PLOS | ONE

RESEARCH ARTICLE

Repeated hapten exposure induces persistent tactile sensitivity in mice modeling localized provoked vulvodynia

Jasmine Landry[1], Tijana Martinov[2], Hanna Mengistu[1], Jyothi Dhanwada[1], Charles J. Benck[1], Jaclyn Kline[1], Beebie Boo[1], Linnea Swanson[1], Elena Tonc[1], Randy Daughters[1], Brian T. Fife[2], Devavani Chatterjea[1]*

1 Biology Department, Macalester College, St. Paul, Minnesota, United States of America, **2** Center for Immunology, University of Minnesota, Minneapolis, Minnesota, United States of America

* chatterjead@macalester.edu

Check for updates

OPEN ACCESS

Citation: Landry J, Martinov T, Mengistu H, Dhanwada J, Benck CJ, Kline J, et al. (2017) Repeated hapten exposure induces persistent tactile sensitivity in mice modeling localized provoked vulvodynia. PLoS ONE 12(2): e0169672. doi:10.1371/journal.pone.0169672

Editor: Kottarappat N Dileepan, University of Kansas Medical Center, UNITED STATES

Received: May 31, 2016

Accepted: December 20, 2016

Published: February 3, 2017

Copyright: © 2017 Landry et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: Funding support includes National Institutes of Health grants NIH R15 NS067536-01A1 and NIH R15 AI113620-01A1 (awarded to DC; https://www.nih.gov), a National Vulvodynia Association research grant (awarded to DC; http://www.nva.org), and internal awards from Macalester College. The funders had no role in the study design, data collection and analysis, decision

Abstract

Background

Vulvodynia is a remarkably prevalent chronic pain condition of unknown etiology. Epidemiologic studies associate the risk of vulvodynia with a history of atopic disease. We used an established model of hapten-driven contact hypersensitivity to investigate the underlying mechanisms of allergy-provoked prolonged sensitivity to pressure.

Methods

We sensitized female ND4 Swiss mice to the hapten oxazolone on their flanks, and subsequently challenged them four days later with oxazolone or vehicle for ten consecutive days on the labia. We evaluated labiar sensitivity to touch, local mast cell accumulation, and hyperinnervation after ten challenges.

Results

Oxazolone-challenged mice developed significant tactile sensitivity that persisted for over three weeks after labiar allergen exposures ceased. Allergic sites were characterized by mast cell accumulation, sensory hyper-innervation and infiltration of regulatory $CD4^+CD25^+FoxP3^+$ T cells as well as localized early increases in transcripts encoding Nerve Growth Factor and nerve-mast cell synapse marker Cell Adhesion Molecule 1. Local depletion of mast cells by intra-labiar administration of secretagogue compound 48/80 led to a reduction in both nerve density and tactile sensitivity.

Conclusions

Mast cells regulate allergy-provoked persistent sensitivity to touch. Mast cell-targeted therapeutic strategies may provide novel means to manage and limit chronic pain conditions associated with atopic disease.

to publish, or preparation of the manuscript. CJB and JD received stipends from intra-mural programs supported by science education grants to Macalester College from the Arnold and Mabel Beckman Foundation (CJB) and the Howard Hughes Medical Research Institute (HM, JD). TM and BTF are supported by National Institute of Health grant R01AI106791.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Vulvodynia is chronic vulvar pain of unknown etiology, diagnosed in the absence of obvious infections or overt inflammation [1, 2]. As many as 8% of women in the United States are likely to experience symptoms consistent with vulvodynia by the age of 40 [3] most presenting with provoked, localized pain [4, 5]. Mast cell accumulation and hyper-innervation are the two most consistent features of vestibular biopsies from patients diagnosed with vulvodynia [6, 7]. Women with a history of seasonal allergies are twice as likely to develop vulvodynia compared to allergy-free age-matched controls [8]. We previously provided biological plausibility for this association, demonstrating that single and triple labiar skin exposure to hapten oxazolone in pre-sensitized ND4 Swiss mice led to transient tactile sensitivity and an increase in cutaneous nerve density [9]. As versatile immune regulators, mast cells contribute to a broad range of acute and chronic pain responses [10] and functionally associate with nerves in a variety of patho-physiologies [11]. Whether mast cell-mediated allergic responses can drive tissue changes that provoke prolonged painful sensations remains unknown. Here, we investigated the long-term effects of repeated oxazalone exposure on the labia of ND4 Swiss mice, local and systemic inflammatory changes, and the role of mast cells in the persistence of sensitivity to touch and localized hyper-innervation.

Methods

Animals

Ethics statement. This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Macalester Institutional Animal Care and Use Committee (IACUC protocols B13S1 and B16S2). Mice were euthanized via 100% CO_2 inhalation at predetermined time points, and all efforts were made to minimize suffering.

6–12 week old female ND4 Swiss mice (Harlan Laboratories, Indianapolis, IN) were housed with a 12-hour light/dark cycle and free access to food and water.

Oxazolone treatment. Mice were sensitized with topical application of 100 μL of 2% oxazolone (Ox; 4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one, Sigma-Aldrich, St. Louis, MO) on the shaved flank (applied to 15 mm x 15 mm of skin) and subsequently challenged topically on the shaved genital skin around and including the labia (5 mm x 5 mm) with 40 μL of 1% Ox or ethanol vehicle daily for 10 days beginning on day 5 after sensitization (Fig 1A and 1C) (adapted from [9, 12]). Areas of topical Ox application on flank and genital skin were shaved 5 days prior to sensitization.

In separate experiments where sensitivity was not assessed, mice were sensitized with topical application of 100 μL of 2% oxazolone on a 15 mm x 15 mm area of the shaved back and subsequently challenged daily for 10 days on 25 mm x 25 mm areas of both shaved flanks with 100 μL of 1% Ox or ethanol vehicle. Flank challenges began 4 days after sensitization and were performed to obtain larger cell yields for tissue culture and intra-cellular cytokine/transcription factor staining for flow cytometry (Fig 1B).

Mast cell depletion

Ox-sensitized and -challenged mice received intra-labiar injections of mast cell degranulator compound 48/80 (c48/80; 20 μg/mouse in 40 μl 0.9% saline; 20 μl/labium; Sigma-Aldrich) daily from day 5–8 after the cessation of Ox challenges to locally deplete mast cells in the labiar tissue (adapted from [13]; Fig 1C). Mast cell depletion was confirmed by immunofluorescence 1 day after the last c48/80 injection.

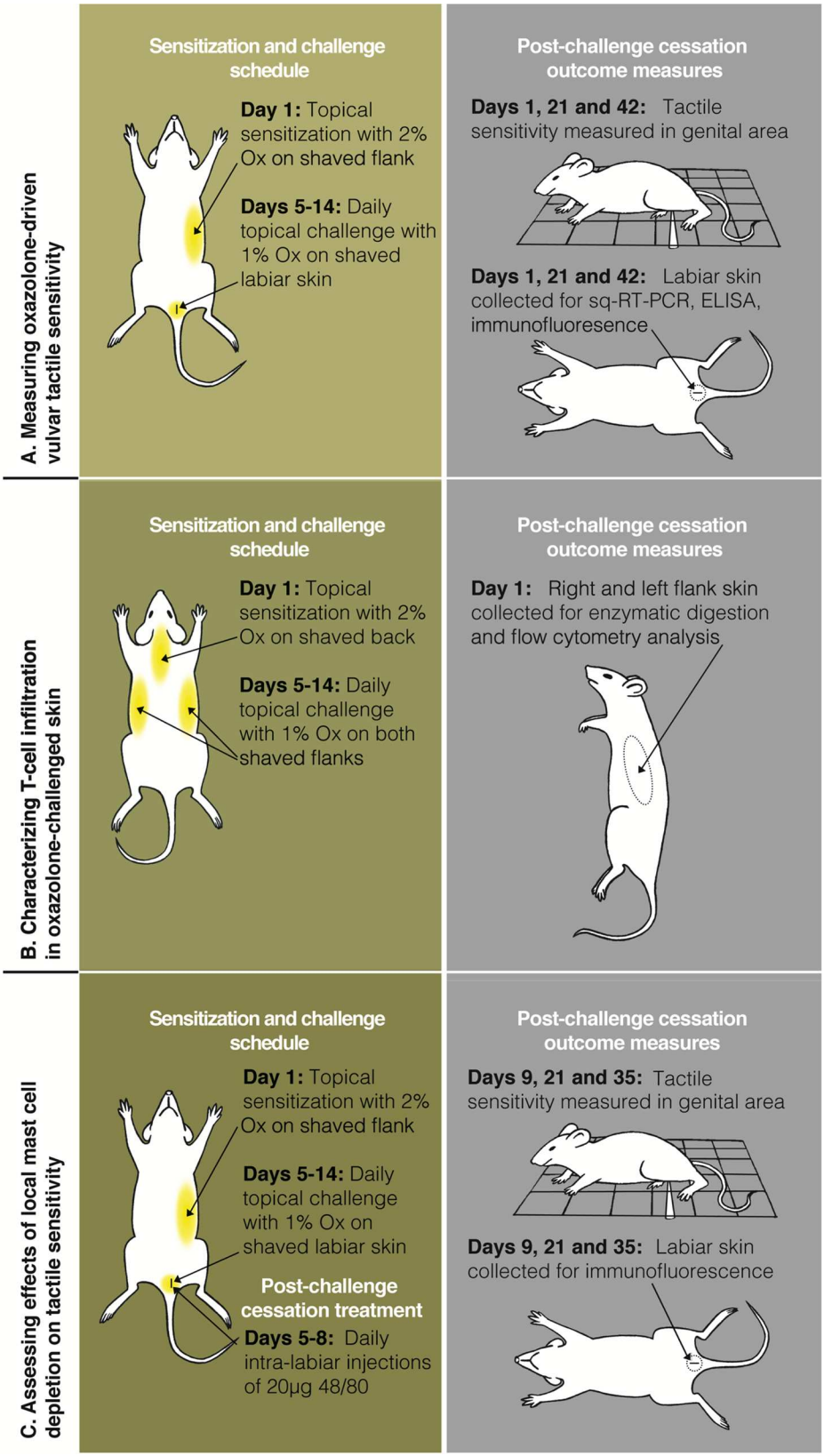
A. Measuring oxazolone-driven vulvar tactile sensitivity
Sensitization and challenge schedule
Day 1: Topical sensitization with 2% Ox on shaved flank
Days 5-14: Daily topical challenge with 1% Ox on shaved labiar skin
Post-challenge cessation outcome measures
Days 1, 21 and 42: Tactile sensitivity measured in genital area
Days 1, 21 and 42: Labiar skin collected for sq-RT-PCR, ELISA, immunofluoresence
B. Characterizing T-cell infiltration in oxazolone-challenged skin
Sensitization and challenge schedule
Day 1: Topical sensitization with 2% Ox on shaved back
Days 5-14: Daily topical challenge with 1% Ox on both shaved flanks
Post-challenge cessation outcome measures
Day 1: Right and left flank skin collected for enzymatic digestion and flow cytometry analysis
C. Assessing effects of local mast cell depletion on tactile sensitivity
Sensitization and challenge schedule
Day 1: Topical sensitization with 2% Ox on shaved flank
Days 5-14: Daily topical challenge with 1% Ox on shaved labiar skin
Post-challenge cessation treatment
Days 5-8: Daily intra-labiar injections of 20μg 48/80
Post-challenge cessation outcome measures
Days 9, 21 and 35: Tactile sensitivity measured in genital area
Days 9, 21 and 35: Labiar skin collected for immunofluorescence

Fig 1. Timeline of oxazolone sensitization, challenge, and post-challenge outcome measures (A) To measure oxazolone-driven vulvar tactile sensitivity, mice were topically sensitized with 2% Ox on the shaved flank (day 1) and subsequently challenged on the shaved labiar skin (days 5–14) with 1% Ox or EtOH vehicle for a total of 10 challenges. Tactile sensitivity was assessed in the ano-genital ridge area 1, 21, and 42 days after challenge cessation. Labiar skin was harvested at these time points from a separate cohort of mice for assessing molecular and cellular changes in the tissue. (B) To characterize T cell infiltration in Ox-challenged skin, mice were topically sensitized on their shaved back with 2% Ox (day 1) and challenged on both shaved flanks with 1% Ox or EtOH (days 5–14). Flank skin was harvested from both sides 1 day after challenge cessation for flow cytometric analysis of T cell infiltration. (C) To assess the effects of local mast cell depletion on Ox-induced tactile sensitivity and hyperinnervation, mice were topically sensitized with 2% Ox on the shaved flank (day 1), challenged on the shaved labia with 1% Ox or EtOH (days 5–14) and treated with intralabiar injection of saline or c48/80 (days 5–8 after Ox challenge cessation). Tactile sensitivity, mast cell levels and innervation were assessed 9, 21, and 35 days after the final Ox challenge.

doi:10.1371/journal.pone.0169672.g001

Tactile sensitivity

We measured tactile sensitivity in a 2 mm x 2 mm area of the ano-genital ridge of mice, the posterior, hairless portion of the vulva located dorsally from the introitus, as previously described in models of vaginal candidiasis [14] and transient labiar contact hypersensitivity [9]. We measured baseline tactile sensitivity of the ano-genital ridge 48 and 24h before Ox sensitization using an electronic Von Frey anesthesiometer (IITC Life Sciences, Woodland Hills, CA) as previously described [9]. Mice were habituated to the testing conditions for 15 minutes before sensitivity measurements, as previously described [15]. In ND4 mice used in these experiments, baseline thresholds ranged from 0.5 to 1.68 g. Baseline and experimental withdrawal thresholds at 1, 21 and 42 days after Ox- and ethanol challenges and at serial time points after mast cell depletion are shown in S1 Table. As previously described [9] mice with thresholds lower than 0.50 g and mice with the two baseline measurements differing by >1.00 g were excluded. Mice were assigned to treatment groups such that the average group baseline values were similar.

Sensitivity was assessed at the same site (ano-genital ridge) and by the same investigator on days 1, 21 and 42 following challenge cessation. Average post-challenge withdrawal threshold values (in grams) were subtracted from average baseline values for each mouse. Percent decrease from baseline was then calculated for each treatment group as (change in withdrawal threshold/baseline withdrawal threshold*100). After mast cell depleting compound 48/80 was injected, sensitivity was assessed on days 9, 21 and 35 after the cessation of Ox challenges.

Immunofluorescent staining and microscopy

Flash frozen labiar skin samples collected from CO_2-euthanized mice on days 1, 9, 21, and 42 after challenges were embedded in Optimal Cutting Temperature compound (Sakura Finetek, Torrance, CA) and cut to obtain 10μm sections. These were fixed and stained with a primary rabbit polyclonal antibody against calcitonin gene related peptide (CGRP; Abbiotec, San Diego, CA; 1:500) and AlexaFluor 488-conjugated secondary antibody (Thermo Fisher Scientific, Wilmington, DE; 1:1000) as previously described [9, 16]. To stain mast cells, slides were incubated for one hour with FITC-Avidin (Vector Laboratories, Burlingame, CA) as previously described [9, 16]. Stained slides were cover-slipped with the anti-fade mounting medium Vectashield containing 1.5 μg/mL 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories); the DAPI stained the nuclei in tissue sections. Composite images of 10 optical 1μm sections projected on the z-axis were taken using a laser scanning confocal microscope (Olympus FV1000) and analyzed using FluoView FV1000 image analysis software (Olympus Corporation, Center Valley, PA). Mast cell and $CGRP^+$ nerve density values were determined by fluorescent pixel intensity measurements taken in four representative 5000μm^2 regions of interest in each of the three sections per slide for three slides per mouse and 4–6 mice per treatment group.

Flow cytometry

For these experiments, mice were sensitized on the back with Ox and challenged on the flank as described above. Leukocytes were isolated from flank skin samples from CO_2-euthanized mice as previously described [12] 1 day after the cessation of challenges (Fig 1B). Cells were washed, blocked with supernatant from a 2.4G2 hybridoma cell line (50μl, HB197TM, American Type Culture Collection, Manassas, VA), stained with fluorochrome-conjugated monoclonal antibodies (S2 Table) at 1:100 dilutions for 30 minutes and analyzed on a LSR Fortessa X-20 (Becton Dickinson, Franklin Lakes, NJ) flow cytometer. Acquired data were analyzed using FlowJo software (Version X, FlowJo LLC, Ashland, OR). Dead cells were excluded using Ghost Dye-BV510 (Tonbo Biosciences, San Diego, CA). Flank skin cells were stained with antibodies against surface proteins, fixed and permeabilized (eBioscience, San Diego, CA; manufacturer's directions) before staining with anti-FoxP3-FITC (S2 Table). Flank skin cells were cultured in RPMI (Thermo Fisher Scientific) supplemented with 10% fetal calf serum (Serum Source International, Charlotte, NC), glutamine and antibiotics (Thermo Fisher Scientific) with PMA/ionomycin and Brefeldin A (Tonbo Biosciences) for 18 hours. Cells were then washed, surface stained as described above, fixed and permeabilized (Cytofix/Cytoperm kit, BD Biosciences, San Jose, CA) before intracellular staining with anti-IFN-γ-PE (S2 Table).

RNA isolation and quantification of gene expression

Total RNA was extracted from flash frozen labiar skin samples collected at indicated time points using the Total RNA Mini Kit (Midwest Scientific, St. Louis, MO), quantified on a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific) and reverse-transcribed using the Superscript III First-Strand Synthesis System (Thermo Fisher Scientific) in a 2720 Thermal Cycler (Thermo Fisher Scientific). Relative abundances of transcripts of interest were quantified by running 40 cycles of semi-quantitative polymerase chain reaction on a StepOnePlus thermocycler (Thermo Fisher Scientific) using quality controlled TaqMan Gene Expression Assay Primer/Probe Sets (S3 Table) and Master Mix (Thermo Fisher Scientific) and normalized to expression of housekeeping gene β-2 microglobulin to determine fold expression values for genes of interest [17].

Protein and histamine quantification

Flash-frozen skin samples collected at indicated time points were homogenized in cell lysis buffer (Cell Signaling Technology, Beverly, MA) with protease inhibitor (EMD Millipore, Billerica, MA) using a Tissue-Tearor (BioSpec Products, Bartlesville, OK), incubated on ice for 20 minutes, strained through a 40 μm filter, and centrifuged for 10 minutes at 2000 rpm at 4˚C. IFN-γ content in labiar tissue lysates was measured using an IFN-γ DuoSet ELISA kit (R&D Systems, Minneapolis, MN) and normalized to total protein content determined using the detergent-compatible DC Protein Assay (Bio-Rad, Hercules, CA). Total serum Immunoglobulin E (IgE) content was measured by ELISA (BD Biosciences) in serum isolated from blood collected either from the submandibular vein or by post-mortem heart puncture, or in vaginal lavage fluid collected by gently flushing the vaginal canal with 60μl of phosphate buffered saline. We quantified histamine content of skin lysates by ELISA (Neogen Corporation, Lansing, MI). Absorbances were recorded with a PowerWave XZ microplate spectrophotometer (BioTek Instruments, Winooski, VT).

Statistical analysis

Data were processed using Excel (Microsoft, Redmond, WA) and graphed using PRISM 5.0 (GraphPad, San Diego, CA). One-way ANOVA, *post hoc* Tukey HSD analyses, or unpaired

Student's t-test were run using JMP Software to compare treatment groups at designated time points (v. 10, SAS, Cary, NC). Two-way ANOVA with interaction and repeated measures was also performed to determine the effect of time, treatment, and interaction of time and treatment using JMP software.

Results

Ten oxazolone challenges to the labiar skin of sensitized ND4 swiss mice provoke a heightened and persistent tactile sensitivity

One day after cessation of ten daily allergen challenges, Ox-sensitized female ND4 Swiss mice had a 70% decrease in withdrawal threshold to light pressure applied with an electronic Von Frey meter when compared to their baseline sensitivity (Fig 2A). Both shaved, untreated mice and sensitized mice challenged with ethanol (EtOH; vehicle) were significantly less sensitive to touch than Ox-treated mice, with only a 20% decrease in withdrawal thresholds compared to their baselines. Individual withdrawal thresholds for each mouse in all treatment groups are

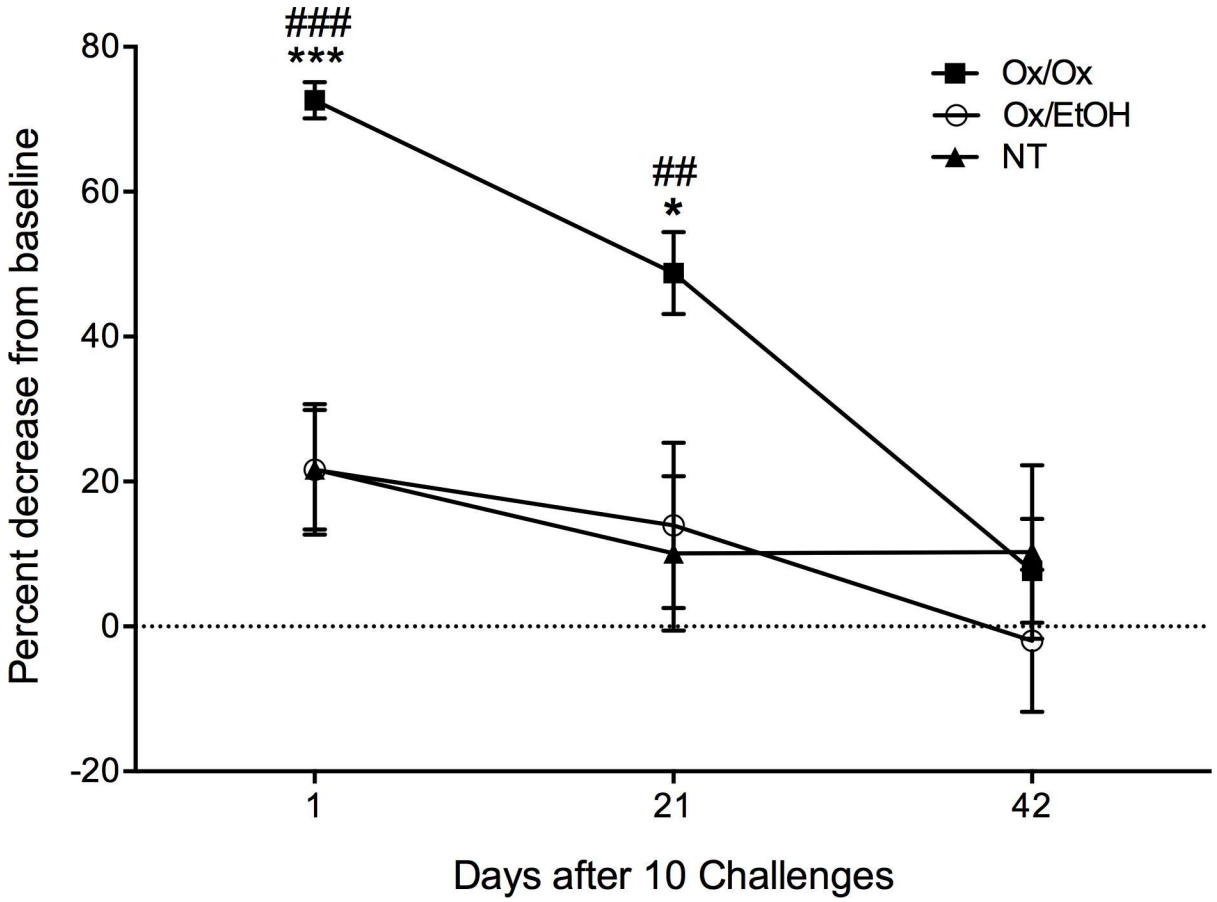

Fig 2. Ten oxazolone challenges provoke tactile sensitivity that persists for 21 days after cessation of challenges. Sensitized mice that received ten daily Ox challenges on the labiar skin had increased tactile sensitivity in their ano-genital ridge area compared to controls. Percent decrease in labiar withdrawal threshold for each treatment group is displayed as mean ± SEM. Significance at each time point was determined using one-way ANOVA and Tukey Kramer *post hoc* analysis based on comparisons to previously sensitized mice challenged with EtOH (Ox/EtOH; * = $p<0.05$, *** = $p<0.001$) and untreated controls (NT; ## = $p<0.01$, ### = $p<0.001$). n = 9–12 mice per treatment group; data represent two independent experiments. Raw withdrawal thresholds at baseline and post Ox-challenge cessation for each animal are shown in S1 Fig and summarized in S1 Table.

doi:10.1371/journal.pone.0169672.g002

shown in supporting data (S1A–S1C Fig). Ox-challenged mice were significantly more sensitive to pressure compared to control groups at 1 and 21 days after the final challenge; all groups returned to baseline sensitivity by 42 days after cessation of allergen challenges (Fig 2A); the same mice were evaluated at all time points. When we fit the data to a two-way repeated measures ANOVA model, we found that both Ox-treatment ($p<0.0001$) and time ($p<0.0001$) had a significant effect on sensitivity. No significant random effects were found. We also investigated an interaction of time and treatment. The interaction term approached, but did not reach, statistical significance ($p = 0.0538$). This pronounced, persistent sensitivity was localized to the site of allergen challenge; tactile sensitivity at a distal site (the hind paw) remained unchanged after labiar Ox challenges (S1D Fig, S4 Table). Previously, we showed that baseline tactile sensitivity is not modulated by stages of the estrus cycle [9]. Here, we also show that Ox-triggered tactile sensitivity is not affected by estrus cycle stage (S1E Fig, S4 Table). Furthermore, we detected no overt signs of inflammation or tissue injury by visual inspection 21 days after 10 Ox challenges, when sensitivity was still detectable and significant over vehicle-challenged controls (S1F Fig).

Ten oxazolone challenges to the labiar skin of sensitized ND4 swiss mice result in sustained sensory hyper-innervation at the site of allergen exposure.

ND4 female mice showed an increase in $CGRP^+$ sensory neurons at the site of Ox challenge along with heightened sensitivity to touch. Ox-challenged mice presented a significant, >3-fold increase in cutaneous labiar CGRP intensity compared to vehicle-challenged and untreated controls 1 day after 10 Ox challenges (Fig 3A). Significant hyper-innervation persisted in Ox-challenged mice at least until day 21 (Fig 3A–3D); all groups returned to baseline levels by day 42. Thus, significant hyper-innervation was present for three or more weeks after allergen challenges had ceased, but it was no longer evident by six weeks after the end of Ox treatment. Transcripts encoding nerve growth factor (NGF) were elevated ~10-fold in Ox-challenged mice over EtOH-treated controls in the labiar skin tissue 1 day after 10 Ox challenges, suggesting that the local tissue environment in the allergic site supported increased growth and maintenance of neurons; this increase was less pronounced by day 21 (Fig 3E).

Ten oxazolone challenges to the labiar skin of sensitized ND4 swiss mice induce local accumulation of mast cells.

Mast cell numbers in Ox-challenged skin were 4-fold higher than in ethanol-challenged skin on 1 day after the cessation of Ox challenges (Fig 4A) and remained elevated until day 21 (Fig 4A–4D); all groups resolved to baseline by day 42. Total histamine levels in the labiar skin of Ox-treated mice were significantly higher than in ethanol-treated mice (Fig 4E) possibly reflecting the increased numbers of histamine containing mast cells at the allergic site. Similarly to the observed pattern of hyper-innervation, increased mast cell accumulation at the site of Ox challenge was clearly present at least for three weeks after allergen challenges were completed, but resolved to baseline levels by six weeks post challenge cessation.

Elevated circulating IgE is a hallmark of atopic disease [18]. Serum IgE levels were significantly increased in Ox- over ethanol-challenged mice (Fig 4F) on day 1 after 10 Ox challenges and remained elevated over two weeks. Total IgE in the vaginal lavage increased slightly but significantly in the Ox-challenged mice 1 day after 10 Ox challenges (Fig 4G). IgE can enhance mast cell survival, acting in part through IL-6 and IL-13 signaling [19]. We therefore tested the levels of *Il6* and *Il13* mRNAs. Both transcripts were elevated in labiar skin ~100-fold 1 day after 10 Ox challenges (Fig 4H). This increase was no longer discernible by day 21 for *Il13* although *Il6* transcripts remained slightly elevated in Ox-challenged mice.

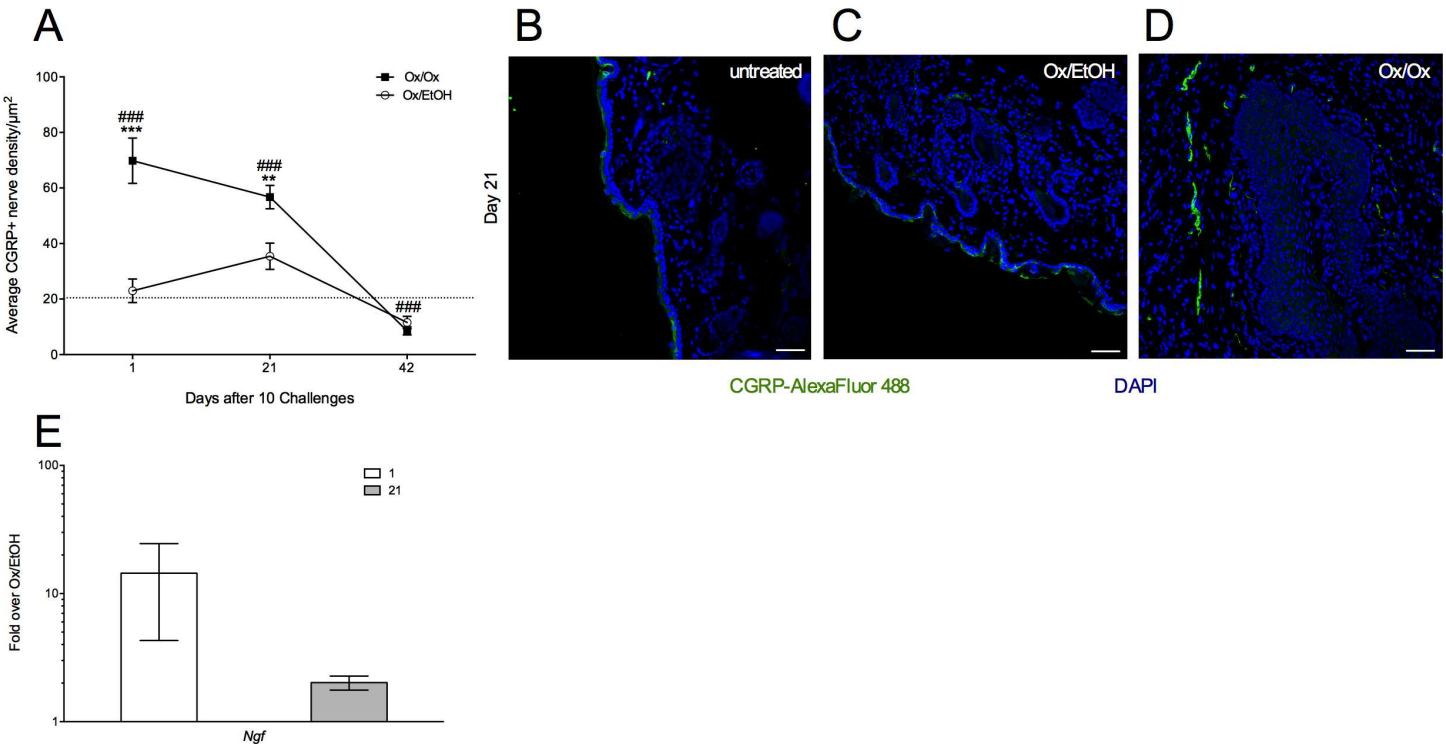

Fig 3. Labiar CGRP⁺ nerve density is increased after oxazolone challenges accompanying an increase in *Ngf* transcripts. (A) Density of CGRP⁺ nerve fibers in 10 μm labiar skin cryo-sections from sensitized mice challenged with Ox or EtOH, displayed as mean ± SEM (n = 3-5/treatment group). Dashed line corresponds to average CGRP⁺ nerve fiber density/μm² in untreated mice. Images are representative from day 21 after cessation of challenges (B-D; 20x magnification; scale bar represents 50 μm). Means compared to Ox/EtOH (** = p<0.01, *** = p<0.001) or untreated controls (### = p<0.001) at each time point; significance determined by one-way ANOVA and Tukey Kramer *post hoc* analysis. (E) Relative abundance of *Ngf* in Ox- vs. EtOH-challenged mice 1 day after 10 challenges displayed as mean ± SEM (n = 5-6/treatment group; two independent experiments).

doi:10.1371/journal.pone.0169672.g003

The cell adhesion molecule 1 (CADM1) regulates mast cell-neuron synapses in atopic dermatitis [20]. *Cadm1* transcripts were elevated ~100 fold in Ox-challenged over vehicle-challenged labiar skin 1 day after 10 Ox challenges supporting the concomitant increase of nerve density and mast cells at the allergic site; this increase was no longer discernible on day 21 (Fig 4H).

Local mast cell depletion after repeated exposure to allergen reduces innervation and tactile sensitivity.

ND4 mice were treated with daily injections of mast cell degranulator c48/80 (10 μg/labium) on days 5–8 after Ox challenge cessation (Fig 1C). To confirm depletion, we assessed mast cell density in the labiar skin on day 9 after 10 Ox challenges i.e. 1 day after c48/80 injections were completed, and detected >5-fold decrease (Fig 5A–5C). Mast cell depletion was accompanied by a significant ~2-fold reduction in the density of cutaneous CGRP⁺ neurons in the allergic skin (Fig 5D–5F) and a significant reduction in tactile sensitivity assessed at individual time points on days 9 and 21 (Fig 5G) compared to Ox-challenged mice that were not treated with c48/80. When we fit the data to a two-way repeated measures ANOVA model, we found that c48/80 injections had significant effect on sensitivity (p = 0.0069). The effect of time was not statistically significant, nor did we find evidence of a random effect. We investigated an interaction of time and c48/80 treatment, but the interaction term did not reach statistical significance. Mice given intra-labiar injections of saline (vehicle) had indistinguishable sensitivity

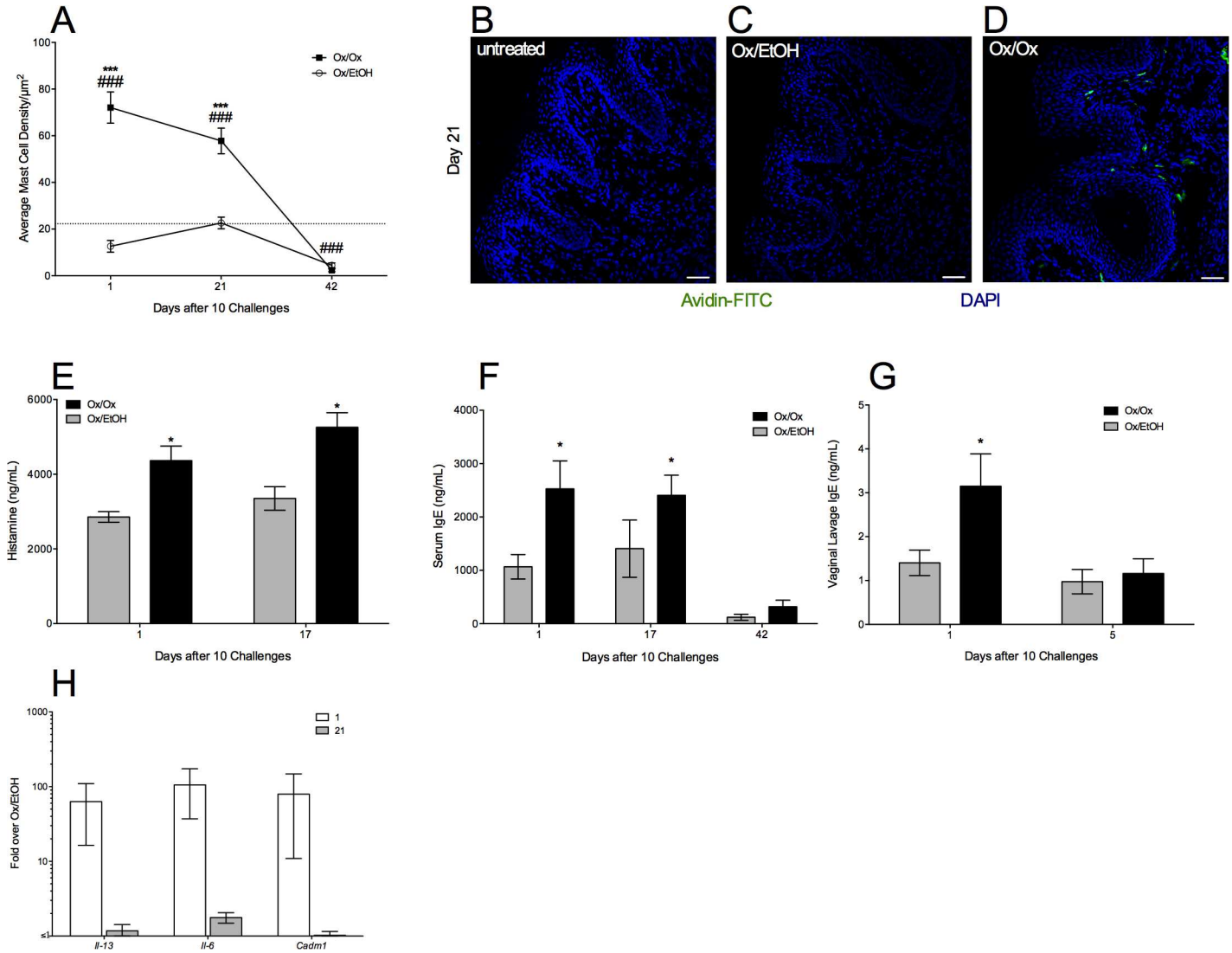

Fig 4. Labiar mast cell density is increased after oxazolone challenges accompanying an increase in modulatory factors. (A) Density of Avidin$^+$ mast cells in 10 μm labiar skin cryo-sections from sensitized mice challenged with Ox or EtOH, displayed as mean ± SEM (n = 4-6/treatment group). Dashed line corresponds to average mast cell numbers/μm^2 in untreated animals. Images are representative from day 21 after challenge cessation (B-D; 20x magnification; scale bar represents 50 μm). Means are compared to Ox/EtOH (*** = p<0.001) or untreated controls (### = p<0.001) at each time point; significance determined using one-way ANOVA and Tukey Kramer *post hoc* analysis. (E) Tissue histamine content in labiar skin of Ox- and EtOH-challenged mice 1 and 21 days after cessation of oxazolone challenges. Total IgE content in serum (F) and vaginal lavage (G) in Ox vs. EtOH-challenged mice at indicated time points after sensitization. (H) Relative abundance of *Il13*, *Il6*, and *Cadm1* in Ox- vs. EtOH challenged mice 1 day after 10 oxazolone challenges displayed as mean ± SEM (n = 5-6/treatment group; two independent experiments).

doi:10.1371/journal.pone.0169672.g004

from Ox-sensitized and challenged controls 9 days after the cessation of Ox challenges (S2 Fig).

Regulatory CD4 T cells and IFN-γ producing CD8 resident memory cells accumulate in the affected skin after multiple Ox challenges.

Regulatory T cells (T_{reg}) recruit mast cell progenitors to mouse airways after intra-nasal ovalbumin challenge [21] and promote focal mastocytosis in a mouse model of hereditary colon cancer [22]. With this in mind, we examined T_{reg} accumulation in Ox-challenged skin. As

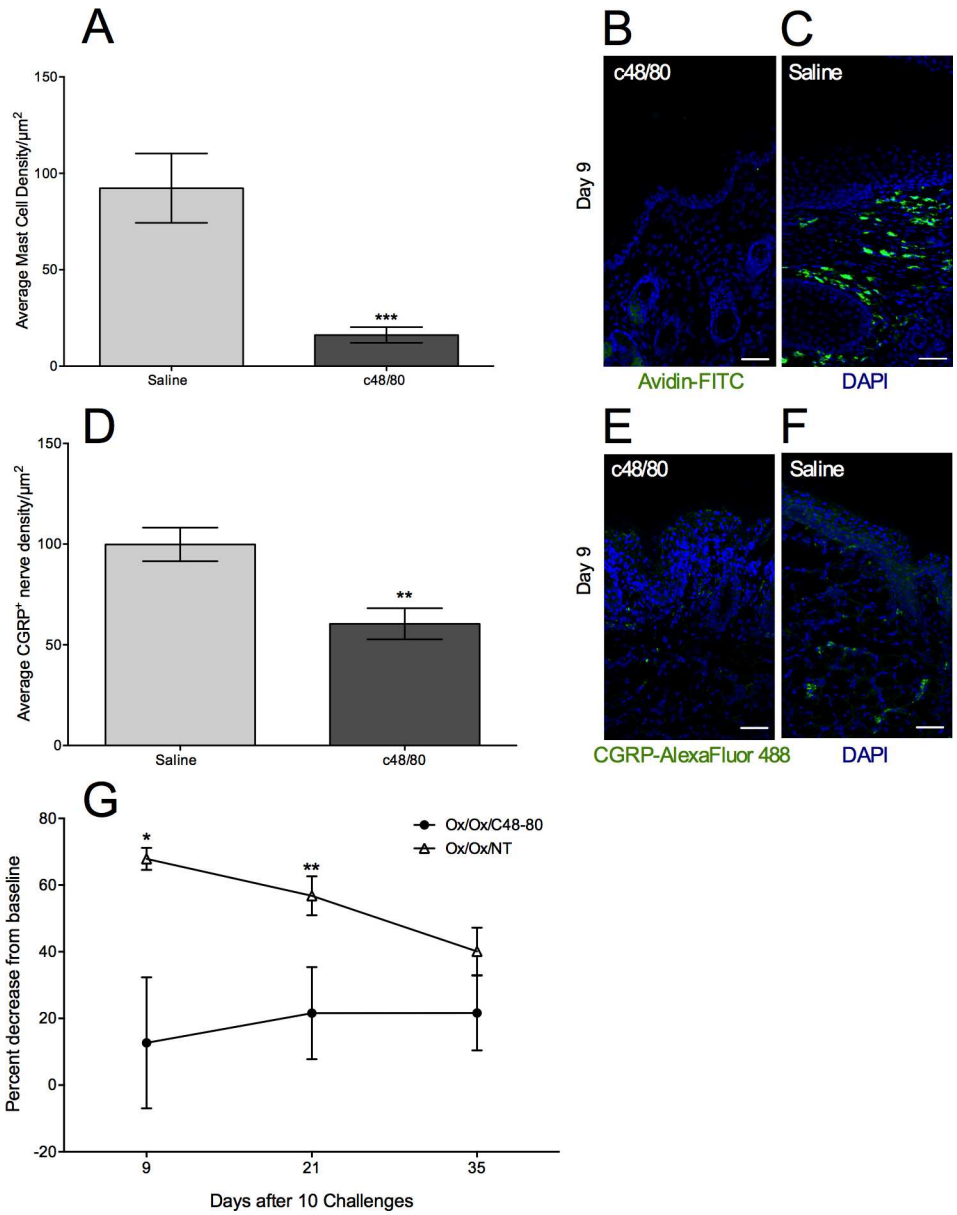

Fig 5. Injection of c48/80 after challenges depletes mast cells and reduces CGRP⁺ nerve density and sensitivity. Density of Avidin⁺ mast cells (A) and CGRP⁺ cutaneous nerves (D) on day 9 after 4 treatments with c48/80 or saline (administered on days 5–8 after cessation of 10 Ox challenges) in 10 μm labiar cryo-sections, displayed as mean ± SEM (n = 2-3/treatment group). Representative images for mast cells (B-C) and nerves (E-F); 20x magnification; scale bar represents 50 μm. Means are compared to Ox/EtOH (** = p<0.01, *** = p<0.001); significance determined using one-way ANOVA and Tukey Kramer *post hoc* analysis. (G) Tactile sensitivity in mice treated with either saline or c48/80 (n = 6–9 mice per treatment group; two independent experiments). Means are compared to Ox/Ox/Saline (* = p<0.05, ** = p<0.01, *** = p<0.001); significance determined using an unpaired Student's T test at each time point.

doi:10.1371/journal.pone.0169672.g005

Ox-challenged labiar skin yields very few cells after enzymatic digestion, we set up experiments where mice were sensitized on the back with 2% Ox and challenged on both flanks with 1% Ox (Fig 1B) to obtain larger Ox-challenged skin samples. One day after 10 Ox challenges, flow cytometric analysis of flank skin from Ox-sensitized and challenged ND4 mice showed an accumulation of $CD3^+CD4^+$ cells, ~32% of which were $CD25^+FoxP3^+$. Few

$CD4^+CD25^+FoxP3^+$ cells were detected in vehicle-challenged mice (Fig 6A–6D). Approximately 625 $CD4^+CD25^+FoxP3^+$ cells were recovered from 25 mm x 25 mm of flank skin of each mouse challenged with Ox compared to <60 $CD4^+CD25^+FoxP3^+$ cells detected in skin samples from vehicle-challenged mice.

IFN-γ signaling drives mast cell contributions to chronic asthma-like tissue remodeling seen in ovalbumin-challenged mice [23]. Transcripts encoding IFN- were elevated ~500-fold in Ox vs. vehicle-challenged labiar skin 1 day after 10 Ox challenges and remained elevated ~10-fold over controls at day 21 (Fig 6E). *Tbx21* mRNA encoding T-bet, a known driver of IFN-γ production in multiple cell types [24], was also increased ~100-fold in Ox-challenged over vehicle-challenged labiar skin on day 1, and remained elevated ~10-fold at day 21 (Fig 6E). On day 1, total IFN-γ protein levels were also significantly increased in Ox-challenged vs. untreated labiar skin (Fig 6F). $CD3^+CD8^+CD103^+CD44^+$ resident memory cells [25] isolated from the flank skin after 10 Ox challenges and cultured for 18 hours with and without PMA/ionomycin stimulation produced detectable IFN-γ, suggesting they could be potential sources of IFN-γ *in vivo* (Fig 6G and 6H).

Discussion

Ten labiar Ox challenges in sensitized ND4 mice provoked increased tactile sensitivity that lasted over three weeks after challenge cessation, and resulted in a marked accumulation of mast cells and overgrowth of $CGRP^+$ sensory neurons at the site of allergen challenge. Painful sensitivity to touch, mast cell increases, and nerve overgrowth in the absence of overt inflammation are consistent with the most common characteristics of localized, provoked vulvodynia [2]. A diagnosis of vulvodynia is made in the clinic by reporting painful sensations in response to palpation of the vestibule, mons, labia, perianal and perineal areas by applying light consistent pressure with a cotton swab, similar to our method of measuring responses to pressure in the ano-genital ridge of mice [5]. While some vulvodynia patients report referred peripheral sensitivity in non-vestibular sites [26], we did not find altered sensitivity in the hind paw tissue of mice with lowered labiar withdrawal threshold for pressure. Kakurai et al. reported mast cell-dependent nerve elongation after a single Ox challenge in sensitized C57BL/6 mice [16]. Earlier, we showed that nerve density increases within 48 hours after one or three Ox challenges [9]. Here, we found that after 10 Ox challenges, local nerve density increased 3–4 fold and remained elevated for over three weeks. This allergen-provoked local hyper-innervation in mice sensitive to touch and pressure parallels the increase in vestibular innervation seen at the painful sites of women diagnosed with vulvodynia [7].

Mast cells accumulate in affected tissues of C57BL/6 mice following repeated Ox challenges [16, 27, 28]. We noted a concomitant ~4-fold increase in the abundance of tissue mast cells at day 1 and a ~2-fold increase in total histamine content that persisted through day 21 after 10 labiar Ox challenges. Increases in mast cell numbers and degranulation [6, 29] as well as secondary mast cell hyperplasia [30] have been reported in vulvar biopsies of women diagnosed with vulvodynia. We are currently investigating the extent of mast cell activation and degranulation at the sensitive sites.

Mast cell-nerve interactions contribute to multiple inflammatory pathologies [10, 11]. Mast cells can produce NGF in both rodents [31] and humans [32]. Increased CADM1 on mast cells has been identified in atopic dermatitis and pulmonary emphysema [33] and has been shown to enhance nerve-mast cell interaction in a mouse model of trinitrochlorobenzene-driven contact hypersensitivity [20]. Here, we found significantly elevated *Ngf* and *Cadm1* mRNAs in touch-sensitive, allergen-challenged mice over vehicle-treated controls on 1 day after 10 Ox challenges. However, these differences were less pronounced by day 21, suggesting that these

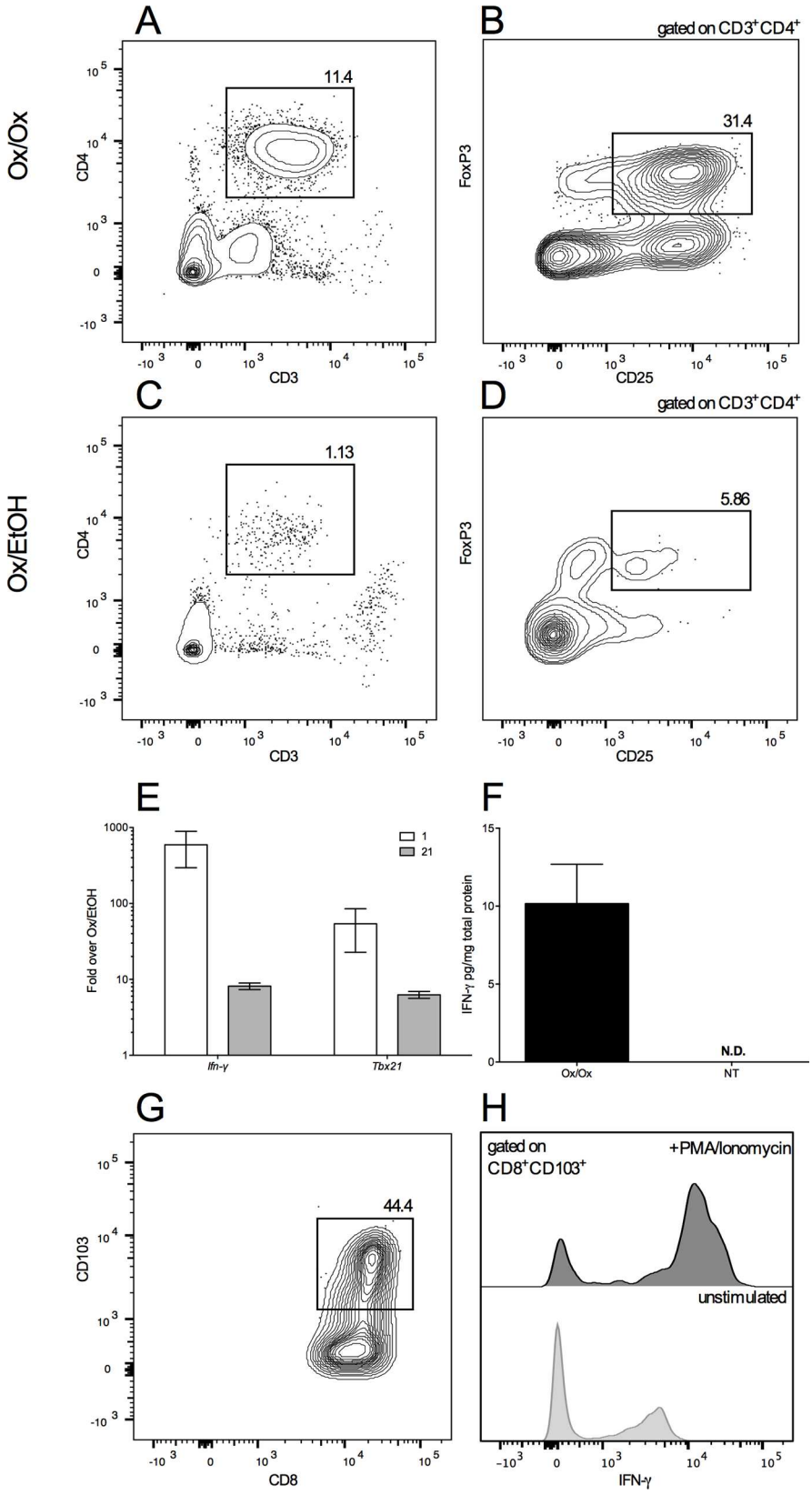
A
B
gated on CD3+CD4+
Ox/Ox
11.4
31.4
CD4
FoxP3
CD3
CD25
C
D
gated on CD3+CD4+
Ox/EtOH
1.13
5.86
CD4
FoxP3
CD3
CD25
E
F
1
21
Fold over Ox/EtOH
Ifn-γ
Tbx21
IFN-γ pg/mg total protein
N.D.
Ox/Ox
NT
G
H
44.4
CD103
CD8
gated on
CD8+CD103+
+PMA/Ionomycin
unstimulated
IFN-γ

Fig 6. $CD4^+CD25^+FoxP3^+$ T cells and IFN-γ producing $CD8^+CD103^+$ memory T cells accumulate in oxazolone challenged skin. Flow cytometric analysis of collagenase-digested, gradient-separated flank skin cells from Ox- (A-B) or EtOH- (C-D) challenged mice 1 day after the cessation of 10 challenges. Cells in (A) and (C) are scatter-gated for lymphocytes, single cells, live, and $CD45^+$. Data are pooled from 10 mice per treatment group. Relative abundance of *Ifn-γ* and *Tbx21* (E) and total IFN-*γ* protein content (F) in labiar skin of Ox- vs. EtOH challenged mice after 10 challenges displayed as mean ± SEM (n = 5-6/treatment group). (G-H) Flow cytometric analysis of collagenase-digested, gradient-separated flank skin cells collected 1 day after 10 challenges from Ox-challenged mice and cultured for 18 hours with or without PMA/ionomycin; cells in (G) are live, singlet-gated, scatter-gated for lymphocytes, and $CD45^+CD3^+CD8^+$.

doi:10.1371/journal.pone.0169672.g006

are early markers of tissue changes that support the increased innervation facilitating prolonged sensitivity. Local changes in NGF and CADM1 have not been reported in vulvodynia patients. However, patient samples are usually acquired after symptoms have been established for some time, and these molecular changes may not be detectable later. Levels of total IgE antibodies were significantly increased in both the serum and vaginal lavage of Ox-challenged mice. In one study, ~30% of vulvodynia patients presented elevated vaginal IgE including 16 women with IgE antibodies against seminal fluid antigens [34]. Differences in serum IgE have not been assessed in vulvodynia patients to date. Circulating IgEs are a survival factor for mast cells acting via IL-6 and IL-13 [16]. We detected increased levels of transcripts encoding both of these cytokines in the labiar skin of Ox-challenged but not vehicle-challenged mice.

The characteristics of inflammatory pathways that contribute to vulvodynia pathology remain poorly understood [35]. TNF-α and IL-1β are elevated in vestibular tissue homogenates from vulvodynia patients [36]. Additionally, vulvar fibroblasts derived from patients as opposed to controls produce larger amounts of IL-6 and IL-8 when challenged with yeast antigen [37], and are responsive *in vitro* to low doses of *Candida albicans* in a Dectin-1-dependent manner [38]. Yeast infections can predispose to vulvodynia [39] and others have shown increased ano-genital sensitivity in mice following repeated vulvovaginal candidiasis [14]. In our study, transcripts encoding TNF-α and CXCL-2 (the murine homolog of human IL-8) were also increased relative to controls in Ox-challenged mice (S3 Fig). CXCL2 protein levels were elevated in experimental vs. untreated mice (S3 Fig). Given the high expression of *Cxcl2* mRNAs and protein in Ox-treated mice, we measured neutrophil influx into the affected skin since CXCL-2 is a potent attractant for these cells [40] but found no changes in levels of activated neutrophils (S3 Fig).

Ectopic germinal center-like structures containing T cells, B cells, macrophages and dendritic cells have been reported in vestibular tissue from vulvodynia patients [41]. We detected T cell accumulation in Ox-challenged murine skin suggesting an overall inflammatory profile. Regulatory T cells have been shown to be required for mast cell progenitor recruitment into allergic airways of ovalbumin-challenged mice [21]. Ox-challenged but not vehicle-treated mice in our experiments show distinct infiltration of $CD4^+CD25^+FoxP3^+$ cells that could, in turn, recruit mast cells into affected labiar skin. Mast cells also require IFN-γ signaling to mediate immune infiltration and pulmonary remodeling in a mouse model of chronic asthma [23]. IFN-γ transcripts showed the most substantial and sustained increase in Ox-challenged labiar skin over controls in our experiments. One day after the last allergen challenge, IFN-γ protein levels were also increased ~10-fold over untreated controls. *Tbx21* mRNAs encoding T-bet–a potent driver of IFN-γ production–were also increased in labiar tissue at this time. $CD3^+CD8^+CD103^+CD44^+$ resident memory T cells identified in Ox-challenged mice were positive for IFN-γ by intracellular staining both without stimulation and after overnight incubation with PMA/ionomycin, suggesting they could produce IFN-γ *in situ*. These findings are in agreement with a recent report showing IFN-γ^+ resident memory $CD8^+$ T cells in the ear pinnae of mice challenged monthly with Ox (for a total of three challenges) after sensitization

[42]. While it remains to be seen whether mast cell-driven tissue changes in the skin are similar to those described in the airway [20], it appears that IFN-γ is present in the affected tissue, at least at an early stage. Overall, the inflammatory skin microenvironment after repeated Ox exposures has cellular and molecular characteristics that can support the recruitment and activity of mast cells that are potentially critical to the subsequent increases in innervation and tactile sensitivity. Gimenez-Rivera and colleagues showed that mast cell-deficient mice have exacerbated chronic, but not acute, contact hypersensitivity to Ox [28]. It is possible that MC accumulation at allergic sites drives the resolution of dermal inflammation after prolonged allergen exposure while potentially promoting a persistent increase in sensitivity. Farmer *et al.* reported that vaginal candidiasis-induced ano-genital sensitivity in mice was accompanied by hyper-innervation without detectable changes in immune cell status [14]. While we assay labiar skin of ectodermal origin and Farmer and colleagues targeted vaginal epithelium of mesodermal origin, clinical measurements of pain and inflammation are conducted in human vestibular tissue of endodermal origin [42]. Whether embryonic origin of tissue affects tactile sensitivity is currently unknown. In ongoing studies, we are comparing the effects of multiple hapten exposures in the vaginal epithelium and the labia.

Our findings indicate that the local tissue microenvironment supports concomitant increases in nerves and mast cells after repeated allergen challenges. Therefore, it is likely that nerve-mast cell interactions are important in the formation of the localized, provoked sensitivity to touch that ensues. When we depleted local mast cells in the labiar tissue with a short-term administration of c48/80 after challenge cessation, we found a reduction in both local sensory innervation and tactile sensitivity. While therapeutic administration of the mast cell stabilizer sodium cromolyn has been ineffective in idiopathic vulvar vestibulitis [43], we show here that mast cell depletion, rather than stabilization, can potentially reduce hyperinnervation and enhanced sensitivity to touch that occurs after repeated exposures to allergen.

Our work provides strong evidence that repeated allergen-induced mechanical sensitivity is at least in part mediated by *de novo* nerve growth at the site of challenge, and warrants further investigation into mechanisms of allergy-induced nerve sprouting that contribute to pain amplification. Others have shown sprouting of cutaneous sensory neurons in the hind paws of mice under inflammatory conditions associated with thermal and mechanical pain [44] as well as Complete Freund's Adjuvant-induced arthritis and skin inflammation [45].

We have developed a new, clinically relevant murine model of persistent allergy-provoked tactile sensitivity driven by a mast cell-centered tissue response. Early inflammatory events set the stage for long-term mast cell increases in the tissue that then support the overgrowth and stability of sensory neurons. While the current study focuses on the biological plausibility of allergy-driven tissue changes, repeated injury or infection at a particular tissue site may also stimulate similar remodeling responses. Mechanisms underlying recruitment of mast cell progenitors in tissue remodeling are being elucidated in many models of allergic disease [46]. Since increased vestibular mast cell accumulation is one of the most consistent characteristics of vulvodynia, specifically targeting tissue-resident mast cells as well as mast cell recruitment into tissues may be viable therapeutic approaches not only for allergen-provoked vulvodynia but also for other forms of the condition epidemiologically associated with a history of infections [39] or violence-related injury [47]. Such therapeutic strategies may also limit chronic pain associated with increased accumulation and activation of mast cells in a diverse array of pathologies including cystitis [48], migraines [49] and inflammatory bowel disorders [50].

Supporting information

S1 Fig. Labiar challenges do not reduce hindpaw withdrawal thresholds; estrus stage does not affect sensitivity measurements. (A-C) Withdrawal thresholds of (A) Ox-sensitized mice challenged 10x with Ox on the labia, (B) Ox-sensitized mice challenged 10x with ethanol on the labia, and (C) untreated mice. Each colored symbol represents a longitudinally assessed animal. (D) Sensitized mice that received ten daily Ox challenges to the vulvar region have no significant change in tactile sensitivity in the hind paw footpad compared to vehicle-treated controls; percent change in withdrawal threshold of each treatment group is displayed as mean ± SEM. n = 7–8 mice per treatment group. Data fit to a two-way ANOVA with interaction and repeated measures show no significant effect of time, treatment, interaction of the two, or random effects on hindpaw sensitivity after labiar Ox challenge. (E) Vaginal lavage smears were collected from mice for four consecutive days after the cessation of 10 oxazolone challenges to the labia and stained with 0.1% crystal violet as previously described [51]. Relative proportions of nucleated epithelial cells, cornified epithelial cells, and leukocytes were quantified to assign mice to stage of cycle. No differences in Ox-provoked sensitivity were found at day 1 or 21 after challenge cessation between mice in the estrus, pro-estrus, diestrus and metestrus stages. Raw withdrawal thresholds are summarized in S4 Table. (F) Ox-challenged mice (left) show no obvious signs of inflammation at 21 days after challenge cessation, despite enhanced sensitivity to pressure at this time point. Ethanol-challenged mice (right) are shown for comparison.
(TIFF)

S2 Fig. Four intra-labiar saline injections on days 5–8 after challenge cessation do not affect tactile sensitivity. Changes in labiar withdrawal threshold of Ox-challenged mice that received intra-labiar injections of 0.9% saline are not significantly different (p = 0.3766) from those seen in mice challenged with Ox alone (n = 9/treatment group). Shown here is mean ± SEM (Student's t-test).
(TIFF)

S3 Fig. Ox challenges are associated with increasedTNF-α and CXCL-2 mRNA and protein without neutrophil influx. (A) Relative abundance of *Tnf*-α and *Cxcl-2* is increased in Ox- vs. EtOH-challenged mice 1 and 21 days after 10 challenges, displayed as mean ± SEM (n = 5-6/treatment group; two independent experiments). (B) Total CXCL-2 protein content is increased in Ox- vs. untreated mice 1 day after challenge cessation measured by ELISA (R&D Systems, manufacturer's directions) displayed as mean ± SEM (n = 4-6/treatment group). (C) Ox-, EtOH-challenged mice and untreated controls have similar myeloperoxidase activity in the labiar skin. To measure myeloperoxidase activity, samples were frozen at -80˚C in 50 mM K2HPO4 buffer (pH 6.0) with 0.05% hexadecyl trimethylammonium bromide (HTAB), thawed, homogenized in 5x volumes of HTAB buffer, sonicated 3x for 10 s, frozen and thawed 3x, re-sonicated, and centrifuged for four minutes. Absorbance was recorded at 450 nm after a 20-min incubation in 50 mM phosphate buffer (pH 6.0) with 0.025% hydrogen peroxide and 0.167 mg/mL *o*-dianisidine dihydrochloride at room temperature in the dark [52]. Myeloperoxidase levels are normalized to tissue weight and displayed as OD/g of wet tissue. Shown here is mean ± SEM (n = 3/treatment group).
(TIFF)

S1 Table. Labiar withdrawal threshold values of untreated, pre-sensitized Ox challenged, ethanol-challenged, and c48/80-treated Ox-challenged mice.
(DOCX)

S2 Table. Lineage and activation markers used to identify skin-infiltrating cells in oxazolone-challenged labia.
(DOCX)

S3 Table. Accession numbers for Taqman primer-probe sets (Life Technologies, Carlsbad, CA) for semi-quantitative PCR used to measure relative transcript abundance in Ox-challenged labia.
(DOCX)

S4 Table. Hindpaw withdrawal thresholds to pressure in mice challenged on the labia, and labiar withdrawal thresholds at different stages of the estrus cycle after Ox challenge cessation.
(DOCX)

Acknowledgments

The authors thank past and current members of the Chatterjea laboratory, particularly Nita Chai, Alyssa Ashbaugh, Megan Schermerhorn, Hilary McLeland-Wieser, Erica Arriaga-Gomez, and Emily Ewan for their help, Dr. Jason Mitchell in the Fife laboratory for his assistance, Dr. Vittorio Addona for biostatistics advice, Jamie Atkins for animal care, Patty Byrne Pfalz for administrative support, Kim David and Amy Pelz for graphic design, and Dr. David Matthes and Dr. Bernard Harlow for helpful discussions and feedback on the manuscript.

Author contributions

Conceptualization: DC JL TM.

Formal analysis: DC JL TM HM JD CJB JK BB LS ET.

Funding acquisition: DC RD BTF.

Investigation: DC JL TM HM JD CJB JK BB LS ET.

Methodology: DC TM.

Project administration: DC.

Resources: DC RD BTF.

Supervision: DC.

Validation: DC JL TM HM JD CJB JK BB LS ET.

Visualization: DC JL TM.

Writing – original draft: DC JL TM.

Writing – review & editing: DC JL TM.

References

1. Wesselmann U, Bonham A, Foster D. Vulvodynia: Current state of the biological science. Pain. 2014; 155:1696–1701. doi: 10.1016/j.pain.2014.05.010 PMID: 24858303
2. Pukall CF, Goldstein AT, Bergeron S, Foster D, Stein A, Kellogg-Spadt S, et al. Vulvodynia: Definition, Prevalence, Impact, and Pathophysiological Factors. J Sex Med. 2016; 13:291–304. doi: 10.1016/j.jsxm.2015.12.021 PMID: 26944461

3. Harlow BL, Kunitz CG, Nguyen RH, Rydell SA, Turner RM, MacLehose RF. Prevalence of symptoms consistent with a diagnosis of vulvodynia: population-based estimates from 2 geographic regions. Am J Obstet Gynecol. 2014; 210:40.e1–8.
4. Reed BD, Harlow SD, Sen A, Legocki LJ, Edwards RM, Arato N, et al. Prevalence and demographic characteristics of vulvodynia in a population-based sample. Am J Obstet Gynecol. 2012; 206:170.e1–9.
5. Sadownik LA. Etiology, diagnosis, and clinical management of vulvodynia. Int J Womens Health. 2014; 6:437–449. doi: 10.2147/IJWH.S37660 PMID: 24833921
6. Bornstein J, Goldschmid N, Sabo E. Hyperinnervation and mast cell activation may be used as histopathologic diagnostic criteria for vulvar vestibulitis. Gynecol Obstet Invest. 2004; 58:171–178. doi: 10.1159/000079663 PMID: 15249746
7. Goetsch MF, Morgan TK, Korcheva VB, Li H, Peters D, Leclair CM. Histologic and receptor analysis of primary and secondary vestibulodynia and controls: a prospective study. Am J Obstet Gynecol. 2010; 202:614.e1–8.
8. Harlow BL, He W, Nguyen RH. Allergic reactions and risk of vulvodynia. Ann Epidemiol. 2009; 19:771–777. doi: 10.1016/j.annepidem.2009.06.006 PMID: 19825458
9. Martinov T, Glenn-Finer R, Burley S, Tonc E, Balsells E, Ashbaugh A, et al. Contact hypersensitivity to oxazolone provokes vulvar mechanical hyperalgesia in mice. PLoS One. 2013; 8:e78673. doi: 10.1371/journal.pone.0078673 PMID: 24205293
10. Chatterjea D, Martinov T. Mast cells: versatile gatekeepers of pain. Mol Immunol. 2015; 63:38–44. doi: 10.1016/j.molimm.2014.03.001 PMID: 24666768
11. Forsythe P, Bienenstock J. The mast cell-nerve functional unit: a key component of physiologic and pathophysiologic responses. Chem Immunol Allergy. 2012; 98:196–221. doi: 10.1159/000336523 PMID: 22767065
12. Benck CJ, Martinov T, Fife BT, Chatterjea D. Isolation of Infiltrating Leukocytes from Mouse Skin Using Enzymatic Digest and Gradient Separation. J Vis Exp. 2016; 107.
13. Piovezan AP, D'Orléans-Juste P, Frighetto M, Souza GE, Henriques MG, Rae GA. Endothelins contribute towards nociception induced by antigen in ovalbumin-sensitised mice. Br J Pharmacol. 2004; 141:755–763. doi: 10.1038/sj.bjp.0705663 PMID: 14744803
14. Farmer MA, Taylor AM, Bailey AL, Tuttle AH, MacIntyre LC, Milagrosa ZE, et al. Repeated vulvovaginal fungal infections cause persistent pain in a mouse model of vulvodynia. Sci Transl Med. 2011; 3:101ra91. doi: 10.1126/scitranslmed.3002613 PMID: 21937756
15. Martinov T, Mack M, Sykes A, Chatterjea D. Measuring changes in tactile sensitivity in the hind paw of mice using an electronic von Frey apparatus. J Vis Exp. 2013; 82.
16. Kakurai M, Monteforte R, Suto H, Tsai M, Nakae S, Galli SJ. Mast cell-derived tumor necrosis factor can promote nerve fiber elongation in the skin during contact hypersensitivity in mice. Am J Pathol. 2006; 169:1713–1721. doi: 10.2353/ajpath.2006.060602 PMID: 17071594
17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (–Delta Delta C(T)). Methods. 2001; 25;402–408. doi: 10.1006/meth.2001.1262 PMID: 11846609
18. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils. J Allergy Clin Immunol. 2010; 125:S73–80. doi: 10.1016/j.jaci.2009.11.017 PMID: 20176269
19. Bax HJ, Keeble AH, Gould HJ. Cytokinergic IgE Action in Mast Cell Activation. Front Immunol. 2012; 3:229. doi: 10.3389/fimmu.2012.00229 PMID: 22888332
20. Hagiyama M, Inoue T, Furuno T, Iino T, Itami S, Nakanishi M, et al. Increased expression of cell adhesion molecule 1 by mast cells as a cause of enhanced nerve-mast cell interaction in a hapten-induced mouse model of atopic dermatitis. Br J Dermatol. 2013; 168:771–778. doi: 10.1111/bjd.12108 PMID: 23106683
21. Jones TG, Finkelman FD, Austen KF, Gurish MF. T regulatory cells control antigen-induced recruitment of mast cell progenitors to the lungs of C57BL/6 mice. J Immunol. 2010; 185:1804–1811. doi: 10.4049/jimmunol.1001146 PMID: 20601599
22. Gounaris E, Blatner NR, Dennis K, Magnusson F, Gurish MF, Strom TB, et al. T-regulatory cells shift from a protective anti-inflammatory to a cancer-promoting proinflammatory phenotype in polyposis. Cancer Res. 2009; 69:5490–5497. doi: 10.1158/0008-5472.CAN-09-0304 PMID: 19570783
23. Yu M, Eckart MR, Morgan AA, Mukai K, Butte AJ, Tsai M, et al. Identification of an IFN-γ/mast cell axis in a mouse model of chronic asthma. J Clin Invest. 2011; 121:3133–3143. doi: 10.1172/JCI43598 PMID: 21737883
24. Lazarevic V, Glimcher LH, Lord GM. T-bet: a bridge between innate and adaptive immunity. Nat Rev Immunol. 2013; 13:777–789. doi: 10.1038/nri3536 PMID: 24113868

25. Schenkel JM, Masopust D. Tissue-resident memory T cells. Immunity. 2014; 41:886–897. doi: 10.1016/j.immuni.2014.12.007 PMID: 25526304

26. Giesecke J, Reed BD, Haefner HK, Giesecke T, Clauw DJ, Gracely RH. Quantitative sensory testing in vulvodynia patients and increased peripheral pressure pain sensitivity. Obstet Gynecol. 2004; 104:126–133. doi: 10.1097/01.AOG.0000129238.49397.4e PMID: 15229011

27. Hershko AY, Suzuki R, Charles N, Alvarez-Errico D, Sargent JL, Laurence A, et al. Mast cell interleukin-2 production contributes to suppression of chronic allergic dermatitis. Immunity. 2011; 35:562–571. doi: 10.1016/j.immuni.2011.07.013 PMID: 21982597

28. Gimenez-Rivera V, Siebenhaar F, Zimmermann C, Siiskonen H, Metz M, Maurer M. Mast Cells Limit the Exacerbation of Chronic Allergic Contact Dermatitis in Response to Repeated Allergen Exposure. J Immunol. 2016.

29. Bornstein J, Cohen Y, Zarfati D, Sela S, Ophir E. Involvement of heparanase in the pathogenesis of localized vulvodynia. Int J Gynecol Pathol. 2008; 27:136–41. doi: 10.1097/pgp.0b013e318140021b PMID: 18156988

30. Regauer S, Eberz B, Beham-Schmid C. Mast cell infiltrates in vulvodynia represent secondary and idiopathic mast cell hyperplasias. APMIS. 2015; 123:452–6. doi: 10.1111/apm.12372 PMID: 25912132

31. Leon A, Buriani A, Dal Toso R, Fabris M, Romanello S, Aloe L, et al. Mast cells synthesize, store, and release nerve growth factor. Proc Natl Acad Sci USA. 1994; 91:3739–3743. PMID: 8170980

32. Xiang Z, Nilsson G. IgE receptor-mediated release of nerve growth factor by mast cells. Clin Exp Allergy. 2000; 30:1379–1386. PMID: 10998013

33. Yoneshige A, Hagiyama M, Fujita M, Ito A. Pathogenic Actions of Cell Adhesion Molecule 1 in Pulmonary Emphysema and Atopic Dermatitis. Front Cell Dev Biol. 2015; 3:75. doi: 10.3389/fcell.2015.00075 PMID: 26636084

34. Babula O, Bongiovanni AM, Ledger WJ, Witkin SS. Immunoglobulin E antibodies to seminal fluid in women with vulvar vestibulitis syndrome: relation to onset and timing of symptoms. Am J Obstet Gynecol. 2004; 190:663–667. doi: 10.1016/j.ajog.2003.09.031 PMID: 15041996

35. Akopians AL, Rapkin AJ. Vulvodynia: The Role of Inflammation in the Etiology of Localized Provoked Pain of the Vulvar Vestibule (Vestibulodynia). Semin Reprod Med. 2015; 33:239–245. doi: 10.1055/s-0035-1554919 PMID: 26132928

36. Foster DC, Hasday JD. Elevated tissue levels of interleukin-1 beta and tumor necrosis factor-alpha in vulvar vestibulitis. Obstet Gynecol. 1997; 89:291–296. doi: 10.1016/S0029-7844(96)00447-4 PMID: 9015038

37. 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.

38. Falsetta ML, Foster DC, Woeller CF, Pollock SJ, Bonham AD, Haidaris CG, et al. Identification of novel mechanisms involved in generating localized vulvodynia pain. Am J Obstet Gynecol. 2015; 213:38.e1–12.

39. Nguyen RH, Swanson D, Harlow BL. Urogenital infections in relation to the occurrence of vulvodynia. J Reprod Med. 2009; 54:385–392. PMID: 19639929

40. De Filippo K, Dudeck A, Hasenberg M, Nye E, van Rooijen N, Hartmann K, et al. Mast cell and macrophage chemokines CXCL1/CXCL2 control the early stage of neutrophil recruitment during tissue inflammation. Blood. 2013; 121:4930–4937. doi: 10.1182/blood-2013-02-486217 PMID: 23645836

41. Tommola P, Bützow R, Unkila-Kallio L, Paavonen J, Meri S. Activation of vestibule-associated lymphoid tissue in localized provoked vulvodynia. Am J Obstet Gynecol. 2015; 212:476.e1–8.

42. Farage MA, Maibach HI. Morphology and physiological changes of genital skin and mucosa. Curr Probl Dermatol. 2011; 40:9–19. doi: 10.1159/000321042 PMID: 21325836

43. Nyirjesy P, Sobel JD, Weitz MV, Leaman DJ, Small MJ, Gelone SP. Cromolyn cream for recalcitrant idiopathic vulvar vestibulitis: results of a placebo controlled study. Sex Transm Infect. 2001; 77:53–7. doi: 10.1136/sti.77.1.53 PMID: 11158692

44. Chakrabarty A, McCarson KE, Smith PG. Hypersensitivity and hyperinnervation of the rat hind paw following carrageenan-induced inflammation. Neurosi Lett. 2011; 495;67–71.

45. Almarestani L, Longo G, Ribeiro-da-Silva A. Autonomic fiber sprouting in the skin in chronic inflammation. Mol Pain. 2008; 4.

46. Dahlin JS, Hallgren J. Mast cell progenitors: origin, development and migration to tissues. Mol Immunol. 2015; 63:9–17. doi: 10.1016/j.molimm.2014.01.018 PMID: 24598075

47. Harlow BL, Stewart EG. Adult-onset vulvodynia in relation to childhood violence victimization. Am J Epidemiol. 2005; 161:871–880. doi: 10.1093/aje/kwi108 PMID: 15840620

48. Rudick CN, Bryce PJ, Guichelaar LA, Berry RE, Klumpp DJ. Mast cell-derived histamine mediates cystitis pain. PLoS One. 2008; 3:e2096. doi: 10.1371/journal.pone.0002096 PMID: 18461160

49. Loewendorf AI, Matynia A, Saribekyan H, Gross N, Csete M, Harrington M. Roads Less Traveled: Sexual Dimorphism and Mast Cell Contributions to Migraine Pathology. Front Immunol. 2016; 7:140. doi: 10.3389/fimmu.2016.00140 PMID: 27148260

50. Zhang L, Song J, Hou X. Mast Cells and Irritable Bowel Syndrome: From the Bench to the Bedside. J Neurogastroenterol Motil. 2016; 22:181–192. doi: 10.5056/jnm15137 PMID: 26755686

51. McLean AC, Valenzuela N, Fai S, Bennett SA. Performing Vaginal Lavage, Crystal Violet Staining, and Vaginal Cytological Evaluation for Mouse Estrous Cycle Staging Identification. J Vis Exp. 2012; 67; e4389.

52. Chatterjea D, Wetzel A, Mack M, Engblom C, Allen J, Mora-Solano C, et al. Mast cell degranulation mediates compound 48/80-induced hyperalgesia in mice. Biochem Biophys Res Commun. 2012; 425;237–243. doi: 10.1016/j.bbrc.2012.07.074 PMID: 22828511

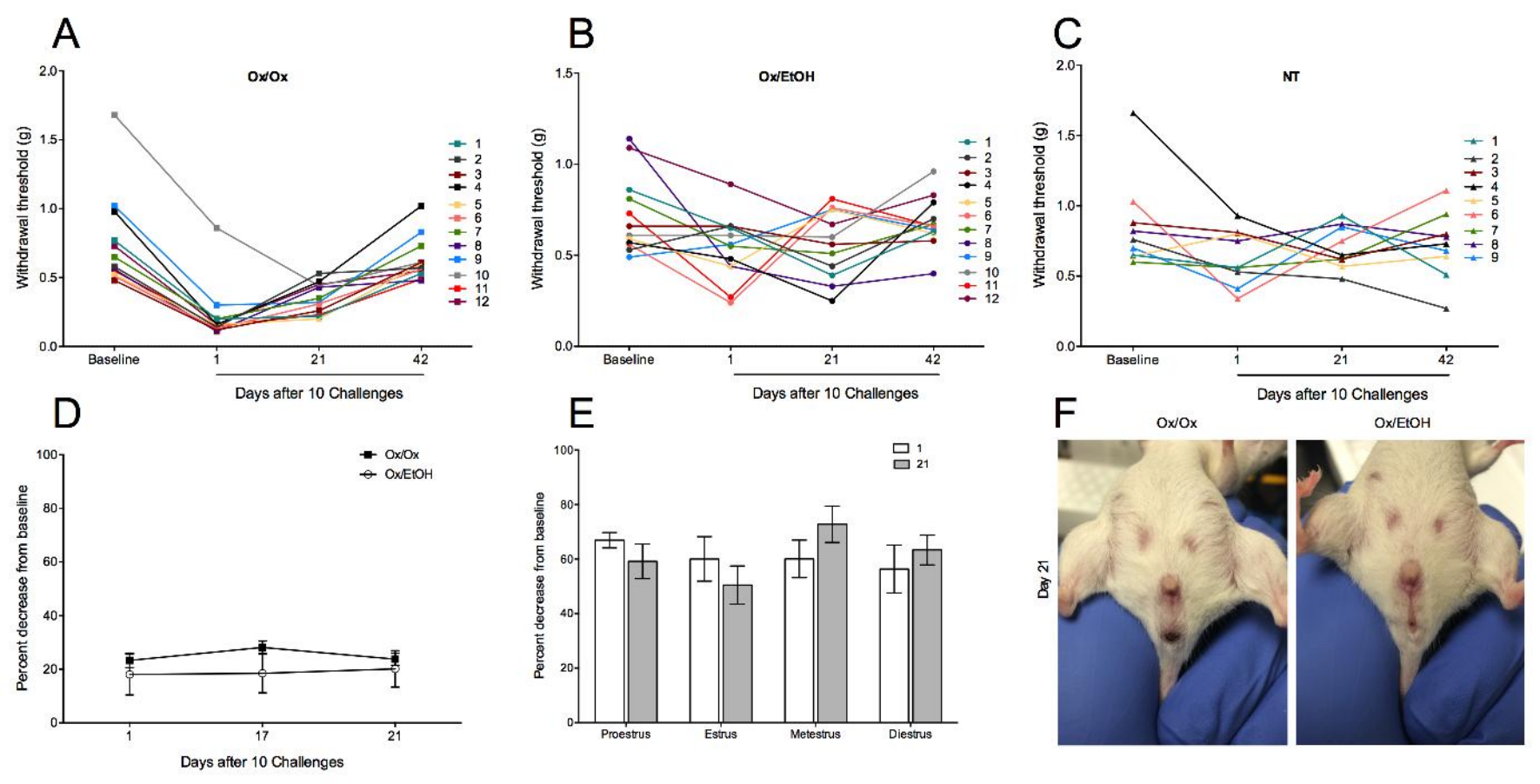
A
Ox/Ox
Withdrawal threshold (g)
2.0
1.5
1.0
0.5
0.0
Baseline
1
21
42
Days after 10 Challenges
1
2
3
4
5
6
7
8
9
10
11
12
B
Ox/EtOH
Withdrawal threshold (g)
1.5
1.0
0.5
0.0
Baseline
1
21
42
Days after 10 Challenges
1
2
3
4
5
6
7
8
9
10
11
12
C
NT
Withdrawal threshold (g)
2.0
1.5
1.0
0.5
0.0
Baseline
1
21
42
Days after 10 Challenges
1
2
3
4
5
6
7
8
9
D
Ox/Ox
Ox/EtOH
Percent decrease from baseline
100
80
60
40
20
0
1
17
21
Days after 10 Challenges
E
1
21
Percent decrease from baseline
100
80
60
40
20
0
Proestrus
Estrus
Metestrus
Diestrus
F
Ox/Ox
Ox/EtOH
Day 21

Table S1. Labiar withdrawal threshold values of untreated, pre-sensitized Ox challenged, ethanol-challenged, and c48/80-treated Ox-challenged mice. (Top) Shown are withdrawal thresholds (mean ± SEM; in grams) for shaved untreated (NT; n = 9), or Ox-sensitized ND4 mice challenged on the labia 10 times with either vehicle (Ox/EtOH (10); n = 12) or Ox (Ox/Ox (10); n = 12) four days after sensitization. Percent change in withdrawal thresholds are shown in Figure 2. (Bottom) Shown are withdrawal thresholds (mean ± SEM; in grams) for Ox-sensitized and challenged ND4 mice that that did not receive intralabiar injection (Ox/Ox/NT), received saline (Ox/Ox/saline), or c48/80 (Ox/Ox/c48-80) on days 5-8 after the last Ox challenge. Percent change in withdrawal thresholds are shown in Figure 5G and Figure S2.

		Day after 10 Challenges		
Treatment	**Baseline**	**1**	**21**	**42**
NT	0.86 ± 0.11	0.63 ± 0.06	0.70 ± 0.05	0.72 ± 0.08
Ox/EtOH (10)	0.72 ± 0.06	0.54 ± 0.05	0.57 ± 0.05	0.68 ± 0.04
Ox/Ox (10)	0.75 ± 0.10	0.22 ± 0.06	0.35 ± 0.03	0.63 ± 0.04
Treatment	**Baseline**	**9**	**21**	**35**
Ox/Ox/NT	0.84 ± 0.05	0.36 ± 0.05	0.36 ± 0.04	0.43 ± 0.05
Ox/Ox/saline	0.83 ± 0.11	0.40 ± 0.09	0.26 ± 0.02	0.50 ± 0.06
Ox/Ox/c48-80	0.66 ± 0.05	0.44 ± 0.10	0.43 ± 0.05	0.46 ± 0.06

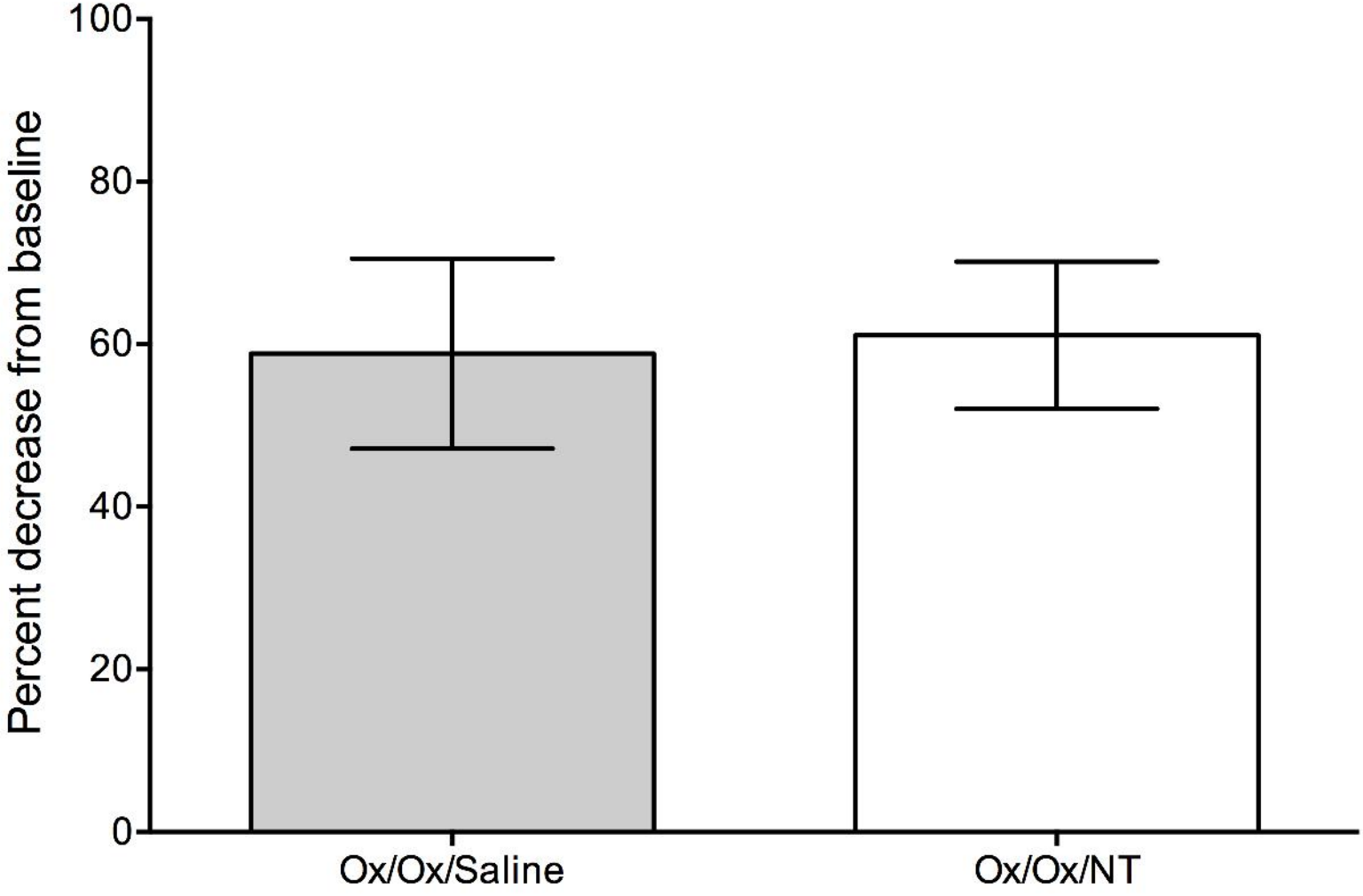
Percent decrease from baseline
100
80
60
40
20
0
Ox/Ox/Saline
Ox/Ox/NT

Table S2. Lineage and activation markers used to identify skin-infiltrating cells in oxazolone-challenged labia

Antigen	Color	Clone	Host	Stable Public Identifier	Vendor
CD3ε	BV650	145-2C11	Armenian Hamster	BD Biosciences cat no. 564378	BD Biosciences
CD44	BV711	IM7	Rat	BD Biosciences cat no. 563971	BD Biosciences
CD4	APC-eFluor780	RM4-5	Rat	AB_1272183	eBioscience
CD11b	eFluor450	M1/70	Rat	AB_1582237	eBioscience
CD11c	eFluor450	N418	Armenian Hamster	AB_1548654	eBioscience
CD25	PE	PC61.5	Rat	AB_465607	eBioscience
CD45R	eFluor450	RA3-6B2	Rat	AB_1548761	eBioscience
CD45RB	FITC	C363.16A	Rat	AB_465064	eBioscience
PD-1	PE-Cy7	J43	Armenian Hamster	AB_10853805	eBioscience
CD103	APC	2E7	Armenian Hamster	AB_1106992	eBioscience
CD8α	BV785	53-6.7	Rat	AB_2562610	BioLegend
FoxP3	FITC	FJK-16s	Rat	AB_465243	eBioscience
IFN-γ	PE	XMG1.2	Rat	AB_466193	eBioscience

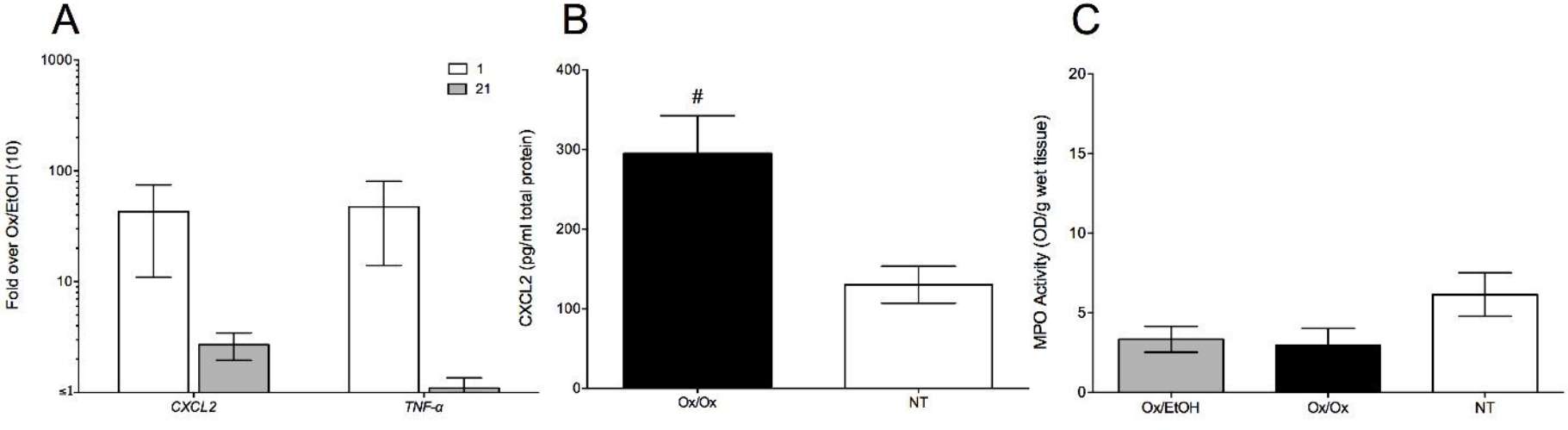
A
1000
100
10
≤1
Fold over Ox/EtOH (10)
1
21
CXCL2
TNF-α
B
400
300
200
100
0
CXCL2 (pg/ml total protein)
#
Ox/Ox
NT
C
20
15
10
5
0
MPO Activity (OD/g wet tissue)
Ox/EtOH
Ox/Ox
NT

Table S3. Accession numbers for Taqman primer-probe sets (Life Technologies, Carlsbad, CA) for semi-quantitative PCR used to measure relative transcript abundance in Ox-challenged labia

Transcript	Genbank Accession number
β-2-microglobulin (β2m)	Mm00437762_m1
Nerve growth factor (NGF)	Mm00443039_m1
Cell adhesion molecule 1 (CADM1)	Mm00457556_m1
Interleukin-13 (IL-13)	Mm00434204_m1
Interleukin-6 (IL-6)	Mm00446190_m1
Interferon-γ (IFN-γ)	Mm01168134_m1
T-box transcription factor (TBX21)	Mm00450960_m1
Tumor necrosis factor alpha (TNF-α)	Mm00443260_g1
Chemokine C-X-C motif ligand 2 (CXCL-2)	Mm00436450_m1

Table S4. Hindpaw withdrawal thresholds to pressure in mice challenged on the labia, and labiar withdrawal thresholds at different stages of the estrus cycle after Ox challenge cessation. (Top) Hindpaw withdrawal thresholds (mean ± SEM; in grams) for Ox-sensitized ND4 mice challenged on the labia 10 times with either vehicle (Ox/EtOH (10); n = 7) or Ox (Ox/Ox (10); n = 8). Percent change in withdrawal thresholds are shown in Figure S1D. (Bottom) Labiar withdrawal thresholds for Ox-sensitized and challenged mice in different stages of the estrus cycle at day 1 and day 21 post challenge cessation (mean ± SEM; in grams; n = 5-6/estrus stage/time point).

		Day after 10 Challenges		
Treatment	**Baseline**	**1**	**17**	**21**
Ox/EtOH (10)	5.34 ± 0.38	4.25 ± 0.26	4.04 ± 0.37	4.35 ± 0.62
Ox/Ox (10)	5.22 ± 0.25	4.97 ± 0.30	4.30 ± 0.34	3.96 ± 0.19

		Day after 10 Challenges	
Estrus Stage	**Baseline**	**Day 1**	**Day 21**
Proestrus	0.67 ± 0.04	0.25 ± 0.05	0.25 ± 0.04
Estrus	0.67 ± 0.04	0.23 ± 0.04	0.33 ± 0.06
Metestrus	0.67 ± 0.04	0.26 ± 0.03	0.21 ± 0.05
Diestrus	0.67 ± 0.04	0.20 ± 0.02	0.23 ± 0.04