Research Paper

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Neuronal–immune axis alters pain and sensory afferent damage during dental pulp injury

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Abstract

Dental pulp tissue is densely innervated by afferent fibers of the trigeminal ganglion. When bacteria cause dental decay near the pulpal tissue, a strong neuronal and immune response occurs, creating pulpitis, which is associated with severe pain and pulp tissue damage. Neuroimmune interactions have the potential to modulate both the pain and pathological outcome of pulpitis. We first investigated the role of the neuropeptide calcitonin gene-related peptide (CGRP), released from peptidergic sensory afferents, in dental pain and immune responses by using Calca knockout ($Calca^{-/-}$) and wild-type ($Calca^{+/+}$) mice, in a model of pulpitis by creating a mechanical exposure of the dental pulp horn. We found that the neuropeptide CGRP, facilitated the recruitment of myeloid cells into the pulp while also increasing spontaneous pain-like behavior 20% to 25% at an early time point. Moreover, when we depleted neutrophils and monocytes, we found that there was 20% to 30% more sensory afferent loss and increased presence of bacteria in deeper parts of the tissue, whereas there was a significant reduction in mechanical pain response scores compared with the control group at a later time point. Overall, we showed that there is a crosstalk between peptidergic neurons and neutrophils in the pulp, modulating the pain and inflammatory outcomes of the disease.

Keywords: Dental pain, Inflammation, CGRP, Neutrophils, Sensory afferent loss

1. Introduction

The pulp tissue resides within the hard tissues of a tooth. On injury, such as bacteria forming deep caries, pulp tissue initiates a robust, often very painful, inflammatory response, the state which is referred to as pulpitis.[29] Clinically, the degree of tissue damage during pulpitis, including the dieback of sensory afferents, is often determined to be irreversible based on the presence of painful symptoms, leading to invasive treatment choices including root canal treatment or tooth extraction.[29] However, the relationship between the severity of pain and degree of tissue damage, including sensory afferent loss, during pulpitis is not clear.[12] Studying the mechanisms of neuroimmune interactions and tissue damage, we can have a better understanding of the relationship between pain and pathological outcomes of pulpitis.

Dental pulp tissue is highly innervated.[10,21] On average, each molar tooth in the mouse is innervated by 50 neurons.[10] The types of neurons innervating dental pulp are diverse and include peptidergic afferents expressing the neuropeptide calcitonin gene-related peptide (CGRP), which constitute about 30% of all pulp neurons.[10,16,41] Calcitonin gene-related peptide mediates headache and trigeminal pain both through peripheral and central mechanisms within the trigeminal system.[9,24,30,57,60] Calcitonin gene-related peptide expression is also higher in the dental pulp of patients with severe dental pain compared with nonpainful cases.[1] However, whether interfering with CGRP signaling from sensory afferents affects pain outcomes in pulpitis is not well established.[4,13] In addition, CGRP released from peptidergic neurons modulates innate immune cell activity through its cognate receptor, receptor-associated modifying protein (RAMP1) and calcitonin receptor-like receptor (Calcrl) expressed by these cell types in different barrier tissues.[7] For example, CGRP has been shown to modulate neutrophil recruitment and antimicrobial signaling in a mouse model of *S. pyogenes* skin and soft tissue infection.[44] Ablation of nociceptors and blockade of CGRP signaling led to increases in neutrophil recruitment, which resulted in decreased dermonecrosis.[44] However, whether sensory neuron-derived CGRP regulates innate immune cells in dental pulp is unknown.

Neutrophils and monocytes are crucial players of innate immune response, serving as the first line of defense against tissue injury and pathogen invasion, being equipped with different antimicrobial mechanisms.[52,53] Neutrophils also are critical for clearance of tissue debris and wound healing.[26] Their dysregulation can contribute to continuous tissue damage in chronic inflammatory diseases.[6,52] Pain is coupled to both wound healing and the innate immune response.[40,45,47,54] Innate immune cells release multiple proinflammatory cytokines such as interleukin-1

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beta (IL-1β) and tumor necrosis factor alpha (TNF-α), which can sensitize nociceptors to drive inflammatory and neuropathic pain.[19,36,45,58] On the other hand, neutrophils can be important for suppression of long-term development of chronic pain: in the complete Freund adjuvant (CFA) inflammatory pain model, it has been shown that while withdrawal threshold was initially higher in animals where neutrophils were reduced, for these animals it took them significantly longer to return to baseline withdrawal thresholds.[40] Investigating the role of neutrophils and/or monocytes in dental pain and sensory afferent damage can improve our understanding of relationship between pain, innate immunity, and pathological outcomes during pulpitis.

2. Materials and methods

2.1. Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School. C57BL/6 mice were purchased from Jackson (Jax) Laboratories (Bar Harbor, ME). *Calca-GFP-DTR*[32] and *Calca*$^{-/-}$ *and Calca*$^{+/+}$[39] mice were bred in house (Harvard Medical School). Age-matched 7- to 12-week-old littermate mice of both sexes were used for experiments. For pain behavior assays, only female mice were used.

2.2. Dental pulp injury procedure

Clinically, as dental caries progress by degradation of dental hard tissues, the pulp tissue gets exposed to caries microbiome and oral environment and the process of progressive tissue degradation begins, the stage referred as pulpitis. Dental pulp injury, drilling of hard tissues of the tooth to expose the pulp to oral environment and bacteria, is a model that has been widely used to model pulpitis.[17] To perform dental pulp injury, mice were anesthetized using intraperitoneal injection of 100 mg/kg ketamine + 10 mg/kg xylazine in sterile phosphate-buffered saline (PBS). The pulp exposures were performed in accordance with published protocols.[17,28] In brief, the maxillary first molar was drilled with a 1/4-round bur at low speed in a way that only the middle pulp horn was drilled until the pulp was exposed which allowed for bacteria inoculation and penetration through the injured site (Supplementary Figure 1, available at http://links.lww.com/PAIN/B905). The contralateral maxillary first molar served as the uninjured control. Sham mice received the same anesthesia and had their mouths held open in a similar manner and duration. For pain behavior and histology experiments, only the maxillary first molar for each animal was injured. For flow cytometry experiments, maxillary first, second, and mandibular first molars were injured on each animal and teeth from 5 mice were pooled. All procedures were performed by 1 investigator (OE).

2.3. Gr-1 neutrophil/monocyte depletion

Mice received 250 μg of Gr-1 antibody (anti-mouse Ly6G/Ly6C [Gr-1], clone RB6-8C5, (BioXCell, NH) 2 hours before the dental pulp injury procedure and on day 3, whereas the control mice received 250 μg of isotype control antibody (rat IgG2b isotype control, anti-keyhole limpet hemocyanin, (BioXCell) by intraperitoneal injections.

2.4. Spontaneous pain-like behavior measured by the mouse grimace scale

The mouse grimace scale is validated for capturing spontaneous pain-like behavior in mice.[22,27,48,61] Animals were acclimated in clear acrylic chambers (8 W × 8 H × 8 L cm) a day before baseline testing for 20 minutes. Animals were videotaped for 10 minutes at baseline and selected time points (day 1, day 3, and day 7 or day 2, day 4, and day 6) with individual cameras (GoPro, Inc, CA). The first image with a clear view of the animal's face of every minute of the video was extracted using iMovie (Apple, Inc, CA), in total leading to 10 images per video. Scoring of the images was performed by investigators blinded to treatment allocation and timing relative to injury. As previously described for each image, orbital tightening, nose bulge, cheek bulge, and ear position were scored (0 "not present," 1 "moderately visible," and 2 "severely visible"). Because whiskers were not clearly visible in some images, whisker scoring was excluded. A mouse grimace scale score for each image was then obtained by the averaging of given scores to each facial feature. The final score for each animal was calculated by averaging the scores of 10 images.

2.5. Mechanical sensory response measured by facial Von Frey stimulation

For this assessment, animals were placed in modified confined chambers with adjustable openings as previously described.[48] The animals would put their face out of the opening, but because they were elevated from the floor, they also did not intend to escape. In this way, we were able to perform the facial stimulation using von Frey filaments. The maxillary first molar teeth are adjacent to the periorbital area beneath the eyes, mechanical stimulation was performed there, ipsilateral to the injury, by blinded single investigator (O.E.). The 0.008, 0.02 g, 0.004, 0.07 g, 0.16 g, and 0.4 g filaments were tested at baseline and at selected time points (day 1, day 3, and day 7) once for experiments performed with *Calca*$^{-/-}$ *and Calca*$^{+/+}$ mice. For Gr-1 depletion experiment, based on our data from *Calca*$^{-/-}$ *and Calca*$^{+/+}$ experiments, we only used 0.02 g (nonpainful stimulus), 0.07 g (allodynia response stimulus), and 0.16 g (painful stimulus) filaments once as the mechanical response score differences between injured and noninjured animals were clear with these filaments. The animals' response to the stimulus on the face was scored by using a scoring system slightly modified from the previous publication in which the same experimental setup was used (**Table 1**).[48] We calculated an overall mean mechanosensory response score by averaging the scores given by the mice to each filament for each time point. All measurements were performed by 1 blinded investigator (O.E.).

2.6. Dental pulp cell suspension preparation

Animals were humanely euthanized by CO_2 inhalation. Maxillae and/or mandibles were dissected and put in minimum essential medium (MEMα) culture media on ice. Any tissues surrounding the teeth

Table 1

Mechanical response scoring.

Score	Response
0	No response
1	Orientation to the stimulus or a slower head turn away from the stimulus
2	A rapid withdrawal
3	Rapid withdrawal followed by facial wipes or when mice stop taking the head out

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were removed. The crowns of teeth were separated at the crown–root interface which allowed both for exposure and collection of coronal pulp tissue. The molar crowns with pulp tissues were then incubated in digestive solution (4 mg/mL collagenase II and 4 mg/mL dispase II) at 37°C for 1 hour. Later, the tissues within the digestive solution were pipetted vigorously for the pulp tissue to be detached from dentin and filtered using a 70-μm mesh. The samples were spun down at 300*g* for 5 minutes and washed with flow buffer (2% FBS, 1μM EDTA, and 0.1% sodium azide).

2.7. Splenocyte cell suspension preparation

Spleens were collected in culture media on ice and were homogenized using a 70-μm cell strainer and culture medium (RPMI). The cell suspension was washed using culture medium, and splenocytes were centrifuged at 1500 rpm, at 4°C for 5 minutes and resuspended in red blood cell lysis buffer for 10 minutes. The splenocyte cell suspension was washed once again, and finally, cells were resuspended in culture media.

2.8. Flow cytometry

The cell suspension was spun down at 300*g* for 5 minutes and resuspended in flow buffer. The cell suspension was first incubated with FcR blocking reagent (Miltenyi Biotec, MA, 130-092-575) for 10 minutes, washed with flow buffer, and then incubated with the following antibodies (Biolegend, CA, USA) Zombi Aqua-KO525 (1:1000), CD45-Cy7 (1:100), CD11b-PE (1:100), Ly6G-APC (1:100), Ly6C-AF700 (1:100), CD3-FITC (1:100), CD4-PE-cy7 (1:100), CD8-perpCy5.5 (1:100), and F4/80-PB (1:100) for 30 minutes. Cell suspensions were washed again, centrifuged, resuspended in 1% paraformaldehyde (PFA), and filtered through a 40-μm mesh. Counting beads (BioLegend, CA) were added to each sample. Flow cytometry was performed on a CytoFLEX flow cytometer (Beckman Coulter Life Sciences, IN)), and data were analyzed using FlowJo software (FlowJo LLC, OR)). Absolute cell count per 100 μL in each sample was calculated using counting beads and bead count.

2.9. Tissue processing for histology

Animals were perfused with PBS with heparin, followed by 4% PFA. Maxillae were dissected. All tissues were postfixed with 4% PFA overnight. Decalcification of maxillae using 18% EDTA at 4°C was performed for 3 to 4 weeks. For paraffin embedding, tissue processing including dehydration process was performed using a tissue processor (TP1020, Leica, Germany). Maxillary jaws were then embedded with paraffin and sectioned (6 μm). For all the other immunofluorescence staining experiments, the maxillary jaws were processed by incubating with 30% sucrose for 1 day, followed by 50% sucrose for 1 day at 4°C, then were cryopreserved by embedding in optimum cutting temperature compound (OCT) for frozen tissue storage and sectioning, at 30 μm thickness.

2.10. H&E staining

Rehydration, hematoxylin and eosin staining, dehydration, and mounting of the slides were completed.[38] Sections of the coronal pulp were examined by a board-certified oral pathologist, blinded to treatment allocation (L.A.). Injured (middle) and noninjured (mesial and distal) coronal pulp horns were investigated and scored individually for extent of necrosis (0-3) and extent of odontoblast loss (0-3) (0: no changes, 1: 1of 3 of the area of interest is affected, 2: 2 of 3 of the area of interest is affected, and 3: 3 of 3 of the area of interest is affected) and edema (0: absent and 1: present). The overall score for each section was calculated by averaging mesial and distal and middle pulp horns. Three sections per animal were scored and averaged to obtain the individual animal score.

2.11. Immunofluorescence staining

Cryosections were washed with PBS. Sections were blocked with 5% goat serum (source) and 1% triton for 1 hour at room temperature. Sections were then incubated with the primary antibodies overnight at 4°C. Primary antibodies were rabbit anti-beta tubulin (tuj1) (1:500, Abcam Cambridge, United Kingdom), rat anti-Ly6g/Ly6C (1:200, Abcam), and rat anti-F4/80 (1:200, Abcam). Sections were then washed with 1% goat serum and 1% triton and were incubated with the secondary antibodies for 2 hours. Secondary antibodies were goat anti-rabbit Alexa Fluor 546 (1:500, Thermo Fisher, MA) and anti-rat Alexa Fluor 647 (1:500, Thermo Fisher). Image acquisition was performed by a confocal microscope (Leica, Stellaris). An equal number of stacks were imaged using the same acquisition settings. Image processing was performed using Fiji[51] with the same settings across experiments. To quantify the area with sensory afferent loss, we divided the pulp chamber into 2 areas as injured middle pulp horn with the connection parts to noninjured pulp horns and noninjured pulp horns. For both areas, we first measured the whole area stained with DAPI, then the area without tuj1 staining and calculated the percent area with sensory afferent loss. Quantification of 1 section from each animal (5 from isotype and 5 from Gr-1 groups) was performed using Fiji.

2.12. Fluorescence in situ hybridization

Sections were deparaffinized using xylene and rehydrated using 100% ethanol. Half of the sections in 1 slide were incubated with 1:200 Cy3-conjugated nonsense eubacterial probe (5′-/5Cy3/CGACGGAGGGCATCCTCA-3′), whereas the other half of the same slide was incubated with 1:200 Cy3-conjugated eubacterial probe (5′-/5Cy3/GCTGCCTCCCGTAGGAGT-3′) in 20% SDS, 1:10 formamide, and 9:10 bacterial hybridization buffer overnight in 50°C in a tissue culture incubator. Sections were washed first with hybridization buffer followed by PBS before mounting.

2.13. Statistical analysis

Statistical comparisons of 2 groups for a single variable with normal distributions were analyzed by the unpaired Student *t*-test. Statistical comparisons of 3 or more groups at a single time point were analyzed by 1-way analysis of variance (ANOVA) with Tukey post hoc tests. Outcomes of pain behavior assays were analyzed by 2-way ANOVA with repeated measures. Between-subject effects were used to determine whether there had been any significant effects of time, experimental group, or a significant interaction, followed by post hoc Dunnett and Sidak tests where necessary. A P value less than 0.05 was considered statistically significant. Statistical analyses were conducted using Prism 9 (GraphPad Software, LLC) and Excel (Microsoft, WA).

3. Results

3.1. CGRP+ neuronal afferents reside at the dentin–pulp junction and dieback as the disease progresses

We first wanted to capture the spatial distribution of CGRP+ fibers within the pulpal tissues in relation to all nerves of the

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pulp, marked by beta tubulin 3 (tuj1), and how the neuronal architecture was affected after the pulp injury. CGRPα is the main isoform of CGRP expressed by nociceptive afferents.[49] Calca-GFP reporter mice have been used to study CGRP+ peptidergic sensory innervation of the skin and other barrier sites.[2,32,42] Using these mice and anti-beta tubulin III (tuj1) antibody to stain for all nerve fibers, we observed that CGRP+ fibers extend along the dentin–pulp junction through all 3 pulp horns in healthy, intact pulp tissue (**Fig. 1A**). On day 0, 1 hour after mechanical injury of the dental pulp, we observed minimal afferent loss located at the middle, injured pulp horn (**Fig. 1B**). On day 1, we observed continuity of Tuj1-expressing afferents along with CGRP+ fibers between the 3 pulp horns and damage localized to the injury site (**Fig. 1C**). At days 3 and 7, the extent of the afferent dieback increased and we observed loss of the continuity of tissues connecting the pulp horns and partial loss of afferents within the other noninjured pulp horns (**Figs. 1D and E**). Qualitatively, we observed that especially on day 7, the loss of CGRP+ fibers was prominent, even in relation to all Tuj1+ nerve fibers (**Fig. 1E**).

3.2. Calcitonin gene-related peptide increases spontaneous pain-like behavior, but does not contribute to mechanical hypersensitivity

To assess the contribution of CGRP to pain during pulp injury, we investigated whether facial mechanical hypersensitivity responses and spontaneous pain-like behavior captured by grimace scoring were different in Calca$^{-/-}$ mice compared with wild-type mice. We found that the mechanical response scores were similar for both genotypes across each time point in noninjured animals (**Figs. 2A and B**). Both Calca$^{-/-}$ and Calca$^{+/+}$ mice exhibited a higher mechanical response score at day 1 ($P = 0.015$, $P = 0.015$), day 3 ($P = 0.090$, $P = 0.001$), and day 7 ($P = 0.001$, $P = 0.01$) after the injury compared with baseline measurements (**Figs. 2A and B**), supporting that dental pulp injury produces mechanical hypersensitivity in the facial skin. When we determined the change in mechanical response scores relative to baseline values for each individual mouse, the largest increase in mechanical hypersensitivity was on day 7 in injured animals compared with noninjured animals (**Fig. 2C**). However, we did not find any difference in mechanical response scores

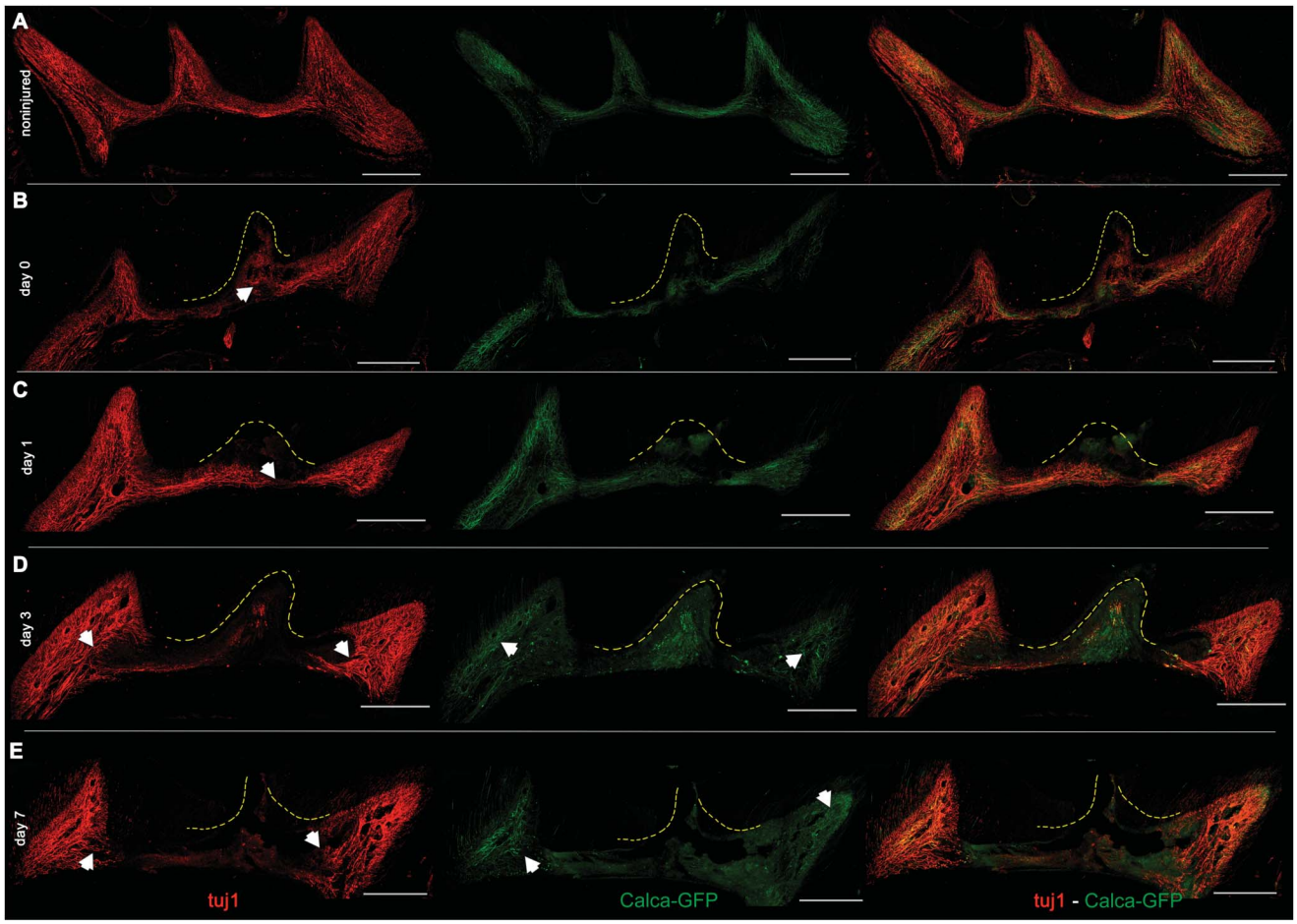

Figure 1. CGRP+ neuronal afferents are an important neuronal subpopulation of the pulp and dieback as the disease progresses. (A) Noninjured pulp. CGRP+ fibers extend along the dentin–pulp junction. (B) Pulp immediately after injury. The arrow shows the presence of intact afferents within the injured middle pulp horn. (C) Pulp 1 day after injury. The arrow points out the continuity of intact fibers just right after their interruption. Most of the CGRP+ fibers are also still present. (D, E) Pulp 3 and 7 days after injury. The arrows point out the extent of dieback by marking the border of remaining intact afferents within the noninjured pulp horns. CGRP+ fibers are scarcer, mostly present at the very distant ends of the pulp chamber. The middle pulp horn is the mechanically injured pulp horn in each image (the dotted yellow lines mark the injury site of the middle pulp horn). The first column includes images showing beta tubulin III (tuj1) immunofluorescence; the second column shows images of CGRP+ fibers, traced by Calca-GFP reporter mice; and the third column shows co-localization of tuj1 with Calca-GFP signal. Scale bars are 200 μm. CGRP, calcitonin gene-related peptide.

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between Calca$^{-/-}$ and Calca$^{+/+}$ mice at any time points (**Figs. 2A and C**).

We also analyzed grimace scores as a measure of spontaneous pain-like behaviors in Calca$^{+/+}$ and Calca$^{-/-}$ injured animals and noninjured animals. There were statistically significant differences in grimace scores for Calca$^{+/+}$ and Calca$^{-/-}$ injured animals and noninjured animals (**Fig. 2G**). We found that there was no difference at baseline among groups ($P = 0.96$, $P = 0.64$) (**Fig. 2G**). On day 1 after injury, grimace scores of wild-type mice were significantly higher compared with noninjured animals ($P = 0.022$), whereas there was no difference between noninjured animals and Calca$^{-/-}$ mice (**Fig. 2G**). On day 7 after injury, we observed a similar trend, where grimace scores of wild-type mice were higher compared with noninjured animals ($P = 0.11$), whereas grimace scores of noninjured and Calca$^{-/-}$ mice were similar ($P = 0.99$) (**Fig. 2G**). These data suggest that CGRP might contribute to spontaneous pain-like behaviors due to dental pulp injury.

3.3. Calcitonin gene-related peptide contributes to recruitment of immune cells at day 1, but does not alter tissue damage

Next, we looked at whether CGRP modulated the innate immune response after pulp injury. One day after injury, the pulp tissue

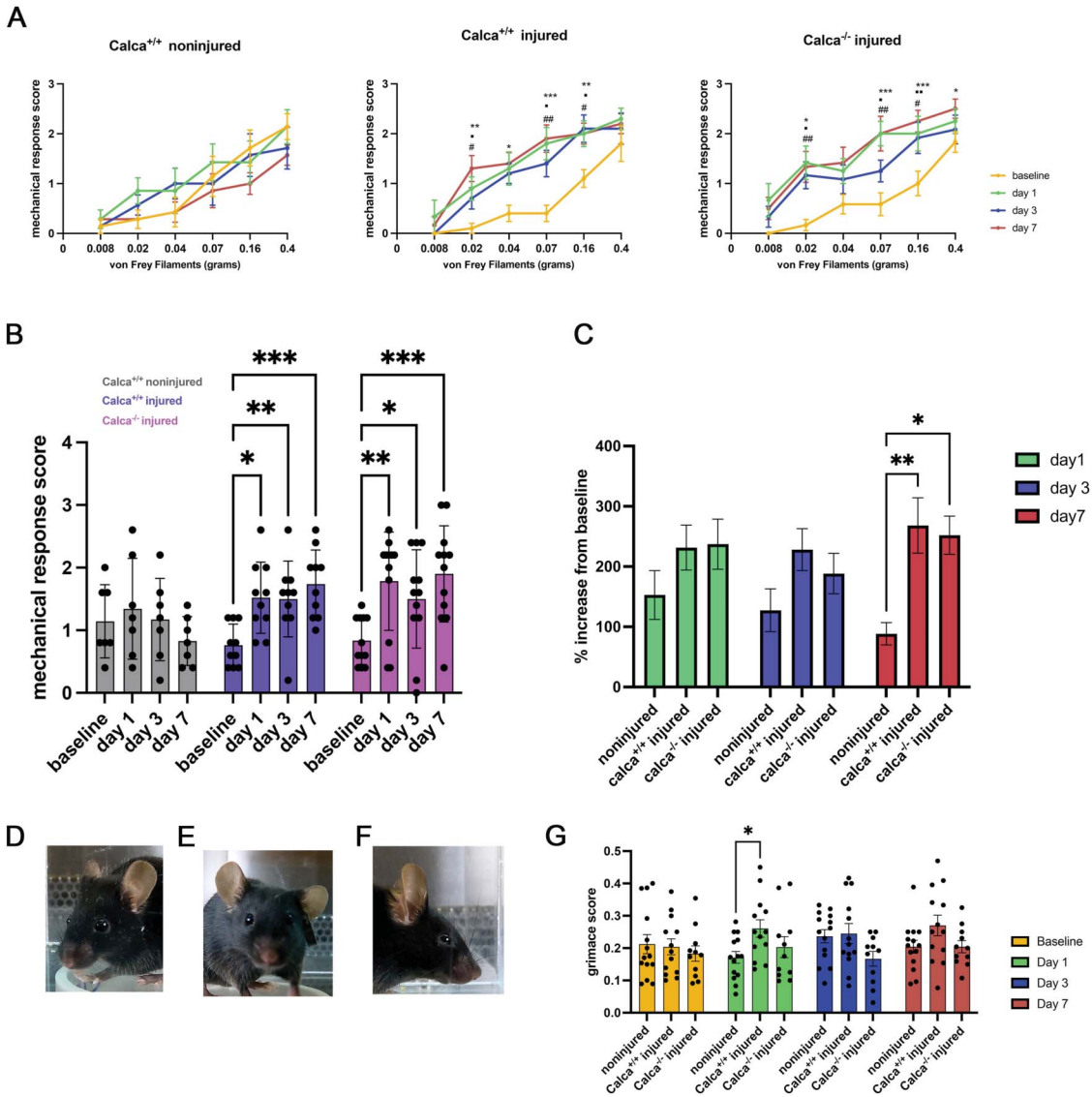

Figure 2. CGRP may contribute to spontaneous pain-like behavior but not to mechanical hypersensitivity after dental pulp injury. (A) Plots present the mechanical response score of noninjured (Calca$^{+/+}$ and Calca$^{-/-}$ grouped together), Calca$^{+/+}$ injured, and Calca$^{-/-}$ injured animals to von Frey filaments with different weights at different time points. (B) Comparison of mean mechanical response scores across different time points. In this plot, each data point represents the mean mechanical response score of 1 animal obtained by averaging the mechanical response scores given to each von Frey filament (2-way ANOVA: across groups: $P = 0.24$, F = 1.49, DOF = 2; time: $P = 0.0001$ F = 9.9, DOF = 3; interaction: $P = 0.004$, F = 3.6, DOF = 6). (C) Percent increase in mean mechanical response scores from baseline values of Calca$^{+/+}$ and Calca$^{-/-}$ noninjured and injured animals (2-way ANOVA: across groups: $P = 0.001$, F = 7.3, DOF = 2; time: $P = 0.7$ F = 0.4, DOF = 2; interaction: $P = 0.6$, F = 0.6, DOF = 4). (D–F) Representative extracted images of mouse used to examine facial expression changes for grimace scoring. Baseline facial features (D), nose bulge and cheek bulge (E), and eye squinting (F). (G) Comparison of grimace scores between noninjured (Calca$^{+/+}$ and Calca$^{-/-}$ grouped together) and injured Calca$^{+/+}$ and Calca$^{-/-}$ across different time points (2-way ANOVA: across groups: $P = 0.009$, F = 4.8, DOF = 2; time: $P = 0.64$ F = 0.5, DOF = 3; interaction: $P = 0.3$, F = 1.2, DOF = 6). (A, B) Repeated measures of ANOVA with Dunnett multiple comparisons. (A) # comparison of day 1 to baseline, ■ comparison of day 3 to baseline, and *comparison of baseline to day 7. (C, G) Two-way ANOVA with Dunnett multiple comparison tests. # ■ *$P < 0.05$, ## ■■ **$P < 0.01$, ### ■■■ ****$P < 0.0001$. Mean ± SEM. ANOVA, analysis of variance; CGRP, calcitonin gene-related peptide; DOF, degree of freedom.

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was collected from Calca$^{-/-}$ and Calca$^{+/+}$ mice and flow cytometry analysis was performed. We found that CGRP contributed to recruitment of neutrophils and monocytes one day after injury. We found that there were fewer total CD45^{+} immune cells ($P = 0.011$) and fewer CD45^{+}CD11b+ myeloid cells ($P = 0.017$) in Calca$^{-/-}$ mice compared with wild-type mice after injury (**Figs. 3B, C, G**). Among myeloid immune cells, we found that there were fewer CD11b+F4/80− myeloid cells which were neutrophils and monocytes in Calca$^{-/-}$ mice compared with wild-type mice ($P = 0.02$) (**Figs. 3D, E, G**). Although cell numbers differed, the overall proportions of immune cells in the dental pulp were similar between Calca$^{-/-}$ and Calca$^{+/+}$ mice (**Fig. 3F**). These data suggest that CGRP regulates overall recruitment of immune cells, of which most were neutrophils and monocytes. We then investigated whether the immune cell recruitment difference in Calca$^{-/-}$ mice compared with wild-type mice resulted in differences in the severity of tissue pathology. As day 1 would be an early time point to assess histologic changes, we investigated histologic necrosis, odontoblast loss, and edema in the 2 groups at day 7. We found that histologically there was no difference in tissue necrosis, odontoblast loss, or edema between Calca$^{-/-}$ mice compared with wild-type mice at this time point (Supplementary Figure 2, available at http://links.lww.com/PAIN/B905).

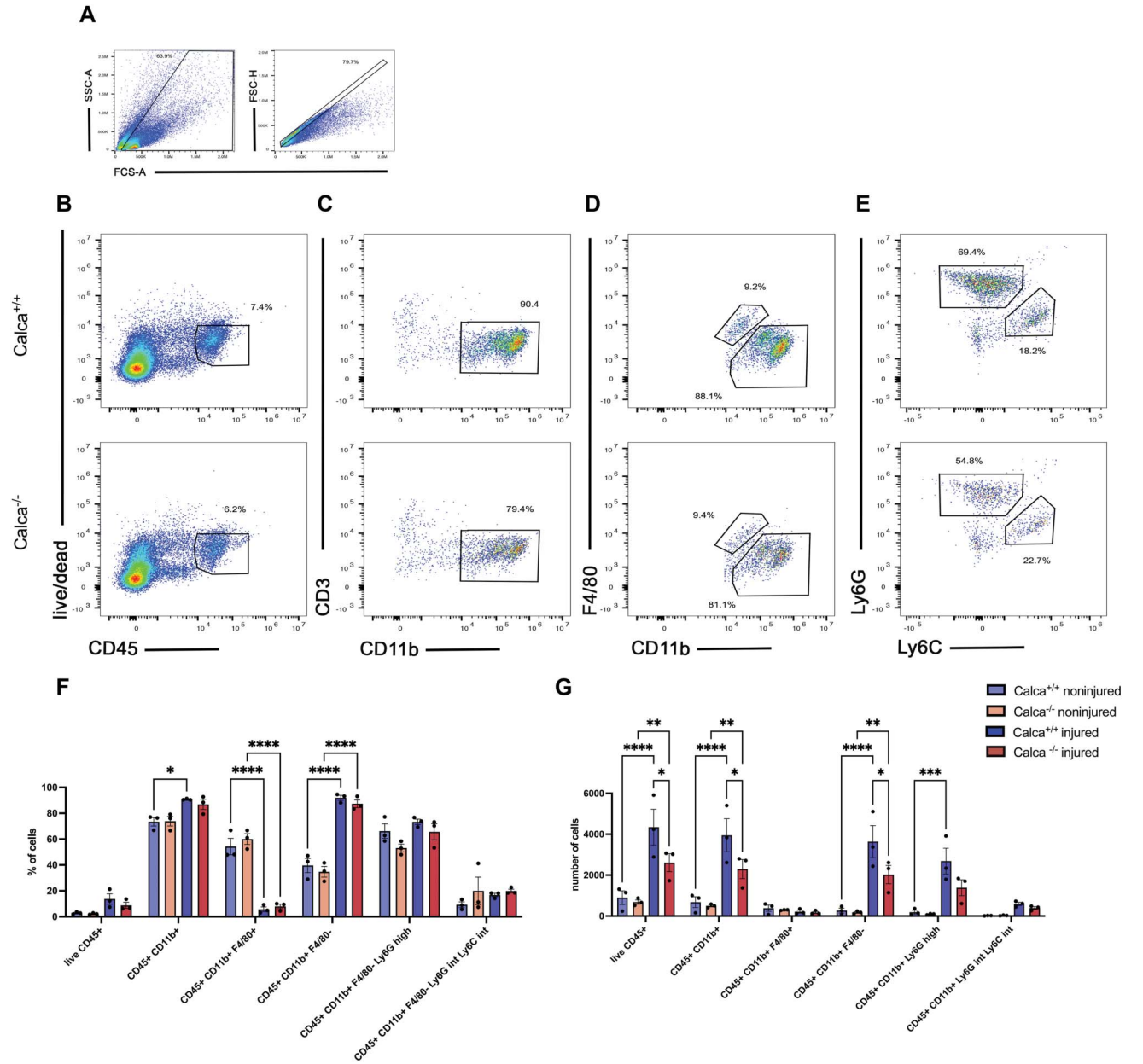

Figure 3. CGRP contributes to recruitment of neutrophils and monocytes at one day after injury. (A) Representative flow cytometry (FC) plots showing initial steps of the gating strategy. (B) Representative FC plots to gate CD45^{+} live immune cells. (C) Representative FC plots to gate CD11b+ myeloid cells. (D) Representative FC plots to gate F4/80+ macrophages. (E) Representative FC plots to gate F4/80− myeloid cells into Ly6G high and Ly6G int Ly6C int neutrophils and monocytes. The first row includes representative FC plots of Calca$^{+/+}$ animals and the lower row includes representative FC plots of Calca$^{-/-}$ animals. (F) Percentage changes of immune cell populations plotted from 3 independent experiments of Calca$^{-/-}$ and Calca$^{+/+}$ animals. (G) Number of immune cell populations plotted from 3 independent experiments of Calca$^{-/-}$ and Calca$^{+/+}$ animals. (F, G) Two-way ANOVA with the Sidak multiple comparisons test. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$. Mean ± SEM. ANOVA, analysis of variance; CGRP, calcitonin gene-related peptide.

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3.4. Neutrophils are recruited into the dental pulp and invade denervated areas

Neutrophils are critically involved in bacterial clearance, tissue injury, and wound healing. We next wanted to determine the kinetics of neutrophil recruitment during pulp injury and how this relates with CGRP+ afferents. We used anti-Ly6C/6G to stain for neutrophils and monocytes. We found that in the noninjured healthy pulp, the presence of neutrophils and monocytes was scarce where intact CGRP+ fibers were visible (**Fig. 4A**). On day 1 after injury, we observed a very significant increase in neutrophils and/or monocytes at the injury site, where we concurrently observed the loss of CGRP+ fibers (**Fig. 4C**). On day 3, we observed that neutrophils and monocytes were extending toward the other noninjured pulp horns. We observed that the presence of intact CGRP+ fibers was mostly outside of the area invaded by these immune cells (**Fig. 4D**). On day 7, the presence of neutrophils and/or monocytes seems to be more spread out within the pulp chamber and their rounded clear morphology became less distinct. At this time point, the presence of CGRP+ fibers was very scarce, mostly localized at the distant ends of the noninjured pulp horns (**Fig. 4E**).

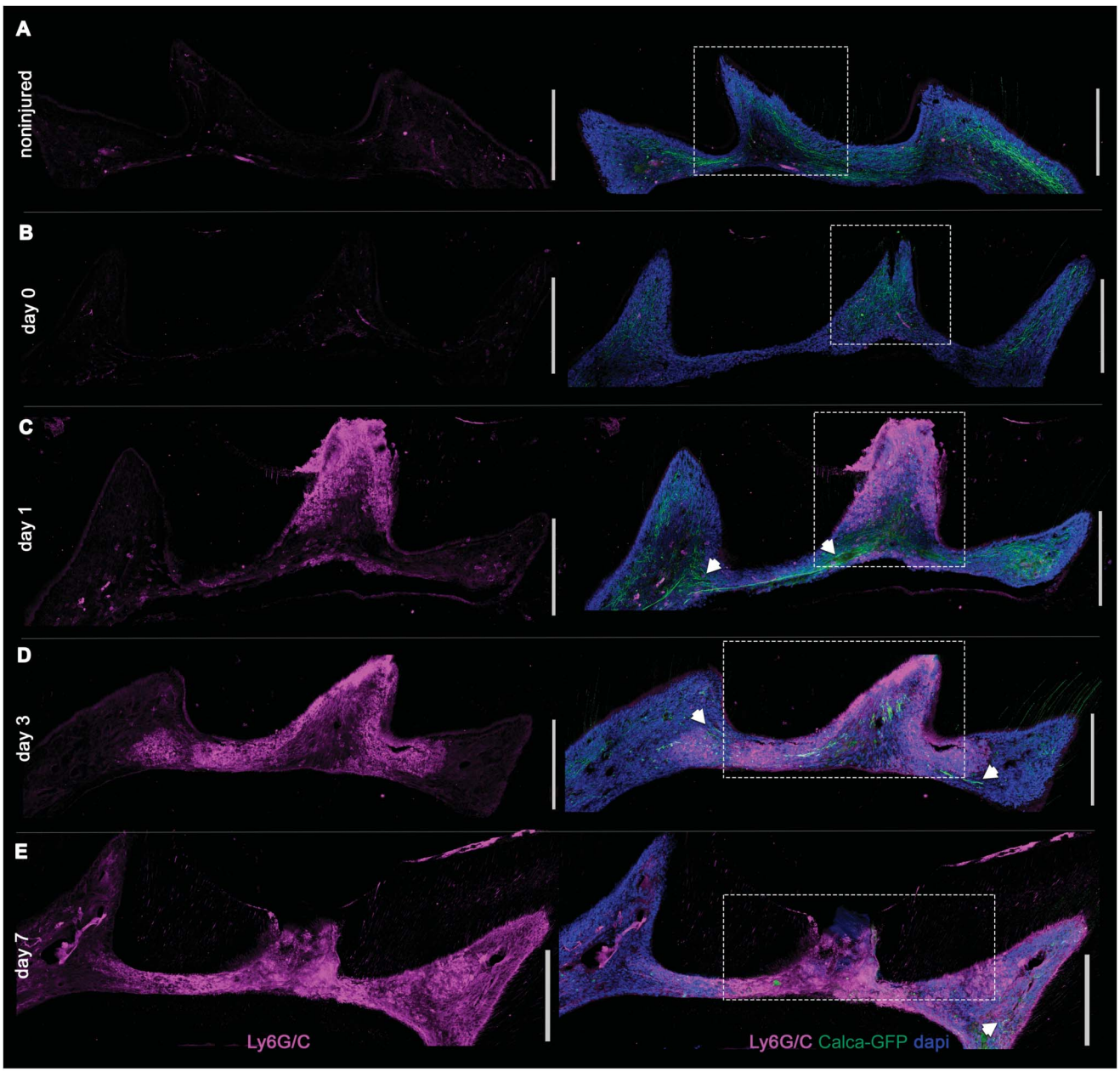

Figure 4. Spatial Investigation of neutrophils and monocytes with CGRP+ afferents within the pulp chamber. (A) Noninjured pulp. The box shows the intact middle pulp horn with intact CGRP+ fibers and very few neutrophils/monocytes. (B) Pulp just right after injury. The box shows the injured middle pulp horn where most of the CGRP+ fibers are still intact and with very few neutrophils and/or monocytes. (C) Pulp one day after injury. The box shows the injured pulp horn densely populated by neutrophils and monocytes. The arrows point out the presence of CGRP+ fibers where there are fewer neutrophils and/or monocytes. (D, E) Pulp 3 and 7 days after injury. The arrows point out areas where the distinct CGRP+ fiber morphology is visible, just adjacent to but outside of where neutrophils and monocytes are densely present. The middle pulp horn is the mechanically injured pulp horn in each image. The first column includes images showing Ly6G/C immunofluorescence and the second column shows Ly6G/C co-localization with Calca-GFP and DAPI. Scale bars are 200 μm. CGRP, calcitonin gene-related peptide.

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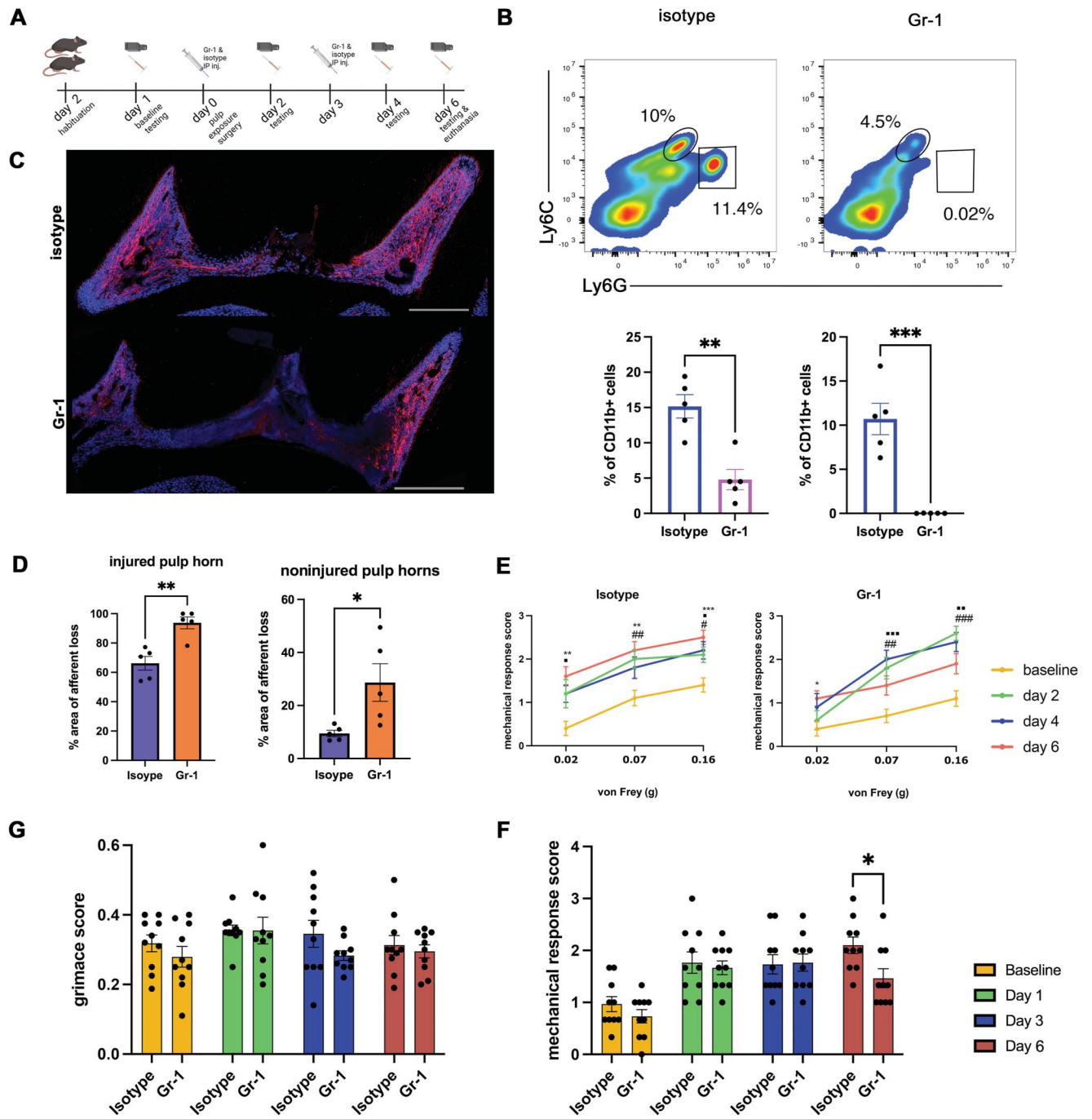

Figure 5. neutrophils and monocytes depletion (Gr-1) increases sensory afferent loss while contributing to delayed reduction in mechanical pain response scores. (A) Schema shows experiment design and timeline for Gr-1 and isotype treatments. (B) Flow cytometry representative plots and analysis to validate neutrophil and monocyte depletion using splenocytes. Graphs show % changes of Ly6C high (monocytes) and Ly6G high (neutrophil) cells among total CD11b+ myeloid cells. Unpaired *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$. Mean ± SEM. (C) Representative immunofluorescence staining images showing extent of sensory afferent loss. Red, tuj1 and blue, DAPI. Scale bar = 200 μm. (D) Analysis of extent of sensory afferent loss 6 days after injury of Gr-1 and isotype treatment groups. In each plot, each point represents an analysis of a section from 1 animal. Unpaired *t* tests. $^{*}P < 0.05$, $^{**}P < 0.01$. (E) Mechanical response score of animals of the isotype group and Gr-1 group to different von Frey filaments at different time points. (F) Plot summarizes the mean mechanical response score in isotype and Gr-1 treatment groups at different time points. In this plot, each point represents the mean mechanical response score of 1 animal obtained by averaging the mechanical response scores given to each von Frey filament (2-way ANOVA: across groups: $P = 0.048$, F = 4.0, DOF = 1; time: $P = 0.0001$, F = 15.1, DOF = 3; interaction: $P = 0.2$, F = 1.5, DOF = 3). (G) Comparison of grimace scores between animals in isotype vs Gr-1 treatment group at different time points (2-way ANOVA: across groups: $P = 0.15$, F = 2.3, DOF = 1; time: $P = 0.17$, F = 1.8, DOF = 3; interaction: $P = 0.69$, F = 0.49, DOF = 3). (E) Repeated measures of ANOVA with Dunnett multiple comparisons. # comparison of day 2 to baseline, ■ comparison of day 4 to baseline, *comparison of baseline to day 6. (F) Two-way ANOVA with the Sidak multiple comparisons test. (G) Two-way ANOVA with the Sidak multiple comparison tests. # ■ $^{*}P < 0.05$, ## ■■ $^{**}P < 0.01$, ###■■■, $^{****}P < 0.0001$. Mean ± SEM. ANOVA, analysis of variance. DOF: degree of freedom.

3.5. Depletion of neutrophils and monocytes leads to more sensory afferent loss and further bacterial invasion

Because we observed that during pulpitis, the intact CGRP+ fibers were mostly located in areas that were not densely populated by eutrophils and/or monocytes, we wanted to investigate whether continuous neutrophil and monocyte accumulation, after initial recruitment, contributed to tissue and sensory afferent damage in this enclosed environment of the pulp. We used Gr-1 antibody to

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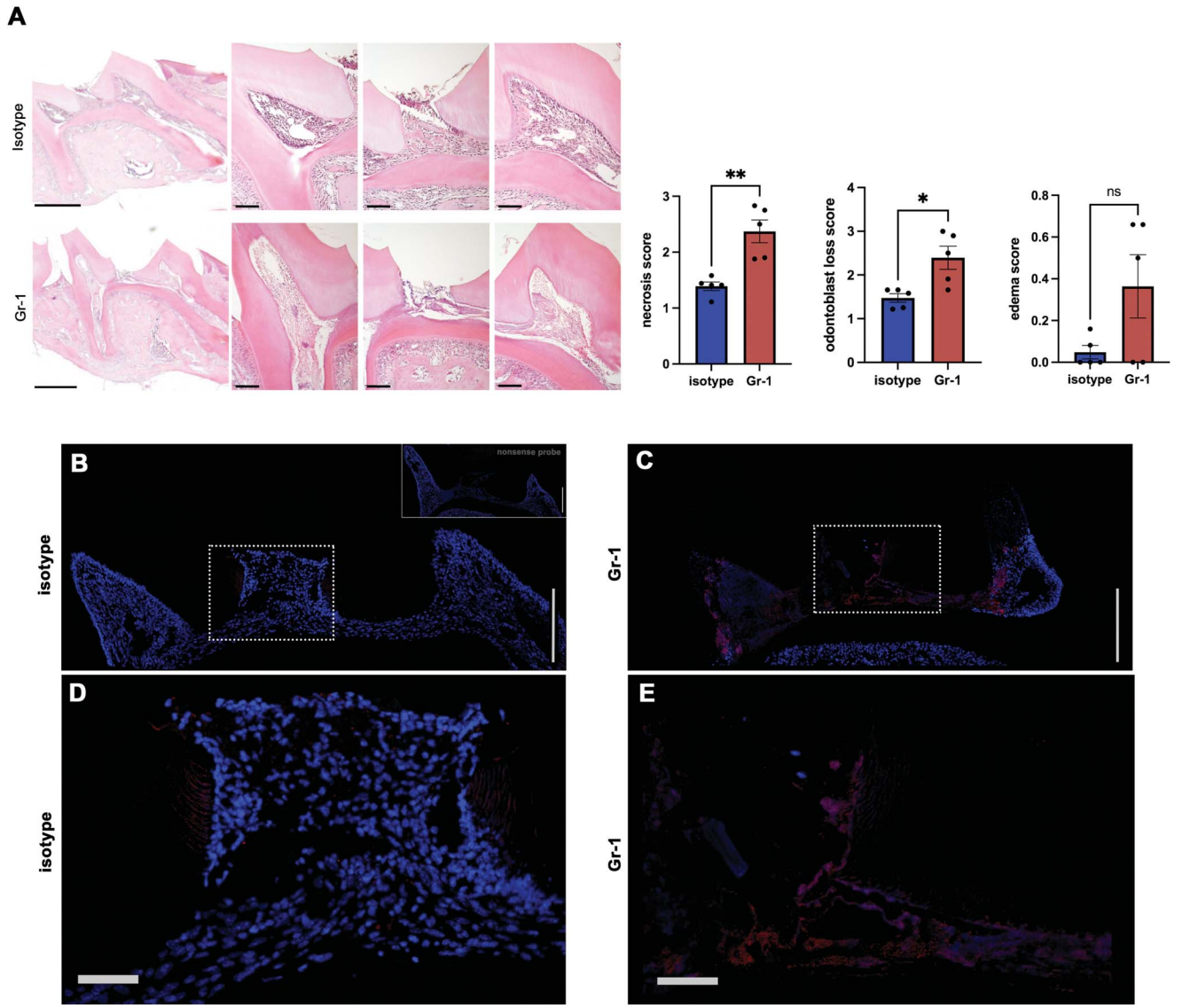

Figure 6. Depletion of neutrophils and monocytes leads to increased tissue necrosis and further bacterial progression. (A) Representative H&E staining images and analysis of extent of tissue damage 6 days after injury of Gr-1 and isotype treatment groups. Scale bar = 500 μm (low magnification) and 100 μm (high magnification). In each plot, each point represents an average score obtained from analysis of 3 sections for 1 animal. Unpaired *t* tests. $^{*}P < 0.05$, $^{**}P < 0.01$. (B) Representative image of 16S FISH from the isotype group. The box shows the injured middle pulp with few bacteria present. The image on the top right corner is a section where the nonsense probe was used as a negative control. (C) Representative image of 16S FISH from the Gr-1 treatment group. The box shows that most of the bacteria not only are densely present at the injury site but also extend to the noninjured pulp horns. (D) Close-up view of the middle-injured pulp horn in (B), marked by the dotted box. (E) Close-up view of the middle-injured pulp horn in (C), marked by the dotted box. Scale bar = 200 μm (low magnification) and 50 μm (high magnification). Red, 16S and blue, DAPI.

deplete neutrophils and monocytes.[5,15] We first confirmed that we substantially reduced the percentage of neutrophils and monocytes by performing a flow cytometry experiment using splenocytes collected at day 6 (**Fig. 5B**). We found that, at day 6 after injury, histologically, in the Gr-1 group, there was significantly more sensory afferent loss at the injured site ($P = 0.002$) and at the noninjured pulp horns ($P = 0.028$) (**Figs. 5C and D**). In addition, there was more tissue necrosis and odontoblast loss ($P = 0.020$, $P = 0.011$) and more edema formation in the Gr-1 group compared with the isotype antibody-injected control group (**Fig. 6A**). By also performing a 16S FISH experiment, we found that after neutrophil depletion there was greater bacterial accumulation, extending to the noninjured pulp horns, whereas in the isotype group, the bacteria remained localized to the injured area (**Figs. 6B–E**).

3.6. Depletion of neutrophils and monocytes reduces facial mechanical hypersensitivity

Finally, we wanted to examine whether reduction in numbers of neutrophils and monocytes altered the pain-like behaviors after dental pulp injury. We saw that the mechanical response score remained similarly high on day 2, 4, and 6 in the isotype group, whereas in the Gr-1 group, there was a reduction of mechanical response on day 6 compared with days 2 and 4 (**Figs. 5E and F**). When we compared the mean mechanical response scores, we found that on day 6, the mean mechanical response score in the Gr-1 group was less compared with the isotype group ($P = 0.03$) (**Figs. 5E and F**). We also compared mechanical response scores in groups to each von Frey stimulus separately as described in the methods and found that there was reduced allodynia in the Gr-1 group which was more clearly present on day

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6 when a 0.07-g filament was tested (Supplementary Figure 3, available at http://links.lww.com/PAIN/B905). We also investigated spontaneous pain-like behavior by grimace scoring, and there was no difference between the 2 groups across all time points (**Fig. 5G**). These findings indicate that when neutrophils and monocytes were significantly reduced, there was more tissue damage, including more sensory afferent loss, whereas there was a reduction in mechanical response scores compared with the control group later point (day 6).

4. Discussion

Pulpitis can be a debilitating painful experience that is driven by both peripheral and central mechanisms within the trigeminal system.[21,62,63] In mice, CGRP injection causes spontaneous pain-like behaviors and activation of CGRP+ fibers increases mechanical sensitivity in mice.[23,31,46,59] By both inducing neuroinflammation at the periphery and sensitization within the trigeminal ganglion, CGRP is likely to play a role in painful pulpitis.[3,35,50] In this study, we assessed spontaneous pain-like behavior and mechanical sensitivity in Calca$^{-/-}$ and Calca$^{+/+}$ animals by using the mouse grimace scale and facial von Frey stimulation, respectively. We observed at the earliest time point that CGRP might have contributed to spontaneous pain-like behavior and there was a similar trend at a later time point. Histologically, we saw significant loss of CGRP+ fibers 3 days after injury which was even more pronounced 7 days after injury. This could be the reason why in this model, the effect of CGRP in the mouse grimace scale was more evident at the early time point but not clearly present at later time points. When we performed facial von Frey testing, we found that there was no difference in mechanical response scores in Calca$^{+/+}$ and Calca$^{-/-}$ injured animals at different time points. Further studies are needed to investigate the mechanisms by which CGRP might be contributing to spontaneous pain-like behavior while not leading to mechanical sensitivity during pulpitis.

We found that in the pulpitis model, CGRP also contributed to immune cell recruitment, mostly neutrophils at the early time point. As neutrophils are known to contribute to acute inflammatory pain,[5,54] it is possible that in this pulpitis model, early time point contribution of CGRP to spontaneous pain-like behavior could

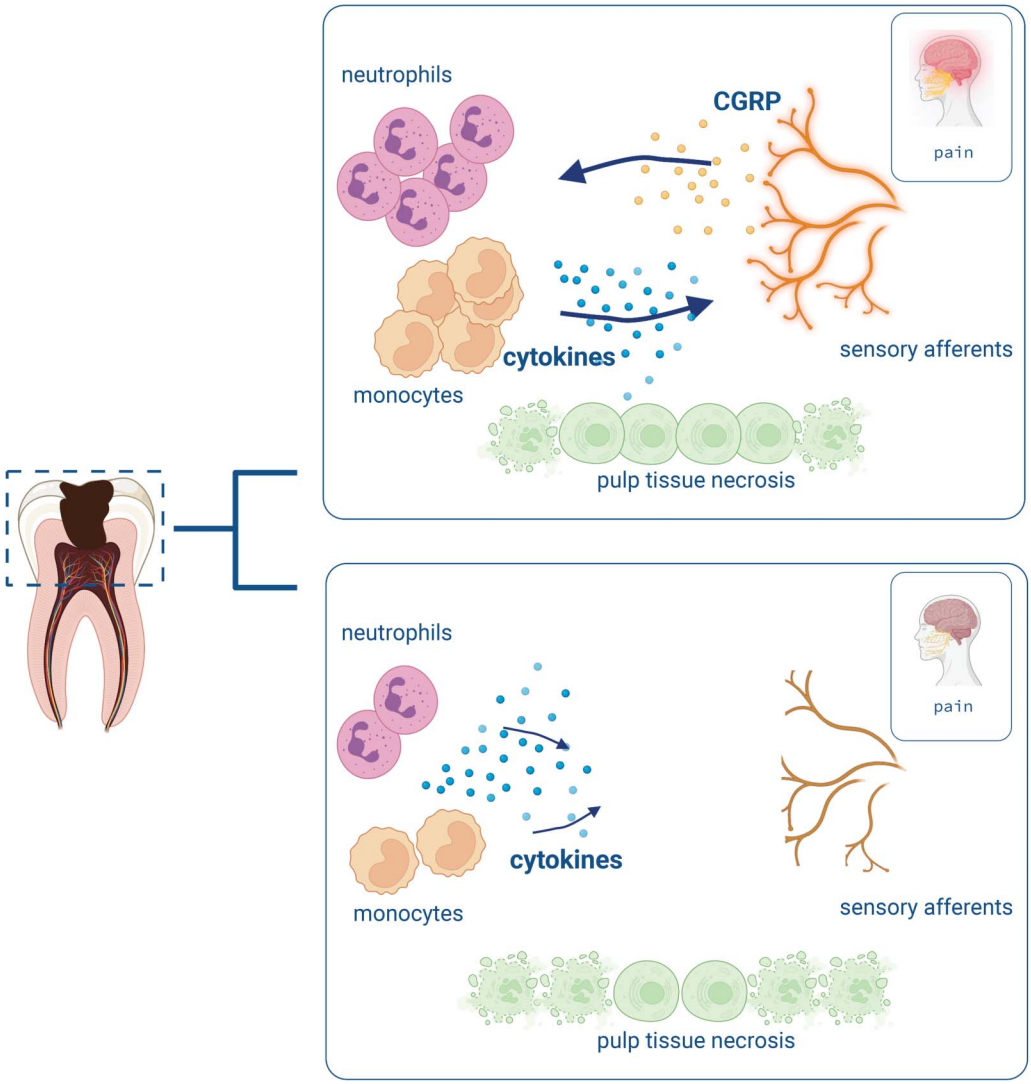

Figure 7. Impact of neuroimmune interactions on pain and tissue necrosis during pulpitis. CGRP is involved in neutrophil and monocyte recruitment while contributing to spontaneous pain-like behavior during pulpitis. CGRP's contribution to inflammation through neutrophil recruitment could contribute spontaneous pain-like behavior. Neutrophils and monocytes are crucial in protecting dental pulp tissue from further bacterial penetration and tissue damage, including sensory afferent loss while contributing to mechanical hypersensitivity. Prepared using biorender.com. CGRP, calcitonin gene-related peptide.

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involve CGRP-mediated recruitment of neutrophils. Furthermore, by investigating the spatial relationship between CGRP+ fibers and neutrophils and/or monocytes at different time points after injury, we saw that as the innate immune cells were becoming more widespread within the pulp, there was more abundant loss of CGRP+ fibers, especially in the regions densely populated by neutrophils and/or monocytes. Neutrophils critically perform acute inflammatory duties including bacterial clearance and removal of dying cells, which is protective of tissues. Conversely, neutrophils can contribute to tissue damage in inflammatory diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE).[6,43,52] To understand whether neutrophils and monocytes are protective or contribute to the progression of tissue damage in pulpitis, we performed a targeted depletion experiment of gr-1+ cells. We found that in the absence of continuous recruitment of neutrophils and monocytes, there was increased sensory afferent loss, tissue damage, and more bacterial invasion. This suggests that in this model, neutrophils and monocytes are crucial for the clearance of bacteria and protect the pulpal tissue, including the sensory innervation.[25,37,53]

In our investigations on the role of neutrophils and monocytes in pulpitis pain, we found that on day 6, at a later time point, there was a rescue of hypersensitive mechanical response scores in neutrophil-depleted and monocyte-depleted animals. Our findings were similar to another study which found that a hypersensitive mechanical response in CFA-induced inflammatory pain model was reduced on day 6 when neutrophils and monocytes were depleted.[54] Putting these findings together with our histologic examination, we found that in neutrophils and monocyte-depleted animals, despite observing more profound sensory afferent dieback and tissue damage, the mechanical nociceptive response was reduced. It is possible that the relationship of pain and sensory afferent loss is complex in pulpitis. In patients experiencing painful pulpitis, there is no clear evidence of a correlation between the severity of pain and the extent of tissue damage.[14,33,55] Surprisingly, about 30% of the patients report very mild or even no pain, although the extent of the pathology is similar to the cases with severe pain.[20,34] Similarly, in patients with diabetic neuropathy with pathology evidenced by sensory afferent dieback, there may or may not be pain coincident with the sensory disturbances.[18,56] The mechanisms that could explain how sensory afferent dieback is painful for some patients while it is nonpainful for others are not clearly understood in both diseases. It is possible that in our mouse pulpitis model, the sensitization of the nerve fibers and mechanical hypersensitivity could be mainly driven by cytokines released from neutrophils and monocytes, whereas dieback of sensory afferents may not contribute to mechanical sensitivity.[19,34] On the other hand, increased sensory afferent loss when neutrophils were depleted might have led to reduction in the mechanical sensory response. Further studies are needed to understand the inflammatory nature of pulpitis pain and the pain due to dieback of sensory afferents, which in some cases, might have a neuropathic component of the pain.[8,11]

5. Conclusion

In a model of pathogen-mediated pulpitis, CGRP release contributed to spontaneous pain-like behavior and neutrophil and/or monocyte recruitment early in the disease but did not change mechanical hypersensitivity or the progression of pathogen-mediated tissue destruction. When neutrophils/monocytes were depleted, there was more sensory afferent dieback and mechanical hypersensitivity responses were lower at the later stage of pulpitis (**Fig. 7**).

Conflict of interest statement

The authors have no conflict of interest to declare.

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