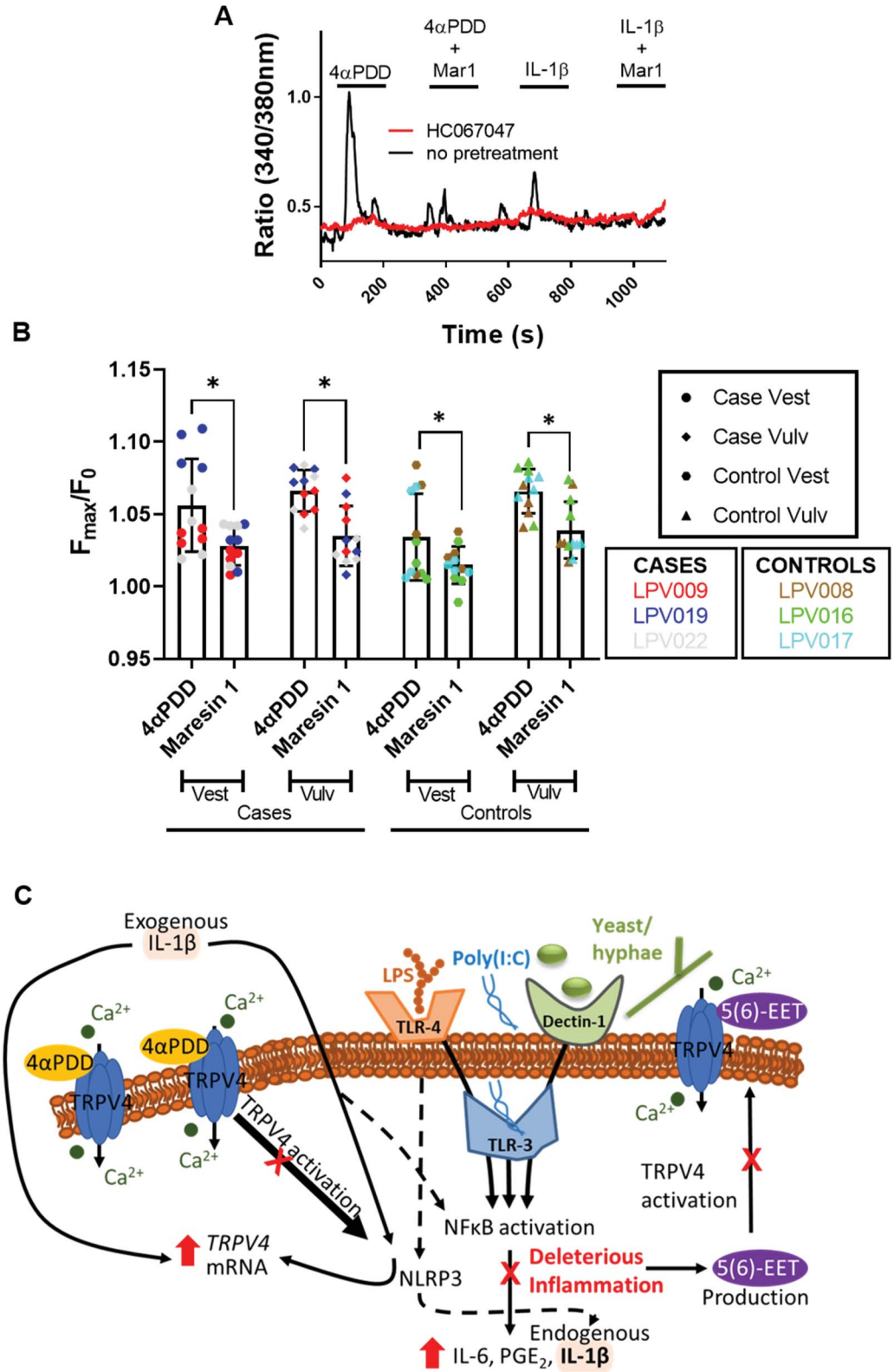

Figure 7. Maresin 1 inhibits TRPV4 signaling, acting on more than one part of the vulvodynia mechanism. (A) Response of a case vestibular strain to TRPV4 activator, 4alpha-phorbol 12,13-didecanoate (4αPDD), in untreated or HC067047 (HC)-treated cells. Cells were pretreated for 24 hours with 50-pg/mL interleukin-1beta (IL-1β) or IL-1β + 10-μM HC067047, then stimulated briefly with 1-μM 4αPDD or 4αPDD + 5-nM maresin 1 (Mar 1). HC067047-pretreated cells did not respond to any stimuli, whereas untreated cells responded, but the response was acutely blunted by Mar 1 treatment. Drugs were applied by superfusion during the period indicated by the application bars. (B) TRPV4 activity initiated by 4αPDD is highest in case fibroblasts. Acute treatment with maresin 1 reduces calcium flux rapidly to levels similar to those in control strains, n = 6, $^*P < 0.05$. (C) Maresin acts on several parts of the vulvodynia mechanism, by compensating for reduced pro-resolving lipids, including MaR1(n-3DPA), quelling deleterious inflammation and inhibiting TRPV4 activation, which is involved in the response to exogenous stimuli and is exacerbated by the production on endogenous IL-1β, which happens downstream of the response to exogenous stimuli. Red Xs denote areas of maresin action, dotted lines denote proposed mechanism, and solid lines denote mechanism supported by data shown herein or previously published. LPS, lipopolysaccharide.

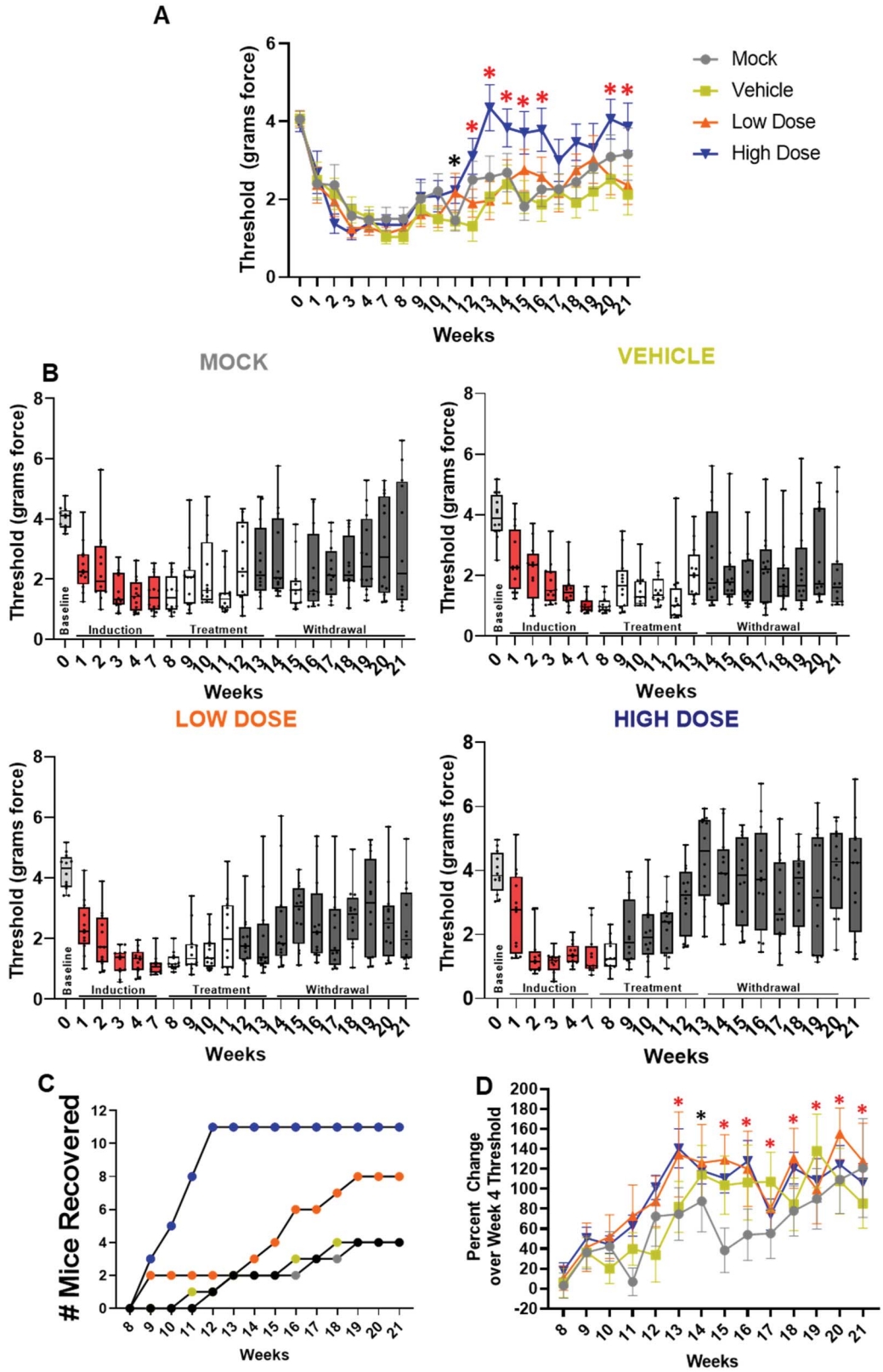

Figure 8. Natural product fish oil containing DHA and SPM precursors is highly effective in alleviating vulvar pain in mice. (A) Weekly threshold values for each group over the course of induction (weeks 1-7) and treatment (weeks 8-21) ± SD, n = 12 mice/group, red * high dose (1.9% Lipinova) outperforms all other treatments, black * high dose outperforms mock, $P < 0.05$. Week 0 = baseline. (B) Box and whisker plots for each group over experimental time course with dots for individual mouse thresholds. Boxes highlighted in dark gray show an increase over the week 7 threshold (final threshold before treatment), n = 12, $P < 0.05$. (C) Number of mice recovering each week based on a restoration of 70% or greater of the starting baseline threshold for 2 consecutive weeks. By the fourth week of treatment, nearly all mice in the high-dose group have recovered. (D) Percent change over the course of treatment, shows that mice improve most rapidly in the treatment groups, especially high-dose treatment, n = 12, red * both high and low dose (0.7% Lipinova) show improvement over week 8, black * low shows improvement, $P < 0.05$. DHA, docosahexaenoic acid; SPM, specialized pro-resolving mediator.

3.4. Exogenous inflammatory stimuli also activate TRPV4 signaling and exacerbate inflammatory signaling in vulvodynia

To investigate whether TRPV4 signaling is specifically activated by IL-1β, an endogenous cytokine, or whether TRPV4 activation is a consequence of challenge with any inflammatory stimuli, we treated cells with exogenous stimuli that mimic bacterial (lipopolysaccharide [LPS]) and viral (polyinosinic:polycytidylic acid [Poly(I:C)]) infection. Both induced an inflammatory response, whereas the response to Poly(I:C) was strongest and was exaggerated in painful areas (Fig. S5, available as supplemental digital content at http://links.lww.com/PAIN/B937), which is consistent with the published literature.[33]

We then tested whether Poly(I:C) could activate calcium flux in several case and control strains; Poly(I:C) elicited activation in all strains, but most robustly in the case vestibule, consistent with enhanced painful signaling in this area (**Fig. 4A**). Treatment with Poly(I:C) increased PGE_2 and IL-6 levels compared with vehicle (**Fig. 4B**), whereas the addition of 4αPDD further increased PGE_2 (**Fig. 4C**), consistent with the feed-forward mechanism illustrated in **Figure 3D**. However, as observed earlier, changes in IL-6 were nominal with the addition of 4αPDD, and the addition of HC067047 was able to inhibit both PGE_2 and IL-6 production (**Fig. 4B**), further evidence of the relationship between TRPV4 signaling and inflammation.

Treatment with the TRPV4 antagonist HC067047 significantly reduced PGE_2 production in response to the combination treatment of Poly(I:C) and 4αPDD (**Fig. 4C**), again supporting the hypothesis that targeting TRPV4 signaling can reduce inflammation in vulvodynia. Therefore, it seems that inflammation, which involves exposure to exogenous stimuli and the production of downstream endogenous stimuli, results in deleterious inflammation that may be connected to the activation of TRPV4 (**Fig. 4D**). 5(6)-EET is believed to gate the channel by binding to TRPV4 directly; 5(6)-EET fails to open the channel when the channel structure is altered.[16] We thus postulated that the elevated 5(6)-EET pools observed in painful areas (**Fig. 1B**) may play an important role in TRPV4 activation in vulvar fibroblasts, based on their function in other cells.[106,107]

3.5. 5(6)-EET directly activates TRPV4 signaling while augmenting inflammatory signaling

To determine whether 5(6)-EET could directly activate TRPV4 signaling and whether there were case–control or site-specific differences, we treated 12 paired strains (6 case and 6 control) with 5(6)-EET alone or 5(6)-EET and the TRPV4 antagonist HC067047 (**Fig. 5A**). We found that calcium flux was enhanced by 5(6)-EET in the case vs nonpainful control vestibule and that treatment with HC067047 significantly reduced calcium flux to levels comparable with controls. These findings show that 5(6)-EET activates calcium flux through TRPV4, which can be significantly attenuated by HC067047. In addition, 5(6)-EET most strongly activates TRPV4 in painful areas, consistent with its potential role in vulvar pain. Furthermore, we found that the addition of synthetic 5(6)-EET to cells treated with IL-1β augments both the production of IL-6 and PGE_2 consistent with the hypothesis that TRPV4 signaling exacerbates inflammation in vulvodynia (**Fig. 5B**). Unlike 4αPDD, this natural TRPV4 activator, 5(6)-EET, augments both IL-6 and PGE_2 production in the painful vestibule. These data demonstrate a direct role for 5(6)-EET in activating TRPV4 and perpetuating inflammation, but does not explain why the active 5(6)-EET pools are elevated in the case vestibule (**Fig. 5C**).

3.6. Treatment with endogenous IL-1β or exogenous Poly(I:C) alter lipid profiles resulting in dysbiosis characterized by increased active 5(6)-EET pools, increased PGE_2, and reduced pro-resolving lipoxin A_4 and maR1(n-3DPA)

To explore the connection between lipid dysbiosis, inflammation, and 5(6)-EET pools, we performed lipidomic analysis on cells treated with the inflammatory stimuli Poly(I:C) and IL-1β and compared these profiles to those from vehicle-treated cells. We found that there was an overall decrease in the breakdown product of 5(6)-EET (5,6-DHET) when vulvar fibroblasts were treated with inflammatory stimuli, with the greatest number of significant decreases occurring in the painful vestibule (**Fig. 6**). With inflammatory treatment (IL-1β or Poly(I:C)), 5(6)-EET levels decreased in control fibroblasts, particularly external vulvar cells, whereas levels in case fibroblasts from the painful vestibule and external vulva remained unchanged with exposure to inflammatory stimuli. Although we did not observe a significant difference in the ratio of active 5(6)-EET over its 5,6-DHET breakdown product as we did in vulvar tissue, active 5(6)-EET levels did not decline in the painful vestibule as they did in the nonpainful external vulva. Sustained levels of 5(6)-EET could naturally augment TRPV4-mediated signaling in the painful vestibule. We observed a concomitant increase in PGE_2 in all cells treated with inflammatory stimuli, regardless of site or case designation. However, pro-resolving lipids that would modulate this inflammation were also reduced, especially in the painful vestibule. Altogether, these data point to deficiencies in pro-resolving mediators in painful areas when inflammation is present. This inflammation causes or at least occurs concomitantly with reductions in pro-resolving lipids and increases in lipids that instigate TRPV4 signaling, which further exacerbates inflammatory signaling. Ongoing deleterious inflammation and lipid dysbiosis translate to sustained 5(6)-EET levels and, with it, enhanced TRPV4 signaling associated with mechanical allodynia. Heat maps of the lipid changes revealed an overall trend towards decreases in pro-resolving and increases in proinflammatory mediators with inflammatory treatments, especially with Poly(I:C) treatment (Fig. S6, available as supplemental digital content at http://links.lww.com/PAIN/B937). The full lipidomic data set may be found in Table S5, available as supplemental digital content at http://links.lww.com/PAIN/B937. We also confirmed that Poly(I:C) and IL-1β treatment generated typical inflammatory responses characterized by increases in PGE_2 and IL-6 levels (Fig. S7, available as supplemental digital content at http://links.lww.com/PAIN/B937). Sample LC-MS traces for maresin are included in figures S8–S11, available as supplemental digital content at http://links.lww.com/PAIN/B937.

3.7. Treatment with pro-resolving maresin acutely inhibits TRPV4 signaling acting on more than one part of vulvodynia pathway

Based on the connection between TRPV4 signaling, pain, lipid dysbiosis, and inflammation, we sought to interrupt this process, which could have therapeutic application. Although the TRPV4 antagonist, HC067047, often reduced inflammatory mediators in cells treated with inflammatory and/or TRPV4 activators (**Figs. 2A, B, 3C, 4B, C**), it did not consistently reduce the levels of both IL-6 and PGE_2 in all samples. Furthermore, we found that acute treatment with HC067047 did not disrupt calcium flux, whereas overnight pretreatment could prevent the response to activators of TRPV4, including 4αPDD and IL-1β (**Fig. 7A**). Based on published reports that other specialized pro-resolving mediators (eg, resolvin D1) could impede TRPV1 signaling,[11,12,53,73,74] we tested the effect of maresin 1 on TRPV4

signaling because to date, we have the most compelling evidence for maresin 1 as a potential analgesic for vulvodynia.[34] We found that maresin 1 reduced calcium flux whether initiated by 4αPDD or IL-1β (**Fig. 7A**). We then compared case–control and site-specific differences in several case and control strain pairs treated with 4αPDD. Activity was highest in cases and was reduced to control levels with concomitant treatment with maresin 1 (**Fig. 7B**). Overall, maresin 1, which we have previously shown is highly effective in reducing signs of vulvar allodynia in a mouse model of LPV,[34] is able to halt TRPV4 signaling on the order of seconds, which might explain its overwhelming efficacy against vulvar pain. This strengthens the rationale for developing maresin 1 as a therapy for LPV and potentially other neuropathic or neuroinflammatory pain conditions because it acts on more than one part of the proposed mechanism by compensating for missing pro-resolving lipids, reducing deleterious inflammation, and impeding pain signaling through TRPV (**Fig. 7C**).

3.8. Pro-resolving lipid treatment rapidly improves pain scores in a mouse model of vulvodynia

We have previously shown that chemically synthesized maresin 1 and DHA purified from fish oil are highly effective in reducing vulvar pain in mice.[34] Although maresin 1 is highly effective, it cannot be purified in sufficient quantities from natural products and will require several lengthy drug development steps before it could be used therapeutically. Docosahexaenoic acid oil is a natural product, but requires significant metabolism into pro-resolving mediators that can quell inflammation. Lipinova is a commercially available dietary supplement that is already in use in humans. It contains DHA and is enriched for several SPM precursors, including 18-hydroxyeicosapentaenoic (18-HEPE), 17-hydroxy-DHA (17-HDHA), and 14-hydroxy-DHA (14-HDHA), the precursor for maresin synthesis. However, we acknowledge that the Lipinova product contains several SPM precursors, such that the effects of this application cannot be directly attributed to maresin. We tested both a high (1.9%) and low (0.7%) dose of Lipinova in our mouse model and found that the high dose restored thresholds to or above starting preallodynia baselines in as little as 4 weeks (**Fig. 8A**). Only Lipinova, particularly the high dose, rapidly, consistently, and significantly increased threshold levels over the last pretreatment threshold (**Fig. 8B**). Mice started recovering in as little as 2 weeks, and 11 of 12 (92%) had recovered by 4 weeks vs 16% or less recovering in the low dose, mock, and vehicle treatment groups at 4 weeks (**Fig. 8C**). This also translated to a more rapid and higher percent recovery in mice receiving the high-dose treatment (**Fig. 8D**). We also observed that vulvovaginal inflammation rose during the induction phase and then tapered off immediately before and remained low during the treatment phase (Fig. S12, available as supplemental digital content at http://links.lww.com/PAIN/B937), suggesting the effects of SPMs go beyond reducing local inflammatory mediator levels. Altogether, Lipinova significantly improved thresholds, whereas mock and vehicle treatments had little to no effect. Lipinova, unlike chemically synthesized SPMs, could be rapidly translated to human use and may have efficacy for numerous inflammatory or neuropathic conditions. To further explore the contributions of TRPV4 signaling to vulvar allodynia, we induced vulvar allodynia in mice with a combination of 4αPDD (TRPV4 agonist) and zymosan and found that sensitivity thresholds were reduced more quickly and to a greater magnitude than with zymosan alone, supporting the hypothesis that TRPV4 signals play a role in vulvar pain (Fig. S13, available as supplemental digital content at http://links.lww.com/PAIN/B937).

4. Discussion

The vulvodynia mechanism is inherently complex[13,14,30–34,39,54,78,80,92,113,115] and, as we have shown here, involves a series of events that culminate in chronic pain and a sharp decline in quality of life.[84] Patients describe themselves as not being in control of their bodies, and LPV is often accompanied by sexual dysfunction, depression, and anxiety.[15,17] Limited knowledge of the disease mechanism has made treatment and even diagnosis challenging. There is currently no clinical test for vulvodynia; diagnosis relies on clinician judgement after exclusion of all other explanations of pain.[17,29,93]

Although there is evidence for the use of the existing therapies, there is no level 1 evidence; most remedies have not been tested in placebo-controlled randomized trials.[30] Treatment typically involves an escalation of interventions[30] often starting with hygiene-related changes, such as washing garments in hypoallergenic soaps and wearing cotton and loose-fitting clothes. When these fail, patients move to topical lidocaine and oral therapies, such as gabapentin and antidepressants. Some patients may undergo nerve block or botulinum toxin injection. Other common treatments involve biofeedback, physical, and psychological therapy. For many patients, treatment culminates in surgical amputation of the affected tissue, often after months or years of suffering.[30,45,46,84,92,93] Vestibulectomy is invasive, but largely curative—evidence that further supports the specialized nature of the vestibule and its role in LPV.[5,80,98–100] Less-invasive medical therapies that address the underlying causes of disease are desperately needed.

Few mechanistic LPV studies have been performed, warranting the development of new models for studying disease.[30–35,39,40,113] Because of the challenges in quantifying pain and the limitations of any one model, we have pursued animal, cellular, and tissue models of vulvodynia.[30–34,39] We have also implemented new technologies for studying LPV, such as targeted lipidomic analysis, which provides a comprehensive assessment of key lipids involved in resolution and inflammation. Using lipidomic analysis, we uncovered differences in the vulvar lipidome, which are linked to pain signaling and could represent a new target for analgesia.

The TRPV family, for which there are 6 members (TRPV1-6), has received attention as an analgesic target, particularly TRPV1.[55,63,66,68–70,85] TRPV1 has been implicated in neuropathic pain, specifically diabetic- and chemotherapy-induced peripheral neuropathy.[63,82,85] Although TRPV1 shows clear merit as a target for analgesic therapy, the role of TRPV4 in pain is less clear.[55,70] TRPV4 has been implicated in visceral and pancreatitis pain, mechanical hyperalgesia, and mechanosensation.[66,69,70] In this article, we provide evidence of a relationship between TRPV4 signaling, inflammation, and vulvar pain. Lipid dysbiosis, characterized by high levels of inflammatory mediators and reduced levels of pro-resolving mediators, perpetuates chronic vulvar inflammation. At the same time, this dysbiosis results in elevated pools of lipid activators of TRPV4, specifically 5(6)-EET. A feed-forward loop exists where inflammation increases TRPV4 expression and activity, whereas increases in TRPV4 activity increase inflammatory mediator production, magnifying the effects of TRPV4 signaling and inflammation, culminating in pain and loss of function.

In this article, we show that inflammation, characterized by elevated levels of IL-6 and PGE_2, is both necessary and sufficient for activation of TRPV4. We offer the first evidence of a neuroinflammatory mechanism in LPV, whereby inflammation triggers neurological signaling and pain. Not only does PGE_2 contribute to inflammation, but also it can directly sensitize peripheral neurons (PNs) and TRPV channels.[90] Although fibroblasts themselves do not transmit pain signals, the increase in TRPV4 activation coupled with

production of mediators that can sensitize TRPV4 channels and PNs in the surrounding nerve-bearing tissue could initiate neuroinflammatory signaling, such that fibroblasts act as "feeder" cells. TRPV4 is more highly expressed and active in the painful vestibule of patients than nonpainful areas from either the vestibule of controls or the external vulva. At the same time, inflammatory signals that perpetuate both TRPV4 signaling and inflammation are elevated at these painful sites. Nonetheless, it is important to keep in context that although vulvar fibroblasts express functional TRPV4, they are not sensory cells and are therefore not directly acting upon nerves in the tissue. There are likely other players in the mechanism we have yet to uncover; analgesia in our mouse model could be the result of the effects of SPMs on various cell types in the mouse vulva. However, it does seem that fibroblasts help to perpetuate a feed-forward mechanism that could be likened to a snowball rolling down a hill, gaining momentum with each revolution. Current evidence suggests targeting TRPV4 is unlikely to result in a severe phenotype[23,66,69,70]; mice treated with HC067047 do not exhibit changes in body weight or temperature or show signs of distress.[24] A TRPV4 antagonist is undergoing clinical trial (NCT02497937)[70] and has potential for clinical translation.

However, SPMs could hasten translation, especially when used topically to limit systemic adsorption. Specialized pro-resolving mediators, especially maresin 1, are highly effective in reducing pain and restoring baseline thresholds, typically within a matter of weeks and often improving the thresholds to above the starting baseline.[34] We previously tested every commercially available SPM in our fibroblast model, further evaluating several promising candidates (eg, lipoxin A_4, resolvin D_2, and maresin 1) in a 3D mouse vulvar biopsy model.[34] We then tested maresin 1 in our mouse model and found it had profound analgesic effects.[34] In this article, we show maresin 1 also halts TRPV4 signaling on the order of seconds. In addition, maR1(n-3DPA) and lipoxin A_4 are deficient in fibroblasts challenged with inflammatory stimuli, suggesting exogenous supplementation with maresin 1 would directly address this deficit. Specialized pro-resolving mediator treatment works on several levels of the vulvodynia mechanism by (1) compensating for reduced or missing SPMs, (2) fostering the resolution of inflammation, (3) reducing PGE_2 levels implicated in sensitization of nerve fibers, and (4) impeding the conductance of pain signals through TRPV4. Although targeting TRPV4 with a TRPV4-specific antagonist, such as HC067047, can reduce pronociceptive signals in our fibroblast model, the effects of SPMs are more pronounced and at lower, nanomolar concentrations. This underscores the utility of SPMs as a future vulvodynia therapy to be pursued in clinical trials.

Maresin 1 has established effects on TRPV1,[37,72,89,109] another member of the TRPV family, which is involved in the response to noxious heat and to capsaicin, the spicy component of chili peppers.[43,52,63,81,94] TRPV1 has been long sought after as a potential analgesic target, and several inhibitors are being investigated in clinical trials.[52,94] However, hyperthermia is a common side effect of targeting TRPV1, which has slowed clinical translation.[43] Maresin 1 has been shown to acutely target TRPV1 channel activation in dorsal root ganglion and trigeminal neurons in several mouse models, while also reducing sensitivity measures and inflammation, all without inducing hyperthermia.[37,72,89,109] Maresin 2 has similar effects other mouse models, including lipopolysaccharide-induced mechanical hyperalgesia.[38] Therefore, our data are consistent with the existing literature, demonstrating a role for maresin in reducing inflammation and pain signaling by inhibiting TRPV channels.

Although we have found promising new avenues for vulvodynia treatment, these are only useful to patients who receive a vulvodynia diagnosis. It is estimated that ~9% to 10% of women in the United States currently suffer from vulvodynia, but most epidemiological studies suggest that this is a drastic underrepresentation.[48,49,77] Women of minority backgrounds are less likely to receive a diagnosis than their Anglo-American counterparts, although vulvodynia may in fact be more prevalent in the Hispanic community.[71] This is part of a greater systemwide problem, but the ability to offer diagnostic testing for vulvodynia stands to improve diagnosis. Another goal of our work is to identify a "vulvodynia signature" that could be adapted to clinical testing. Namely, we have found increases in the 5(6)-EET/5,6-DHET ratio, and decreases in 8(9)-EET, 14(15)-EET, 12-HETE, lipoxin A_4, and maR1(n-3DPA) occur in painful areas, whether directly from biopsy tissue or in fibroblast models. An important next step will be to determine how this signature can be used to identify at-risk patients or patients with active vulvodynia. It could also be used in a clinical trial measure to assess response to treatment or surveil disease progression.

Our research focuses on LPV, but vulvodynia is a complex entity, ie, largely categorized by the clinical presentation of pain, which includes (1) timing (primary or secondary), (2) location (localized or generalized), and (3) nature (provoked or unprovoked) of the pain.[30] There is no mechanistic evidence that these diseases are in fact variations of the same disease entity or entirely separate diseases, and these classifications do not speak to causation or prevention. By investigating the vulvar lipidome, we also anticipate to identify conserved signatures that will help us to parse out these differences. As we are dealing with relatively small clinical populations, this is a gradual process as we collect samples and catalogue deidentified clinical information into our RedCap database.

Specialized pro-resolving mediators and even their precursors are highly effective in reducing inflammation and pain in a mouse model and surrogate measures of pain in a fibroblast model.[34] They are safe, could be translated to clinical use quickly, and most importantly, they directly address the vulvodynia mechanism in 2 distinct ways: (1) They quell the ongoing inflammation, and (2) they interrupt TRPV signaling implicated in pain.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/B937.

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