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Article

Nerve Injury-Induced Immune Responses in the Taste Bud Target Field

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Abstract

Damage to the chorda tympani (CT) nerve through trauma or experimental nerve axotomy results in the degeneration of anterior taste buds and taste loss. Our previous work demonstrated that *Il1r* signaling is required for taste bud regeneration and the recovery of taste function. However, the effects of experimental axotomy on immune responses in the absence of *Il1r* signaling remain unclear. To test this, we performed unilateral CT sectioning in *Il1r* KO or wild-type mice as previously described. We found that CD45⁺ immune cells, CD68⁺ and CD206⁺ M2-like macrophages are significantly increased near anterior taste buds at day two post-injury in wild-type but not *Il1r* KO mice. By day 5, these macrophage responses were slightly elevated in wild-type mice but remained at baseline levels in KO mice, indicating that immune responses to injury were suppressed rather than delayed in the absence of *Il1r* signaling. However, taste buds degenerated at similar time points in both strains. These results suggest that delayed taste bud degeneration in *Il1r* KO mice is not the primary reason for later functional deficits, though suppressed immune response may have other consequences in the injured peripheral taste system.

Keywords: chorda tympani nerve; axotomy; degeneration; Interleukin-1; gustatory; macrophages; regeneration

1. Introduction

Taste buds are heterogenous chemosensory organs that transmit information about chemical stimuli from the oral cavity to the gustatory cortex. Similar to epithelial cells, taste bud cells are not permanent and have a turnover rate of 7-30 days with each taste bud containing 40-100 specialized taste cells. Taste buds are composed of four main taste receptor cell types. Type I cells are "glial-like" with processes that encompass other cell types in the taste bud. Type II and type III cells are directly responsible for the transduction of taste information, with type II responding to bitter, sweet or umami stimuli and type III responding to sour and salty tastants. Post-mitotic type IV cells at the base of the taste bud differentiate into the other three cell types [4,5].

Taste buds are innervated by and receive trophic support from sensory afferent nerve fibers. Anterior taste buds in fungiform papillae are innervated ipsilaterally by one of two chorda tympani (CT) nerves on each side of the tongue. Unlike neurons in the central nervous system, peripheral gustatory axons have the ability to regenerate after injury. In rodents, experimental axotomy of the chorda tympani nerve is followed by degeneration of the distal axons associated with taste buds. However, within four weeks in mice, CT fibers and taste buds regenerate and normal responses to tastants are restored [6-8]. The sense of taste is vulnerable to damage by chemotherapeutic treatment, infection, trauma, or through direct damage to the CT nerve during surgical or dental procedures [9-

14]. Lasting deficits in taste perception remain in some patients, highlighting the need for a better understanding of mechanisms mediating the recovery of gustatory function [15]. The plasticity of the peripheral taste system and bilateral innervation of anterior taste buds make rodent CT injury model ideal for elucidating mechanisms of recovery [14].

Gustatory nerve injury and taste bud degeneration elicits immune responses and activation of inflammatory pathways [16-21]. After CT axotomy in rodents there is a rapid influx of neutrophils in the degenerating taste bud field within 6 hours followed by macrophage responses over the next 48 hours [22-27]. Nerve injury also upregulates chemokine, adhesion molecules, and cytokine responses in the anterior taste field [16-21]. The IL-1 receptor (IL-1R)1 and ligands IL-1 α and IL-1 β are expressed in taste buds, axons, and immune cells responding to axotomy [20,28]. IL-1 α and IL-1 β bind to the IL-1R to trigger intracellular signaling and activate inflammatory responses. In the absence of *Il1r*, these responses are typically blunted in other tissues especially in the context of infection or injury [29-32]. However, lingual mRNA levels of the proinflammatory cytokine, *Il1b*, are upregulated at day 56-60 in *Il1r* KO compared to wild-type, C57BL/6J mice, which could induce inflammation at this late period [33]. This dysregulation, combined with the pleiotropism and redundancy of cytokine pathways, highlights the need to investigate immune responses in the taste bud target field after CT nerve injury.

Il1r KO mice receiving CT axotomy exhibit altered cytokine response dynamics in the injured taste bud field, dysregulated taste progenitor cell proliferation, and incomplete taste bud regeneration. These changes result in absent or minimal neural responses to taste stimuli even at >14 weeks post-injury, compared to the recovery of normal taste function at 4 weeks in wild-type mice [6]. Together, these results support the requirement for IL-1R signaling in taste bud regeneration and functional recovery. Here, we hypothesize that immune responses to CT axotomy are altered in the absence of IL-1R signaling. We performed surgical CT axotomy on wild-type (wild-type) and *Il1r* KO mice as in our previous work [28] then measured innate immune cell populations at different time points post-injury. We also tested whether dysregulated immune responses in *Il1r* KO mice delay taste bud degeneration, potentially preventing later, efficient taste cell regeneration. Identifying the dynamics of specific immune responses will potentially allow for more targeted therapeutic treatments in the restoration of gustatory function after nerve injury, chemotherapy, or infection.

2. Results

2.1. CD45+ Immune Cells Rapidly Infiltrate Axotomized Taste Papillae in Wild-Type But not *Il1r* KO Mice

CD45+, a pan-leukocyte cell marker, labeled cells in fungiform papillae, the lamina propria immediately beneath the lingual epithelium, and throughout the deeper muscle layer of the anterior tongue. We are interested in immune cells that potentially affect taste bud integrity and function, so we spatially limited our analyses to fungiform papillae and nearby lamina propria. CD45+ immune cells were rarely observed within the lingual epithelium or inside taste bud borders in sham-sectioned mice. In response to nerve sectioning, immune cells infiltrated taste papillae and took on an activated morphology with projections extending from the cell (Figure 1A, white arrows). In contrast, CD45+ cells observed in the lingual muscle typically had a more rounded morphology (Figure S1B). NTPdase2, which labels the numerous type I glial-like taste cells, was used to identify taste buds. We note that NTPdase2 expression is also present in lingual nerve fibers as previously reported [34](Figure 1A, open arrows).

We analyzed immune responses at day 2, day 5, and day 60 post-injury. The timing was based on previous studies in rat demonstrating elevated macrophage responses during the first week after CT nerve sectioning. At day 60 post-axotomy, wild-type, C57BL/6J mice have recovered normal taste function for over 5 weeks, while *Il1r* KO mice still exhibit minimal taste responsiveness. We hypothesized that inappropriate immune responses at this later period could underlie the absence of taste bud regeneration and functional recovery in KO mice. We analyzed results with two-way ANOVAs followed by Bonferroni post-tests on selected comparisons: (1) sham-sectioned wild-type vs. *Il1r* KO; (2) CT-sectioned wild-type vs. *Il1r* KO to detect strain-related differences in baseline and injury-induced immune responses, respectively; and (3) sham vs. CT sectioned groups within each

strain to detect the effects of surgical treatment. Non-significant statistics are reported in Appendix A.1.

Given the wispy processes of CD45+ immune cells, we thresholded immunopositive pixels in standard-sized regions of interest as in previous studies [6,16,28]. These analyses revealed early, dramatic differences in CD45+ immune responses to axotomy in wild-type and *Il1r* KO groups (Figure 1C). At day 2 post-injury, there were significant main effects of surgical treatment [$F_{(1,12)} = 20.83$; $P = 0.0006$], mouse strain [$F_{(1,12)} = 7.326$; $P = 0.0191$], and their interaction [$F_{(1,12)} = 20.87$; $P = 0.0006$] on immune cell density in taste cell papillae and lamina propria. Specifically, lingual immune cells were significantly elevated in CT sectioned compared to sham-sectioned control mice ($P = 0.0001$). Notably, CD45+ cell density increased in wild-type compared to *Il1r* KO axotomized mice (Figure 1 A, B; $P = 0.0010$), as immune responses remained at baseline, sham levels in *Il1r* KO mice (Table A.1). As shown in Figure 1D at day 5 post-axotomy, there was a significant main effect of mouse strain [$F_{(1,8)} = 5.484$; $P = 0.0473$], but no difference between groups in post-tests. At day 60 post-injury (Figure 1 A & D), despite variability in the sham, *Il1r* KO group, there was a significant effect of surgical treatment on CD45+ cell density [$F_{(1,9)} = 8.185$; $P = 0.0188$] that was not reflected in post-tests. These results demonstrated that CD45+ leukocyte responses are elevated in wild-type but not *Il1r* KO at day 2 post-nerve injury.

Figure 1

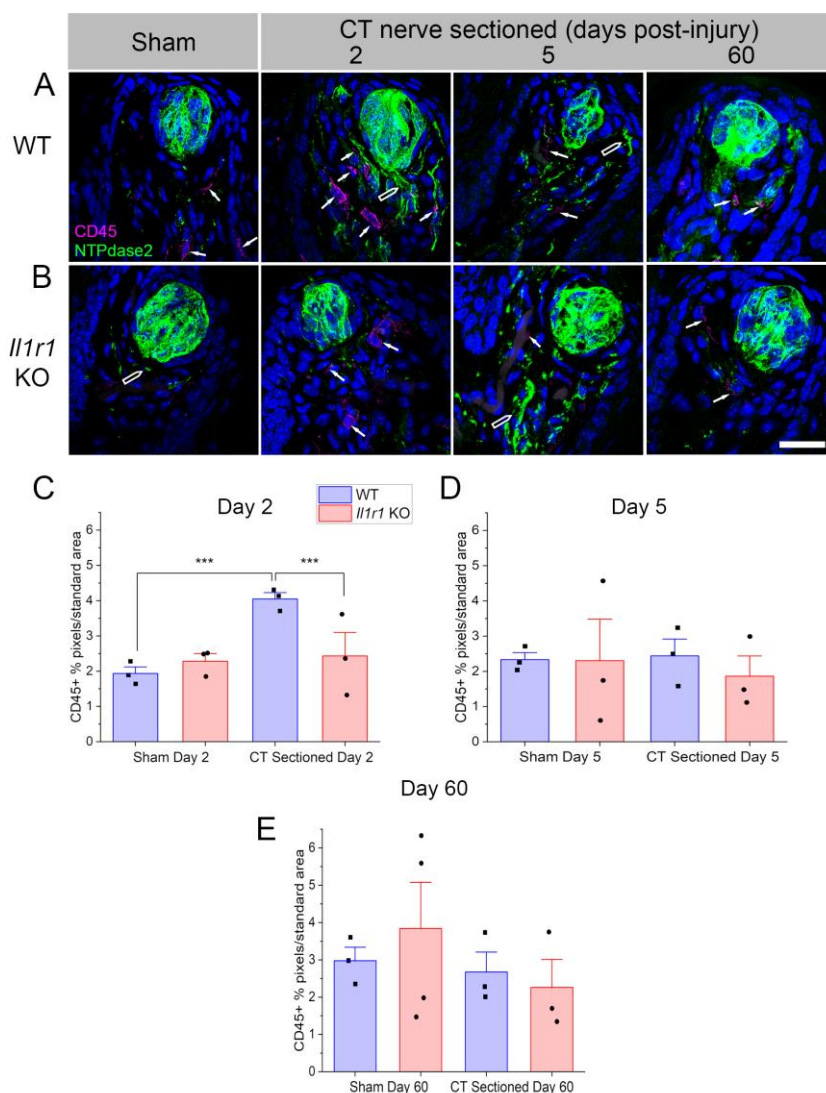


Figure 1. CD45+ immune responses are suppressed in the early post-injury period in *Il1r* KO compared to wild-type mice. (A) The density of CD45+ immune cell responses (magenta, white arrows) increases near

denervated taste buds at day 2 post-injury in wild-type mice. Taste buds are visualized with the type I taste cell marker, NTPdase2 (green), which also labels nerve fibers in the papilla (open arrows). CD45+ immune responses return to baseline at day 5 post-injury and remain at low levels at day 60 in wild-type papillae with regenerated, functional taste buds. (B) In *Il1r* KO mice, few CD45+ immune cells occupy taste papillae at any post-injury period tested. (C) CD45+ pixels were thresholded, expressed as the percent / standard area, and summed at each period (n=3-4 mice/group). There was a significant increase in the mean (+SEM) CD45+ responses at day 2 post-injury compared to sham wild-type mice or axotomized *Il1r* KO mice. Bonferroni post-tests revealed no significant differences in CD45+ immune responses among groups at (D) day 5 or (E) day 60. *** $P < 0.0001$. Scale bar in B = 20 μm .

2.2. CD68+ Macrophage Density Increases in Wild-Type Compared to *Il1r* KO Taste Papillae

In previous and pilot studies we observed robust macrophage responses to CT nerve sectioning in rodents, but few lingual T cells in contrast to human fungiform papillae [16,35,36]. Neutrophil responses were rapid, ending by day 2 after nerve injury [21]. Thus, to identify the subtype of CD45+ leukocytes infiltrating taste tissue we used the standard marker CD68 to quantify macrophage responses. CD68 is expressed in all macrophages isolated from tongue except for epithelial Langerhans cells [37]. Based on the results of the CD45 analysis, we focused on day 2 post-injury since immune responses returned to baseline across all mice strains and treatments at day 5 and 60 post-injury. Identified macrophages were morphologically similar to immune cells identified using the pan-immune marker CD45, and also found in similar locations after axotomy in wild-type mice (Figure 2). Macrophages were sporadically present within (Figure 2A, inset) and often near taste buds and nerve fibers of nerve-sectioned wild-type mice. Few CD68+ macrophages were observed in *Il1r* KO mice (Figure 2B).

At day 2 (Figure 2C), macrophage density significantly increased in CT axotomized compared to sham wild-type mice [$F_{(1,13)} = 5.068$; $P = 0.0423$]. There were also significant main effects of mouse strain [$F_{(1,13)} = 5.068$; $P = 0.0423$], and the interaction between strain and surgical treatment [$F_{(1,3)} = 16.16$; $P = 0.0015$]. Post-tests showed that increased CD68+ macrophage responses to injury in wild-type mice ($P = 0.0013$) were absent in *Il1r* KO mice ($P = 0.0013$). In conjunction with the CD45 analyses, these results demonstrate that early macrophage responses to taste bud denervation are suppressed in the absence of IL-1R signaling.

Figure 2

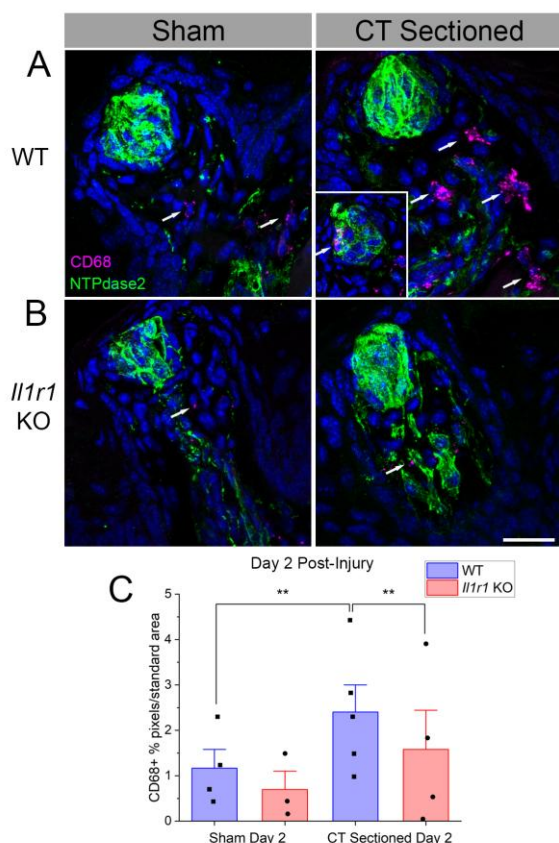


Figure 2. CD68+ macrophage density increases at day 2 post-sectioning in wild-type but not *Il1r* KO mice. (A) Many CD68+ macrophages (magenta, white arrows) are present in wild-type taste papillae at day 2 after axotomy compared to sham controls. Occasionally, macrophages infiltrated denervated taste buds (inset on right). (B) Few macrophages were observed in *Il1r* KO papillae even after axotomy. (C) Quantification of CD68+ pixels/standard area demonstrates a significant increase in the mean (+SEM) level of macrophage responses at day 2 post-injury in wild-type mice. N = 3-4 mice / group. ** $P < 0.001$. Scale bar in B = 20 μ m.

2.3. Immune Responses to Axotomy Are Primarily Composed of M2-Like Macrophages

Macrophages exhibit polarization into M1 and M2 subtypes, though little is known about their phenotype in normal or denervated taste papillae or lingual epithelium. While macrophage responses are more complex in vivo than in vitro [38,39], further phenotyping has provided insights to immune responses to axotomy and sterile tissue injury [40,41]. We refer to these macrophages as “M2-like” and “M1-like” (abbreviated as M2 and M1, respectively) because of this caveat. M2 macrophages are associated with anti-inflammatory responses and tissue repair [42-45]. Since macrophage responses were only elevated in wild-type mice in which taste buds later regenerated, we tested whether M2 responses to axotomy are prevalent in this group. We used CD206 and CD68 to identify M2, double-positive macrophages (denoted as CD206++) in taste papillae and lamina propria based on findings in the tongue and other injured tissues [46-49]. Taste buds were not labeled with NTPdase2 in these experiments because of antibody compatibility, but were easily identified morphologically by DAPI (Figure 3A, B; white dotted lines). In sham-sectioned wild-type or *Il1r* KO mice, few macrophages were present in fungiform papillae and lamina propriae (Figure 3A, B).

At day 2 after axotomy, CD206++ macrophages were numerous in wild-type mice but sparse in *Il1r* KO animals (Figure 3A, B). A majority of CD68+ macrophages (56.6%) expressed the M2 marker, CD206. There were significant effects of surgical treatment [$F_{(1,8)} = 40.30$; $P = 0.0002$], mouse strain [$F_{(1,8)} = 36.09$; $P = 0.0003$], and their interaction [$F_{(1,8)} = 32.23$; $P = 0.0005$](Figure 3D). Double-positive macrophages were significantly elevated in nerve-injured vs. sham-sectioned wild-type mice ($P =$

0.0001). In contrast, there was a significant difference between strains in nerve-injured groups ($P = 0.0001$). Specifically, CD206⁺⁺ macrophages remained at baseline, sham levels in *Il1r* KO mice.

Since CD206⁺⁺ macrophage responses to injury were minimal at day 2, we also analyzed this population at day 5 to check for delayed responses in KO mice. M2-like macrophage responses followed a similar pattern at day 5 post-injury, although at lower levels in wild-type mice compared to day 2. However, there were significant effects of surgical treatment [$F_{(1,8)} = 15.44$; $P = 0.0044$], mouse strain [$F_{(1,8)} = 20.96$; $P = 0.0018$], and their interaction [$F_{(1,8)} = 15.44$; $P = 0.0044$]. As at day 2, responses were elevated in the wild-type group after axotomy ($P = 0.002$), and significantly reduced in *Il1r* KO vs wild-type groups receiving CT nerve sectioning ($P = 0.0013$). In sum, M2-like macrophage responses were robust in control-strain mice, particularly at day 2, but negligible in the system-wide absence of IL-1R signaling.

Figure 3

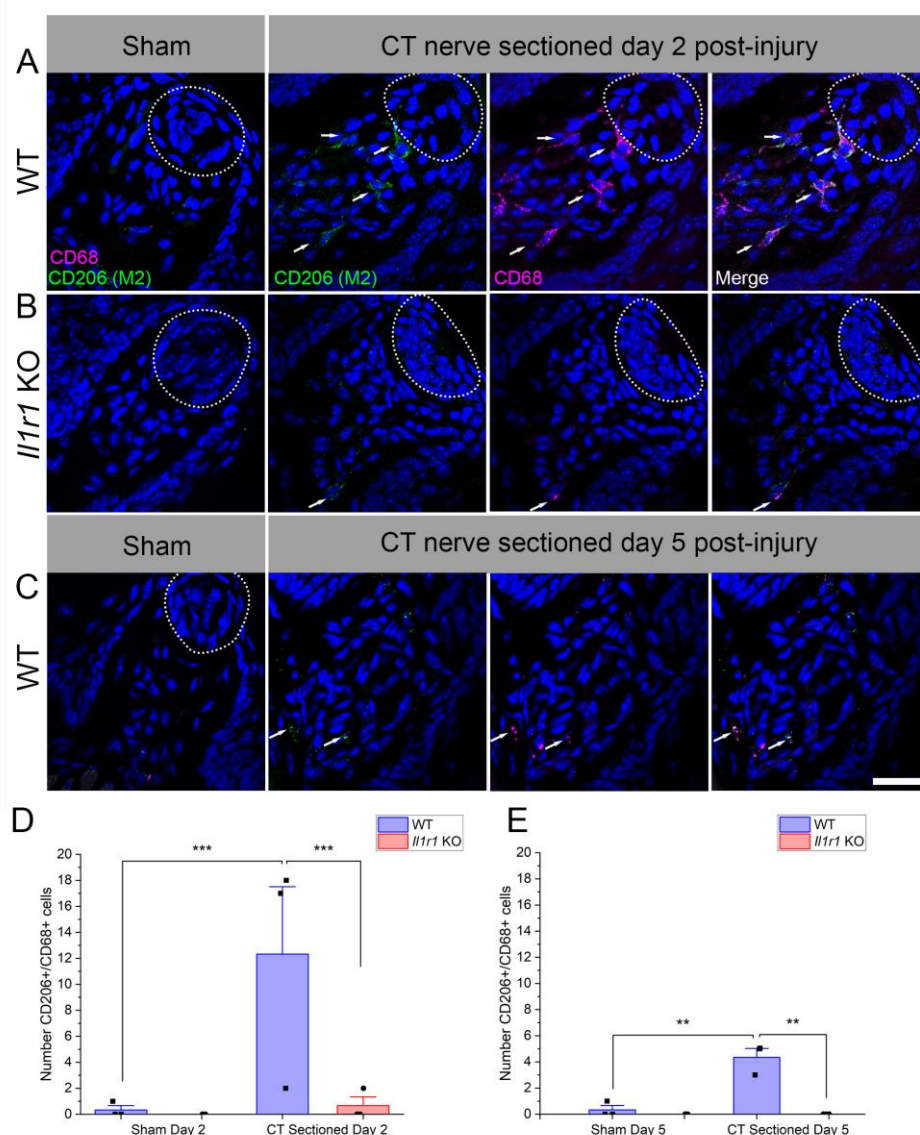


Figure 3. CD68+CD206+ M2-like macrophage responses to nerve injury predominate in wild-type mice. In these analyses, we counted double-positive macrophages, associated with a DAPI+ nucleus, rather than quantifying immunopositive pixels as in Fig. 1-2. (A) Taste tissue, including fungiform papillae, taste buds (dotted white lines) and lamina propria, was populated by few macrophages in sham-sectioned wild-type mice. Double-positive macrophages were prominent in these regions in wild-type mice at day 2 after axotomy (white arrows). (B) At the same time, few macrophages were present in sham- or nerve-sectioned *Il1r* KO mice. (C) CD68+ CD206+ macrophages subsided by day 5 post-injury in wild-type mice, but remained elevated particularly at the base of papillae and in the lamina propria. The mean (+SEM) number of M2-like

macrophages was significantly elevated in wild-type compared to sham-sectioned wild-type and axotomized *Il1r* KO mice at both (D) day 2 and (E) 5 (n=3 mice/group). ** $P < 0.001$; *** $P < 0.001$. Scale bar in B = 20 μm .

2.4. M1-Like Macrophages Were Absent at Day 2 and 5 After CT Nerve Injury

We tested whether a subset of lingual CD68+ macrophages express the proinflammatory M1-like marker, inducible nitric oxide synthase (iNOS) [45]. iNOS is not constitutively expressed, but upregulated by inflammatory stimuli including cytokines, LPS, and peripheral nerve injury [50]. Systemic LPS also induced iNOS in circumvallate taste buds and nerve fibers in previous reports [51]. We observed very limited iNOS expression in CD68+ macrophages deep in the lingual muscle and in ventral glands at day 2 or 5 after CT sectioning in wild-type mice (Figure A1). Macrophages in taste papillae and nearby lingual mucosa remained iNOS-negative, though positive control staining was observed in LPS-treated tongue and inflamed colon (not shown).

2.5. Taste Buds Degenerate in Both Wild-Type and *Il1r* KO Mice

We previously demonstrated similar numbers of taste buds in wild-type and *Il1r* KO mice at day 5 following CT nerve axotomy [6]. However, given the reduced immune responses to injury in *Il1r* KO papillae (Figure 1-3), taste bud degeneration could be delayed as a substrate for later deficits in regeneration and recovery in this strain. To test this, we counted taste buds expressing the marker Keratin (K)8 on the denervated and intact sides of lingual epithelial whole mounts at day 10 post-injury as shown in Figure 4A-D. We then analyzed the percentage of remaining taste buds (number taste buds on right or sectioned side / number on left or intact side X 100). As shown in Figure 4E, taste bud degeneration was similar in wild-type and *Il1r* KO mice. There was a significant main effect of surgical treatment [$F_{(1,8)} = 16.85$; $P = 0.0034$] but not strain [$F_{(1,8)} = 0.1167$; $P = 0.7415$]. There were no significant differences in post-tests, although sham vs. sectioned values approached significance for both wild-type ($P = 0.0877$) and *Il1r* KO ($P = 0.0718$) strains. This is not unexpected in wild-type mice, since CT nerve responses to taste stimuli appear in a subset of animals at day 11-17 post-injury indicating that taste buds are partially regenerated at this period [6]. Thus, taste buds degenerate at the same rate in the absence of IL-1R signaling despite suppressed macrophage responses to injury in these mice.

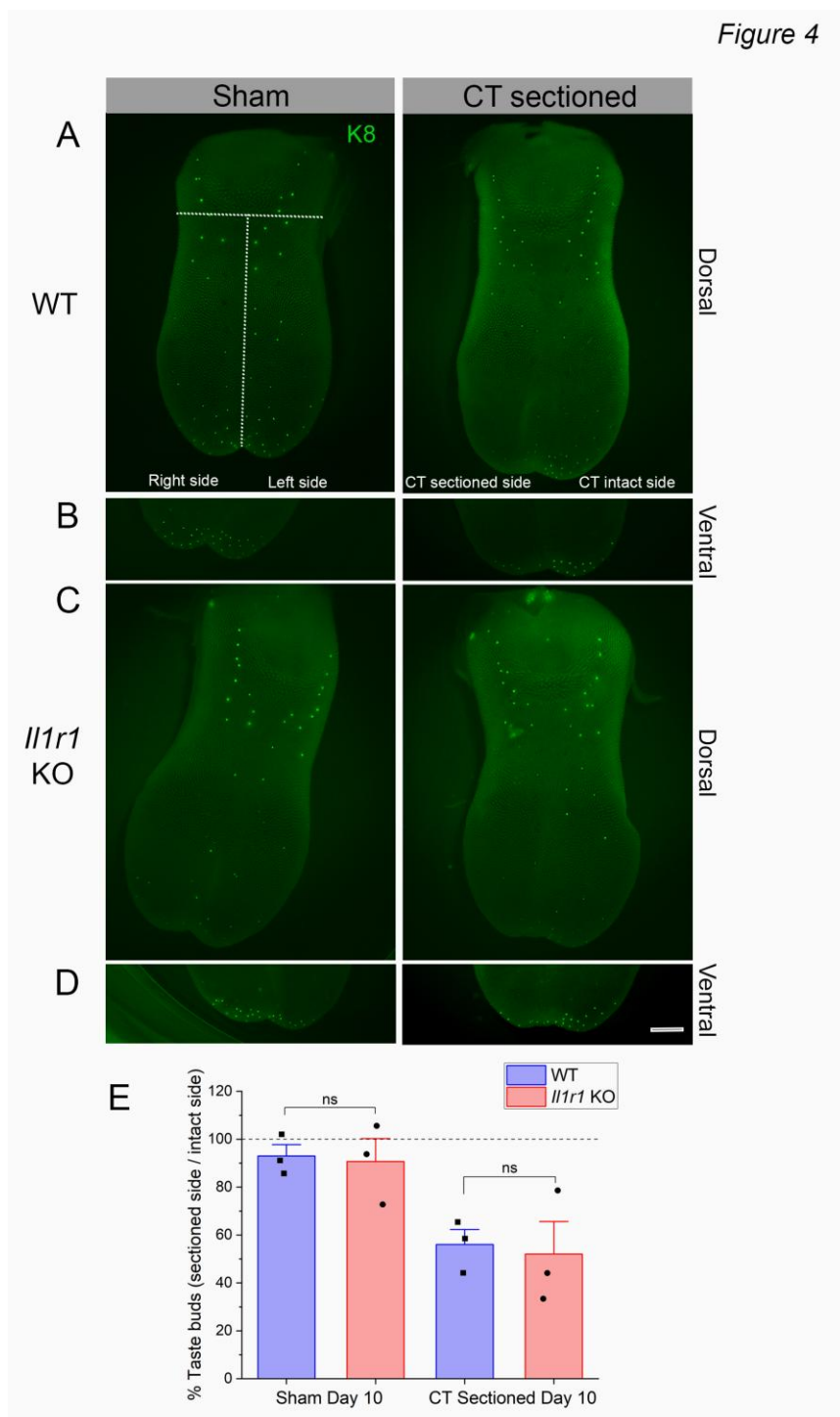


Figure 4. Taste buds degenerate in both wild-type and *Il1r* KO mice at day 10 post-injury. Lingual epithelia were enzymatically and manually dissected and taste buds visualized with Keratin 8 (K8). Taste buds are shown in sham-sectioned (left) or CT nerve injured (right) lingual epithelia. Dotted lines in A, upper left, show regions in which K8⁺ taste buds were counted anterior to the intermolar eminence, and on the right (sectioned) and left (intact) sides of the tongue. We counted taste buds on the (A, C) dorsal epithelium and (B, D) ventral side of the tongue tip, which has a high density of taste buds. Note the reduced density of green taste buds on the right (axotomized) dorsal and ventral tongues of both strains. (E) K8⁺ taste buds on the right side were expressed relative to the left (intact) side to calculate percent degeneration. 100% (horizontal dotted line) represents equal numbers of taste buds on the two sides of the tongue, or no degeneration. There were no significant differences between groups, as detailed in the text. N=3 mice/group. Scale bar in D = 1 mm.

3. Discussion

Immune responses to peripheral nerve axotomy, and the consequences for subsequent axonal regeneration, have been studied in depth [52-55]. Sterile inflammation in denervated target tissue, in contrast, is not well-understood particularly in the taste system [14]. We previously demonstrated that the IL-1R is required for normal taste bud regeneration and the restoration of gustatory function [33]. In the absence of signaling through this pleiotropic cytokine pathway, taste loss persists after experimental axotomy. Here, we report that leukocytes rapidly respond to CT nerve sectioning in control-strain mice that later recover normal taste function. The immune response is dominated by CD206+ M2-like macrophages which infiltrate the denervated anterior taste tissue within days. At the same early post-injury period (i.e., days 2 and 5), CD45+ leukocytes, CD68+ macrophages, and CD206++ M2 macrophages remain at sham-like control levels in the taste tissue of axotomized *Il1r* KO mice. Even 8 weeks after nerve sectioning, immune responses remain minimal in KO mice indicating their absence rather than a delay.

We tested whether taste receptor cell degeneration is delayed in *Il1r* KO mice, but that is not the case. Instead, taste bud numbers are similar at day 5 [33] and 10 post-injury across mouse strains, despite the absence of CD45+ leukocyte responses in KO mice. Neurophysiological responses to taste stimuli return at day 11-17 after CT axotomy in a subset of wild-type mice, indicating that nerve fibers and new taste receptor cells are reconnecting at this period. Partial regeneration at this period likely explains why the reduced number of taste buds in axotomized vs. sham mice approaches but does not reach significance. It is unlikely that *Il1r* KO delays taste bud degeneration beyond day 10, since the CT nerve remains unresponsive to taste stimuli from day 11-60 post-injury in these mice. Our prediction was based on the likelihood that CD45+ immune cells, and particularly macrophages, clear apoptotic taste receptor cells. CD45 is expressed on all cells of hematopoietic lineage, including lymphocytes (e.g., T cells and B cells), innate immune cells, dendritic cells, and at least some fibroblasts.

Our finding that taste bud degeneration proceeds in the absence of infiltrating leukocytes highlights the uncertainty over the fate of denervated, apoptotic taste receptor cells [56]. Suzuki and colleagues reported that macrophages phagocytose nerve fibers while fibroblasts engulf taste receptor cells in posterior mouse taste buds after glossopharyngeal nerve sectioning [57]. This is consistent with our results if CD45-negative fibroblasts phagocytose anterior taste receptor cells while M2-like macrophages clear taste fiber debris. A small subpopulation of CD45+ cells isolated from murine tongue was comprised of fibroblasts in a recent study using single-cell RNA sequencing [37]. However, the sizable population of vimentin-positive stromal cells in other reports suggests that many fibroblasts are CD45-negative [58,59]. Fibroblasts play a role in wound-healing in the tongue, and are suggested to recruit macrophages to the injury site [60]. Taste cells may also be cleared by type I, glial-like taste cells [61,62]. Desquamation through the taste pore has been suggested as a mechanism for taste receptor cell clearance, though it is unclear that this mechanism could accommodate widescale taste cell death after axotomy [63,64].

Two novel, major subpopulations of CD45+ macrophages in uninjured mouse tongue were recently reported. Cx3cr1+ macrophages occupy the taste papillae and lamina propria, while Fcγ2b+ cells reside in the lamina propria and deeper lingual muscle [37]. Both of these resident macrophage populations express CD68 [37]. The spatial segregation (i.e., taste papillae and lamina propria vs. muscle) and morphological differences in the two CD68+ macrophage subsets in the current study may correspond to these populations, though additional immune responses are likely induced by nerve sectioning. The authors suggest that Cx3Cr1+ macrophages may communicate with nearby nerve fibers during homeostasis and after injury as recently reported in skin [65]. Thus, even though taste buds degenerate at the same rate in wild-type and *Il1r* KO mice, we cannot rule out interactions between macrophages in papillae, lamina propria and muscle and CT nerve fibers. Since anterior taste buds are dependent on trophic support from the CT nerve, major changes in the rate that gustatory fibers degenerate are unlikely [14]. In a previous study, we also found that the subset of taste that regenerate (~40%) in *Il1r* KO mice are innervated. In fact, the percent of the taste bud occupied by CT fibers is slightly elevated in the absence of IL-1R signaling at day 56-60 post-injury [33].

IL-1 is a master regulatory cytokine. Knocking out its receptor will inhibit many inflammatory signals that stimulate immune responses to axotomy and subsequent nerve and taste receptor cell degeneration [66,67]. While resident CD45⁺ populations are similar during homeostasis (e.g. sham wild-type vs. KO groups), injury unmasks the critical role of IL-1R cytokine signaling in taste regeneration and recovery [33]. For example, macrophage chemoattractant protein (MCP)-1, intracellular adhesion molecule (ICAM)-1 and vascular adhesion molecule (VCAM)-1 are upregulated at 24 and 48 hours after CT nerve axotomy in rats [18,19]. IL-1 signaling is a major inducer of these recruitment signals, which likely results in the suppressed immune responses to nerve injury reported here [66-68]. One of the challenges in identifying molecular mechanisms by which IL-1 signaling contributes to taste regeneration is the huge number of biological processes that it controls [66-68]. The current study is a step in that direction since we report that M2-like macrophages respond to CT sectioning in control but not *Il1r* KO mice. Importantly, taste buds degenerate at the same rate in both strains. Even though this is a negative result, it shifts the focus of future studies to later regenerative processes.

We suggest that IL-1 signaling may have a growth factor-like role in the injured taste system. This cytokine could target taste progenitor cell proliferation, differentiation, or their ability to attract regenerating CT nerve fibers. One example of a similar non-canonical role for IL-1 occurs in skin wound healing. Keratinocyte-derived IL-1 stimulates growth factor release, including keratinocyte growth factor (KGF)-1/fibroblast growth factor (KGF)-7 from fibroblasts regulating skin wound repair [69]. In the gut, IL-1 and other cytokines induce intestinal stem cell proliferation fostering regeneration and repair [70]. The olfactory epithelium also upregulates *Il1b* and other proinflammatory cytokines in a tumor necrosis factor (*Tnf*)-overexpression model of chronic inflammation. Horizontal basal stem cells recruit macrophages and other leukocytes through an NFκB-dependent mechanism that is acutely pro-regenerative but chronically detrimental to olfactory sensory neuron regeneration and functional recovery [71,72]. The requirement for tightly regulated inflammatory responses might be similar for taste bud regeneration. Importantly, better understanding of neuroimmune mechanisms leading to taste recovery could suggest new strategies to treat the significant clinical problem of taste dysfunction from chemotherapy, injury, and long COVID-19 [73,74].

4. Materials and Methods

4.1. Animals

Adult male and female mice were 8-16 weeks old during all experimental procedures. Wild-type C57BL/6J (WT) and B6.129S7-*Il1r1*^{tm1lmx}/J were obtained from the Jackson Laboratory (000664; #003245, respectively) and bred in-house. Sex differences were not observed in previous studies focused on CT sectioning in rodents [6,16,17,19,22,26]. Genotypes were confirmed using Jackson Laboratory protocols on site or outsourced (Transnetyx). Specified pathogen-free mice were kept on a 12:12 h light: dark cycle with *ad libitum* access to rodent chow (Envigo Tekland) and filtered tap water.

4.2. Chorda Tympani (CT) Nerve Sectioning

A ketamine (50 mg/kg) and xylazine (10 mg/kg) cocktail was administered intraperitoneally to anesthetize mice. Once a surgical plane of anesthesia was achieved, mice were moved to a water-circulating heating pad to maintain body temperature. Mice were given ketoprofen analgesic (5 mg/kg bw) prior to the start of surgery. CT nerve sectioning was done as previously described [33]. Access to the CT nerve was gained ventrally through the neck and transected after bifurcation from the lingual nerve with severed ends being left in place. Sham-sectioned mice underwent the same procedure, however, the CT nerve remained intact.

4.3. Tissue Collection and Immunofluorescence

Two, five, or 60 days after CT nerve sectioning, mice were sacrificed, and tongues were removed with the mandible still attached. Samples were fixed in paraformaldehyde for 5-7 h then tongues dissected free and placed in cryoprotectant overnight [6,75]. Tongues were frozen in O.C.T (Fisher)

and 8 μm sagittal sections were obtained for immunostaining. Samples were rinsed with PBS (7.5 pH) and placed in 5% blocking solution for 45 min for CD45 analysis and 30 minutes for CD68 and CD206 at room temperature. Staining for iNOS followed a similar procedure as described, however, we included a 30-minute permeabilization step in 0.5% triton-x in PBS before placing samples in blocking solution (Sigma-Aldrich 9036-19-5). NTPdase2 (1:1000, Labome, 501943785, rabbit anti-mouse) was used stain Type I taste cells to identify taste buds. We used NTPdase2 instead of Keratin 8 since antigen retrieval interfered with immune cell staining. CD45 (1:100; R&D Systems, AF114, goat anti-mouse) is a pan-immune cell marker while CD68 (1:100; Abcam, ab53444, rat anti-mouse) identifies macrophages. To observe M1 and M2 macrophage subtypes, iNOS (1:100; Abcam, ab3523, rabbit anti-mouse) and CD206 (1:1000; Abcam, ab64693, rabbit anti-mouse) were used, respectively. Tongue sections were incubated in primary antibodies in a humidified box at 4°C overnight. Samples were rinsed with PBS and incubated in donkey anti-goat 594 (1:500; Abcam, ab150136), donkey anti-rabbit 488 (1:1000; Jackson ImmunoResearch, 711-545-152), goat anti-rabbit 488 (1:1000; Jackson ImmunoResearch, 111-545-144) and goat anti-rat 594 (1:500; Abcam, ab53444) in a humidified box at room temperature for 1 hr. Nuclei were stained with DAPI (#D1306; Invitrogen/Fisher Scientific) and slides coverslipped with Fluoromount-G (#0100-01; Southern Biotechnology).

4.4. Whole Mount Lingual Epithelium Analysis

Il1r KO or wild-type mice received CT nerve axotomy then euthanized with isoflurane followed by bilateral thoracotomy 10 days later and whole tongues were dissected from the jaw. We selected this period since we previously found no difference in the number of taste buds remaining at day 5 post-injury in wild-type or *Il1r* KO mice [33]. Tongues were washed in PBS and injected with ~1.5 mL collagenase A (1 mg/mL, catalog no. 10103578001; Roche) and dispase II (2.5 mg/mL, D4693-1G; Sigma). After injection, tongues were incubated in 300mL of PBS with an oxygen bubbler for 45 minutes at room temperature, then placed on a shaker and fixed in 4% PFA for 1 hour. Intact whole epithelial were dissected from the underlying muscle and washed in PBS. Lingual epithelia were placed in blocking solution for 1 hour then incubated in primary antibody K8 TROMA (1:500; DSHB) for 2 days to identify tastebuds. Epithelia were washed with PBS, incubated in secondary antibody (donkey α rat 488, 1:500, 712-545-150; Jackson ImmunoResearch) for 2 days, and counterstained with DAPI in ddH₂O.

We counted taste buds on the right / denervated or left / intact side of the tongue (in sham- or chorda tympani nerve-sectioned mice) on images taken with a Leica M165 FC stereoscope. Separate images were taken of the dorsal epithelium and the ventral tip which has a high density of fungiform taste buds. We expressed the number of taste buds on the right or injured side of the tongue relative to the number on the intact side to obtain the percent degeneration. Brightness and contrast were adjusted to assist in counting taste buds.

4.5. Image Analysis

Non-overlapping images of either taste buds, papilla or in the absence of either, lamina propria, were taken at 40x magnification. CD45⁺ and CD68⁺ pixels were thresholded and calculated as % thresholded area relative to the region of interest (ROI 581.5065 mm²)[27,33,76,77]. The experimenter was blinded to surgical treatment and strain for most analyses. Serial sagittal sections were analyzed using a BX51 microscope with epifluorescence (Olympus), a digital monochrome camera (Cool Snap, Roper Scientific), and MetaMorph software (MDS Analytical Technologies). Quantification of iNOS and CD206 positive cells were done using the same epifluorescence microscope as previously described. Individual cells were considered double positive for CD68 and iNOS or CD206 if colocalization was found around DAPI positive nuclei and could be distinguished from background. For presentation purposes, z-stack images with a step-size of 1 μm were also captured with a Nikon A1R confocal/multiphoton microscope. Images of taste buds were captured at a magnification of 60x with a 3x zoom setting. We then converted images from NIS Elements (version 4.30.01) to maximum Z projections using ImageJ. Images for Figure S1 were captured with a Leica Stellaris confocal system at 40x (2.5x zoom) and a 1 μm step-size, and LAX .lif files converted

to maximum Z projections in ImageJ. Images were minimally adjusted for brightness and contrast in Adobe Photoshop with the same settings applied equally to all panels.

4.6. Statistical Analysis

Two-way ANOVAs were performed in GraphPad Prism (version 10.6.0), using mouse strain (wild-type or *Il1r* KO) and surgical treatment (sham or CT sectioned) as main factors. Bonferroni posttests were used for multiple comparisons. *P* values ≤ 0.05 were considered significant. *F* values, degrees of freedom, and *P* values are reported in the text when significant, and in Appendix A.1 when non-significant.

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Appendix A

Appendix A.1

Table A1. Table showing nonsignificant two-way ANOVA statistics.

Two-way ANOVA Comparison		Main Factor	<i>F</i> -statistic	df	<i>P</i>
Figure 1. CD45+ immune response	Day 5	Surgical treatment	0.7301	1, 8	0.4177
		Interaction	4.102		0.0774
	Day 60	Mouse strain	1.575	1, 9	0.2411
		Interaction	0.4634		0.5132
Figure 4. Taste bud degeneration	Day 10	Mouse strain	0.1167	1, 8	0.7415
		Interaction	0.008495		0.9288

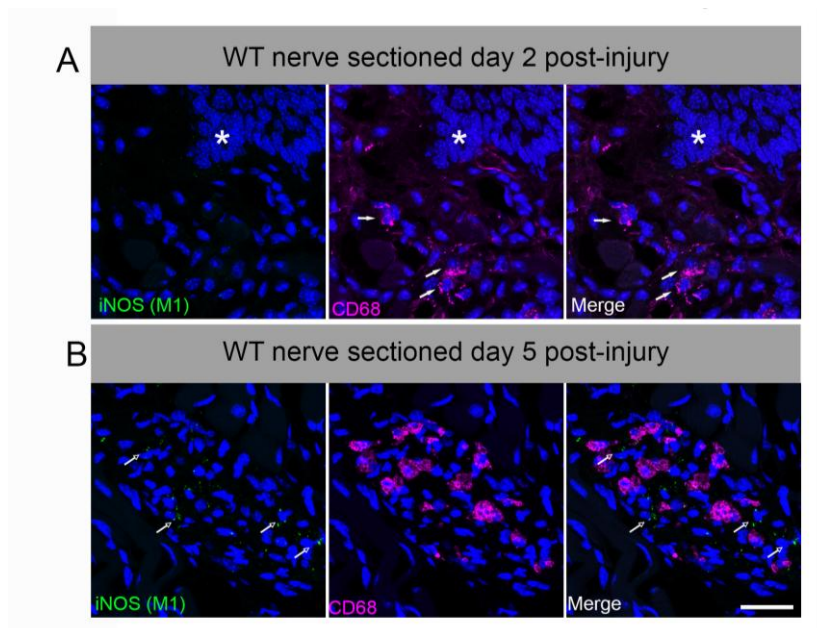


Figure A1. Lingual M1-like macrophages expressing iNOS are rare or absent after axotomy. Macrophages were identified with CD68 (magenta, arrows). **(A)** Macrophages in proximity to taste buds were increased at day 2 post-injury, as in Fig. 2. However, iNOS was not co-expressed with CD68 in taste papillae or lamina propria. White asterisk marks the base of a fungiform papilla. **(B)** At day 5 post-axotomy, CD68+ macrophages are most prominent in the deep lingual muscle. Punctate iNOS was observed nearby, but rarely co-localized with macrophages (green, open arrows). Scale bar in B = 25 μ m.

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