



Fadu head and neck squamous cell carcinoma induces hyperexcitability of primary sensory neurons in an in vitro coculture model

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Abstract

Introduction: Currently, cancer pain is viewed as a process orchestrated by the release of pronociceptive molecules and the invasion of neural structures, referred to as perineural invasion (PNI). Cancer pain resulting from PNI is well-documented, but the mechanisms leading to peripheral sensitization because of tumor growth are not fully known.

Methods: A retrospective study was used to examine how the use of anti-inflammatory medications affected preoperative pain in patients with oral squamous cell carcinoma cancer. We then used an in vitro coculture model in which dorsal root ganglion (DRG) neurons were incubated together with Fadu human head and neck squamous cell carcinoma cancer cells to explore how cancer cells affect the electrical membrane properties of sensory neurons.

Results: We found that inflammation contributes to preoperative pain in patients with oral squamous cell carcinoma. After coculture with Fadu human head and neck squamous cell carcinoma cancer cells, we identified markers of inflammation in coculture media and found evidence of neuronal sensitization, including spontaneous activity, reduced current thresholds, depolarized resting membrane potential, and enhanced responses to current stimulation in human and rat DRG neurons. In rats, these effects were influenced by sex and age: neurons from young adult female rats were resistant to changes in neuronal activity, in contrast to neurons from older adult female rats or male rats of either age group.

Conclusions: Pro-inflammatory substances released in cancer cell–DRG coculture promoted neuronal hyperexcitability and may contribute to cancer pain after PNI, and these effects may differ across age groups and sexes.

Keywords: Perineural invasion, Dorsal root ganglia, Sensitization

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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1. Introduction

Pain in patients with cancer constitutes one of the most prevalent symptoms, accounts for deterioration in the quality of life of patients, and contributes to loss in productivity.¹⁴ Orofacial cancer pain involves spontaneous pain and functional limitations that are distinct from precancer conditions despite the fact that both involve the presence of oral lesions.³² Spontaneous pain is also frequently difficult to manage, with one study showing that 25% of patients did not have a treatment (pharmacological or otherwise) that would consistently relieve spontaneous pain episodes, and 12% of these patients had no effective treatment options for these events.^{12,13,46} However, a recent systematic review of the literature suggests that nonsteroidal anti-inflammatory drugs (NSAIDs) may play a role in malignant pain treatment that highlights the role of inflammation in cancer-related nociception.³⁸

Although perineural invasion (PNI) is one of the most important factors predicting severe pain in patients with head and neck squamous cell carcinoma (HNSCC),¹⁷ the exact mechanisms of peripheral sensitization are still poorly understood. Understanding how interactions between cancer cells and the peripheral

Table 1**Upregulation of cytokines in patient dorsal root ganglion coculture media.**

Target	Fold change vs DRG	Fold change vs Fadu
Adiponectin	1.70 [1.28–1.85]	1.53 [1.31–1.93]
Brain-derived neurotrophic factor (BDNF)	1.29 [1.25–1.34]	1.96 [1.79–2.06]
Beta nerve growth factor (B-NGF)	1.21 [1.10–1.27]	35.56 [32.36–36.68]
Ciliary neurotrophic factor (CTNF)	0.80 [0.77–1.00]	0.84 [0.78–1.30]
C-reactive protein (CRP)	1.10 [1.02–1.43]	1.27 [1.16–1.59]
Eotaxin 1	0.89 [0.78–1.17]	0.88 [0.83–1.25]
Eotaxin 2	1.63 [1.15–1.71]	1.65 [1.19–1.70]
Eotaxin 3	1.13 [1.08–1.29]	1.16 [1.01–1.19]
Fas	2.35 [1.49–2.40]	0.84 [0.81–1.15]
Interleukin 4 (IL-4)	1.06 [1.02–1.15]	1.16 [1.11–1.32]
Glial cell line–derived neurotrophic factor (GDNF)	1.10 [1.02–1.17]	1.13 [0.98–1.18]
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	1.55 [1.30–1.84]	1.25 [1.21–1.43]
Interferon gamma (IFN- γ)	1.26 [1.07–1.32]	1.07 [0.98–1.12]
Interleukin 10 (IL-10)	2.01 [1.70–2.27]	3.65 [2.81–4.60]
Interleukin 18 (IL-18)	1.45 [1.14–1.65]	1.91 [1.70–2.25]
Interleukin 1 alpha (IL-1 alpha)	1.13 [0.95–1.25]	0.87 [0.86–0.96]
Interleukin 1 beta (IL-1 beta)	1.18 [1.05–1.32]	1.35 [1.23–1.36]
Interleukin 6 (IL-6)	2.45 [1.93–2.65]	3.06 [2.69–6.59]
Interleukin 8 (IL-8)	1.41 [1.14–2.06]	1.55 [1.28–1.60]
Leukemia inhibitory factor (LIF)	2.53 [2.16–2.78]	2.32 [2.07–2.56]
Monocyte chemoattractant protein 1 (MCP-1)	1.41 [1.13–1.51]	29.16 [22.25–34.80]
Macrophage inflammatory protein 1 beta (MIP-beta)	2.04 [1.68–2.04]	4.27 [2.87–5.38]
Matrix metalloproteinase 2 (MMP-2)	1.02 [0.96–1.18]	1.24 [1.23–1.47]
Matrix metalloproteinase 3 (MMP-3)	1.27 [1.18–1.33]	1.19 [1.11–1.27]
S100 calcium binding protein B (S100B)	1.08 [0.83–1.36]	1.51 [1.49–2.60]
Thymus and activation regulated chemokine (TARC)	1.03 [0.99–1.23]	1.06 [1.03–1.17]
Transforming growth factor beta (TGF-beta)	1.16 [1.03–1.26]	0.87 [0.82–1.13]
Tissue inhibitor of matrix metalloproteinase 1 (TIMP-1)	1.80 [1.69–2.94]	1.91 [1.87–2.19]
Tumor necrosis factor alpha (TNF-alpha)	2.58 [1.99–2.59]	2.84 [2.04–3.04]
Vascular endothelial growth factor A (VEGF-A)	4.11 [2.75–4.25]	3.22 [2.96–3.60]

Based on human chemiluminescence assays, fold change in optical intensity was calculated for chemiluminescence assays relative to media only and Fadu only for coculture media from 3 patient DRGs. Relative to media only, fold change was ≥ 2 for IL-6 and LIF (both members of the IL-6 family) as well as Fas, IL-10, MIP-beta, TNF-alpha, and VEGF-A. Relative to Fadu only, fold change was also ≥ 2 for IL-6, LIF, IL-10, MIP-beta, TNF-alpha, and VEGF-A, as well as BDNF, B-NGF and MCP-1. Data are expressed as median [25th quartile–75th quartile].

BDNF, brain-derived neurotrophic factor; BNGF, beta nerve growth factor; DRG, dorsal root ganglion; IL-6, interleukin 6; LIF, leukemia inhibitory factor; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; TIMP, tissue inhibitor of matrix metalloproteinase.

nervous system contribute to spontaneous pain could help provide treatment alternatives to lessen this burden. For cancers that promote a highly inflammatory microenvironment,^{6,7} signaling molecules known to cause changes in nociceptor function that lead to sensitization could play a major role in the pathophysiology of cancer pain. Here, we investigated the association between NSAIDs and pain intensity in patients with oral cancers. Then, to determine how HNSCC cells affect neuronal activity, we have developed an in vitro model in which cancer cells are cocultured with dorsal root ganglion (DRG) neurons, enabling us to study the interaction between neurons and supporting cells in close proximity to—but not in direct contact with—cancer cells, approximating the tumor microenvironment. Previous studies have cocultured neurons with cancer cells or cancer conditioned media (CCM) and have identified enhanced growth, neuronal sensitization, changes in expression of norepinephrine, and altered neuron phenotypes^{3–5,8,19,21,28,52}; however, none of these studies examined the direct impact of

exposure to cancer cells on the electrical properties of cultured DRG neurons.

2. Methods

2.1. Research subjects

2.1.1. Retrospective patient studies

After approval from the University of Texas MD Anderson Cancer Center Institutional Review Board (#PA12-1033), we performed a retrospective study that included a cohort of patients with human papilloma virus–negative oral cancers who underwent initial surgery between January 2004 and January 2018 (see Supplementary Methods, available at <http://links.lww.com/PR9/A163>). Patients ≥ 18 years old were included in the analysis, whereas patients with repeated oral procedures and those with missing information regarding preoperative pharmacotherapy were excluded from the study.

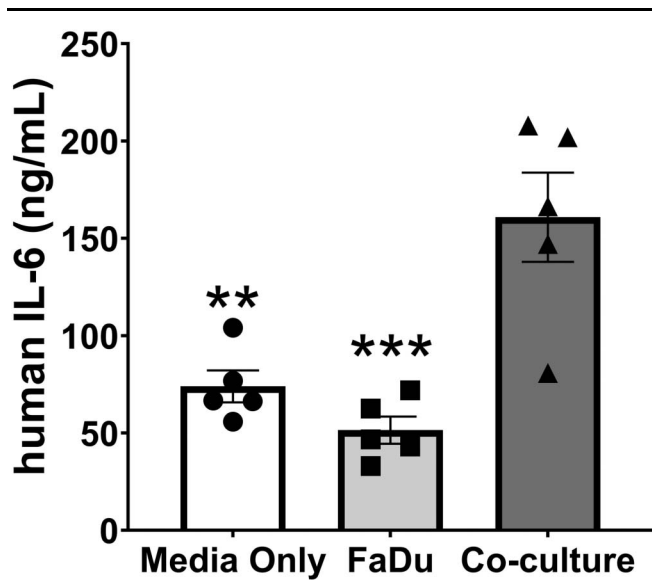


Figure 1. Expression of interleukin 6 was enhanced when human dorsal root ganglion and Fadu cancer cells were cocultured. In studies using patient DRGs ($n = 5$), ELISA of media collected at 24 hours showed that human IL-6 expression in coculture media was significantly higher than media-only DRG or Fadu ($F_{2,12} = 15.6, P = 0.0005$). ** $P < 0.005$, *** $P < 0.001$ vs coculture. DRG, dorsal root ganglion; ELISA, enzyme-linked immunosorbent assay; IL-6, interleukin 6.

2.1.2. Patient dorsal root ganglion tissue studies

All human tissue procurement procedures for DRG collection were approved by the Institutional Review Board (IRB# 2013-0871) at the University of Texas MD Anderson Cancer Center,

and all experiments conformed to relevant guidelines and regulations. Written informed consent for participation, including use of tissue samples, was obtained from each patient ($n = 7$) before inclusion (Supplementary Table 1, available at <http://links.lww.com/PR9/A163>).

2.1.3. Animals

All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas MD Anderson Cancer Center. All studies adhered to the NIH Guide for the Care and Use of Laboratory Animals. Young adult ($n = 26$, 3–5 months) and older adult ($n = 33$, 12–15 months) male and female Sprague-Dawley rats (Harlan, Houston, TX) housed in temperature- and light-controlled (12-hour light/dark cycle) conditions with food and water available ad libitum were used in the study.

2.2. Coculture procedure

The human HNSCC cell line Fadu was purchased from the American Type Culture Collection (HTB-43; Manassas, VA; see Supplementary Methods, available at <http://links.lww.com/PR9/A163>). For coculture with dissociated DRGs, glass coverslips (12 mm diameter) were added to the center of the chambers of a 6-well plate, coated with poly-L-lysine and placed in an incubator overnight. The plate was washed with distilled water. For coculture wells, sterile plastic cylinders (20 mm diameter) were placed over the coverslips to form a temporary barrier. For coculture and Fadu wells, Fadu cells (1×10^5 cells per well) were seeded along the outer edge of the chambers and 2 mL of culture media was added to each well. Media-only wells were not treated

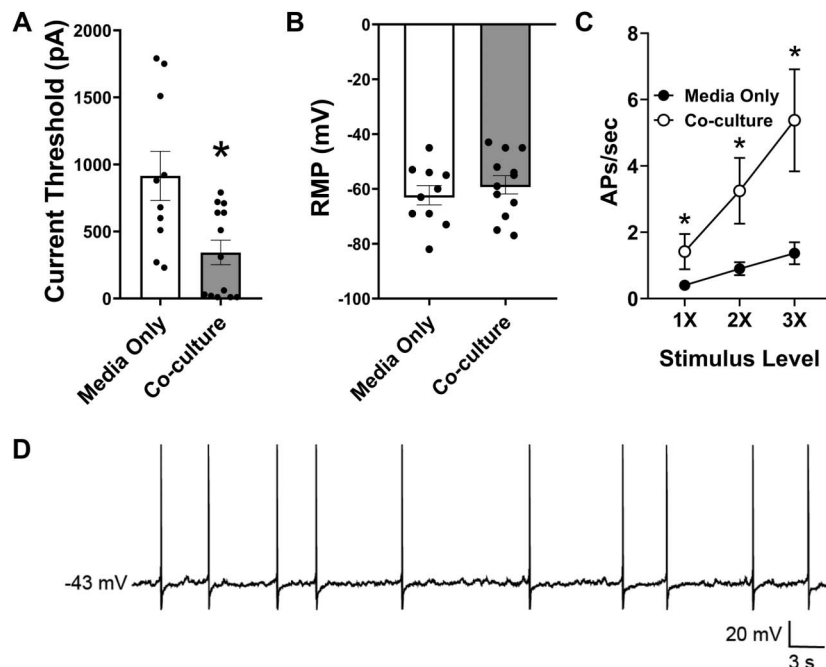


Figure 2. Coculture with Fadu cancer cells led to changes in excitability of human dorsal root ganglion neurons. None of the media-only DRG neurons had SA or DSFs (0 of 10), whereas 8.3% (1 of 12) of coculture neurons had SA ($\chi^2[1] = 0.9, P = 0.350$) and 33.3% (4 of 12) had DSFs ($\chi^2[1] = 4.1, P = 0.044$). DRG neurons ($n = 10$ –12 per group) in coculture for 24 hours with Fadu cancer cells had significantly lower rheobase ($t_{20} = 2.7, P = 0.013$; A), but resting membrane potential (B) did not differ from media-only neurons ($t_{20} = 0.8, P = 0.440$). Responses to current stimulation (number of action potentials per second) at 1 \times , 2 \times , and 3 \times rheobase (C) were significantly higher in coculture DRG neurons ($F_{1,20} = 5.7, P = 0.027$). Post-hoc tests (Bonferroni) showed that responses were higher at all stimulus levels. (D) Raw trace of a DRG neuron with SA after coculture with Fadu cells. Data are expressed as mean \pm SEM. * $P < 0.05$ vs media only. DRG, dorsal root ganglion; DSF, depolarizing spontaneous fluctuation; SA, spontaneous activity.

Table 2**Characteristics of human dorsal root ganglion neurons in media-only or coculture with Fadu cancer cells.**

Characteristic	Media only	Coculture	Significance
Cultured hDRG neurons			
Percent with SA	0% (0/10)	8.3% (1/12)	n.s. ($P = 0.35$)
Percent with large DSFs	0% (0/10)	33.3% (4/12)*	$P < 0.05$
AP amplitude (mV)	120.8 \pm 7.7 (10)	115.2 \pm 4.5 (12)	n.s. ($P = 0.52$)
AP rise time (ms)	3.2 \pm 0.7 (10)	2.4 \pm 0.6 (12)	n.s. ($P = 0.39$)
AP fall time (ms)	16.5 \pm 5.8 (10)	10.0 \pm 1.7 (12)	n.s. ($P = 0.26$)
Width at 0 mV (ms)	9.6 \pm 3.2 (8)	4.2 \pm 0.8 (11)	n.s. ($P = 0.08$)
AP overshoot (mV)	58.7 \pm 5.7 (8)	61.2 \pm 3.0 (11)	n.s. ($P = 0.68$)
AP afterhyperpolarization (mV)	6.2 \pm 2.9 (3)	18.6 \pm 2.9 (6)	n.s. ($P = 0.24$)
Threshold potential (mV)	-6.3 \pm 4.9 (10)	-14.2 \pm 3.6 (12)	n.s. ($P = 0.20$)

Data expressed as percentages or mean \pm SEM with the number of neurons in parentheses.

* $P < 0.05$.

AP, action potential; DRG, dorsal root ganglion; DSF, depolarizing spontaneous fluctuations; .DSF, depolarizing spontaneous fluctuation; SA, spontaneous activity.

after washing. After plating, Fadu cells were incubated for 4 to 6 hours before DRG collection. Human and rat DRG cell suspensions were prepared as described previously.^{35,42} The 6-well plate containing the Fadu cells was removed from the incubator, and the plastic cylinders were removed from coculture wells along with most of the volume of culture media. For media-only and coculture wells, glass cylinders (8 mm height, 6 mm inner diameter, and 8 mm outer diameter) were placed in the center of each glass coverslip, and 100 μ L of cell suspension was added. Warmed culture media (2 mL) was placed into each well outside

the glass cylinder. After 40 minutes, the cylinders were carefully removed, and the plate was incubated overnight. For immunocytochemistry studies only, this procedure was performed using glass-bottom 6-well plates without coverslips (see Supplementary Methods, available at <http://links.lww.com/PR9/A163>).

In experiments using conditioned media, DRG neurons were seeded as described above and 3 mL of either Fadu conditioned media or nonconditioned media was added to the wells.

For experiments using the human interleukin 6 receptor (hIL-6R) antagonist tocilizumab (Selleck Chemicals, Houston, TX),

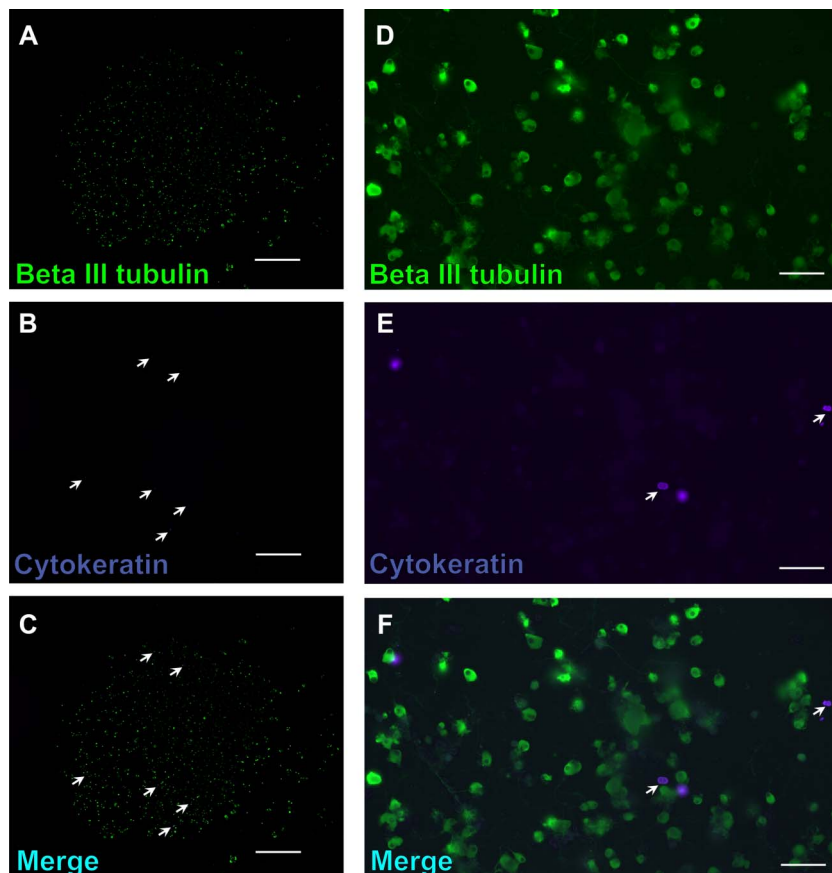


Figure 3. In coculture wells, Fadu cancer cells did not infiltrate areas where dorsal root ganglion neurons were seeded. Immunocytochemistry of rat DRG and Fadu cells using $\times 2$ objective (A–C) and $\times 20$ objective (D–F) showed few (< 10) cytokeratin-positive cells (white arrows) located within the area where DRG cells were seeded. When viewed at $\times 20$, these cells were not found to interact with neurons or neurites and appeared to be non-viable cancer cells. Neurons (green fluorescence) were labeled with neuron-specific beta III tubulin (A and C). Fadu cancer cells (far-red fluorescence) are labeled with cytokeratin (B and E). (A–C) Scale bar = 1000 μ m; (D–F) scale bar = 100 μ m. DRG, dorsal root ganglion.

Table 3
Upregulation of cytokines in rat dorsal root ganglion coculture media.

Target	Fold change vs DRG	Fold change vs Fadu
Adiponectin	1.24 [0.84–1.29]	0.91 [0.60–1.05]
Brain-derived neurotrophic factor (BDNF)	1.21 [1.01–1.28]	0.83 [0.64–1.10]
Beta nerve growth factor (B-NGF)	0.88 [0.73–1.27]	0.88 [0.73–1.20]
Ciliary neurotrophic factor (CNTF)	0.99 [0.87–1.50]	1.24 [0.88–1.38]
C-reactive protein (CRP)	1.07 [0.90–1.29]	1.12 [0.94–1.22]
Eotaxin 1	0.65 [0.57–1.23]	0.84 [0.70–1.26]
Eotaxin 2	1.04 [0.68–1.37]	1.21 [1.01–2.01]
Eotaxin 3	1.26 [1.14–1.36]	1.83 [1.66–2.31]
Fas	1.08 [0.89–1.18]	0.96 [0.63–1.29]
Interleukin 4 (IL-4)	0.88 [0.77–1.56]	1.33 [0.87–1.75]
Glial cell line-derived neurotrophic factor (GDNF)	1.10 [0.89–1.42]	0.92 [0.65–1.44]
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	1.22 [1.07–1.31]	0.92 [0.81–1.09]
Interferon gamma (IFN- γ)	1.03 [0.82–1.18]	1.03 [0.64–1.12]
Interleukin 10 (IL-10)	0.99 [0.96–1.17]	0.88 [0.77–1.10]
Interleukin 18 (IL-18)	1.06 [0.85–1.33]	0.90 [0.76–0.99]
Interleukin 1 alpha (IL-1 alpha)	1.03 [0.99–1.04]	1.22 [0.77–1.39]
Interleukin 1 beta (IL-1 beta)	0.99 [0.83–1.07]	1.00 [0.84–1.54]
Interleukin 6 (IL-6)	2.39 [1.30–14.37]	1.69 [1.60–3.65]
Interleukin 8 (IL-8)	15.54 [6.57–25.23]	5.70 [6.28–19.83]
Leukemia inhibitory factor (LIF)	1.15 [0.86–1.50]	1.27 [0.12–1.80]
Monocyte chemoattractant protein 1 (MCP-1)	1.18 [0.89–1.59]	0.91 [0.87–0.94]
Macrophage inflammatory protein 1 beta (MIP-beta)	0.78 [0.73–0.85]	1.14 [0.62–1.48]
Matrix metalloproteinase 2 (MMP-2)	0.89 [0.64–0.96]	1.22 [0.13–1.57]
Matrix metalloproteinase 3 (MMP-3)	1.03 [0.93–1.60]	1.40 [0.25–1.80]
S100 calcium binding protein B (S100B)	1.01 [0.85–1.14]	1.22 [0.47–2.47]
Thymus and activation regulated chemokine (TARC)	0.82 [0.64–1.00]	1.00 [0.79–1.31]
Transforming growth factor beta (TGF-beta)	1.15 [1.06–1.63]	1.10 [1.07–1.24]
Tissue inhibitor of matrix metalloproteinase 1 (TIMP-1)	7.85 [2.90–17.61]	2.98 [2.16–11.71]
Tumor necrosis factor alpha (TNF-alpha)	0.99 [0.94–1.07]	1.09 [0.07–1.12]
Vascular endothelial growth factor A (VEGF-A)	1.72 [1.05–2.45]	1.06 [1.99–1.39]

Based on human chemiluminescence assays, fold change in optical intensity was calculated for chemiluminescence assays relative to media only and Fadu only for coculture media from 6 older adult rat DRGs. Relative to Media Only, fold change was ≥ 2 for IL-6, IL-8 and TIMP-1 in coculture media. Relative to Fadu only, fold change was also ≥ 2 for IL-8 and TIMP-1 in coculture media. Data expressed as median [25th quartile–75th quartile]. DRG, dorsal root ganglion; IL-6, interleukin 6; LIF, leukemia inhibitory factor; TNF, tumor necrosis factor; TIMP, Tissue Inhibitor of Matrix Metalloproteinase.

stock solution (5 mg/mL in phosphate-buffered saline [PBS]) was diluted to achieve a final concentration of 5 μ g/mL for incubation with DRG only or coculture for 24 hours before recording. Vehicle-treated DRG or coculture wells were treated with an equivalent volume of PBS. For IL-6 experiments, DRG neurons were treated with 60 ng of recombinant rat IL-6 (rrIL-6) or recombinant human IL-6 (rhIL-6; R&D Systems, Minneapolis, MN), using either direct application to the bath solution during recording or a 1-hour incubation period before recording.

2.3. Enzyme-linked immunosorbent assay

Using DRGs collected from adult rats ($n = 16$) and patients ($n = 5$), we used the same plating procedure described above with 3 experimental groups: media only (DRGs alone), Fadu (Fadu cancer cells alone, 1×10^5 cells per well), and coculture (DRG + Fadu cancer cells, 1×10^5 cells per well). Cell culture media samples were collected after 24 hours of incubation. Quantification of IL-6 was performed by enzyme-linked immunosorbent

assay (ELISA) method using commercially available kits (Quantikine ELISA kits; R&D Systems) according to the manufacturer's instructions (see Supplementary Methods, available at <http://links.lww.com/PR9/A163>). Experiments were performed in duplicate or triplicate, and the mean concentration was used for statistical analysis.

2.4. Chemiluminescence assay

We collected media using the same plate procedure described above from older adult male rat DRGs ($n = 6$) and patient DRGs ($n = 3$) with 3 experimental groups: media only (DRGs alone), Fadu (Fadu cancer cells alone, 1×10^5 cells per well), and coculture (DRG + Fadu cancer cells, 1×10^5 cells per well). Cell culture media samples were collected after 24 hours of incubation and processed according to the manufacturer's instructions. Membranes were imaged by using Image Quant LAS 4000 Mini (GE Healthcare Life Sciences, Marlborough, MA) and cytokine spots in the membranes were quantified using ImageJ protein analyzer software.

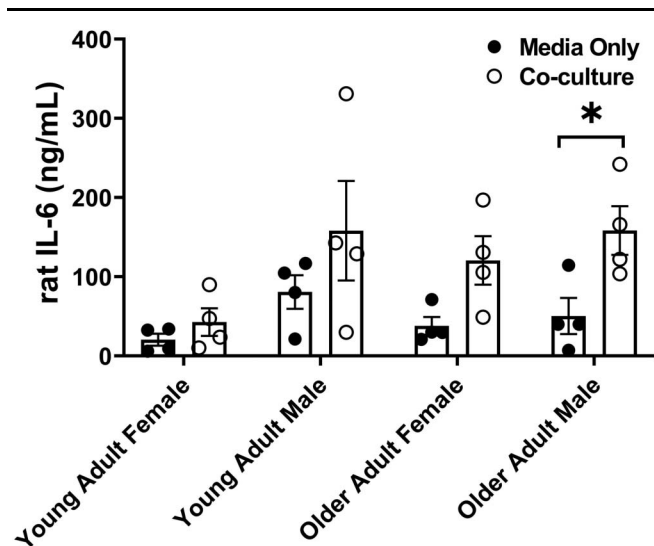


Figure 4. Expression of interleukin 6 was enhanced when rat dorsal root ganglion and Fadu cancer cells were cocultured. In studies using rat DRGs ($n = 16$), ELISA for rat IL-6 in media collected at 24 hours showed a significant main effect for treatment group (media only vs coculture, $F_{1,24} = 11.5$, $P = 0.002$) and sex ($F_{1,24} = 7.0$, $P = 0.014$) but not age ($F_{1,24} = 0.6$, $P = 0.452$). Rat IL-6 expression in media from coculture DRGs was higher than that of media-only DRGs overall, and post-hoc tests (Bonferroni) showed that this was significant for older adult males. Rat IL-6 expression was also higher among males than females overall, and among young adult rats, rat IL-6 expression in coculture media from young adult male rats was significantly higher than in young female rats ($P < 0.05$). * $P < 0.05$. Data are expressed as mean \pm SEM. DRG, dorsal root ganglion; ELISA, enzyme-linked immunosorbent assay; IL-6, interleukin 6.

2.5. Electrophysiology

Whole-cell patch recording was performed as described previously (see Supplementary Methods, available at <http://links.lww.com/PR9/A163>).³⁶ Dorsal root ganglion neurons were held at 0 pA to record spontaneous activity (SA) for 5 minutes, followed by a series of 500 ms depolarizing current injections in 10-pA steps from -50 pA until an action potential (AP) was evoked. The current that induced the first AP was defined as the current threshold ($1 \times$ rheobase). Neurons were then stimulated with 2-second current injections at $1 \times$, $2 \times$, and $3 \times$ rheobase. Only neurons with a resting membrane potential (RMP) of at least -40 mV, stable baseline recordings, and evoked spikes that overshoot 0 mV were used for further experiments and analysis. We preferentially recorded from DRG neurons of small diameter ($\leq 30 \mu\text{m}$).

2.6. Statistical analysis

For coculture or drug treatment experiments, all treatments were used for each subject and the treatment conditions were randomly assigned. Where appropriate, researchers performing experiments were blinded to experimental conditions of coverslips and conditioned media samples. All data are expressed as percentages, mean \pm SEM, or median (25th quartile–75th quartile). For all analyses, $P < 0.05$ was considered significant. See Supplementary Methods for detailed analyses (available at <http://links.lww.com/PR9/A163>).

3. Results

3.1. Inflammation contributes to oral preoperative pain in patients with human head and neck squamous cell carcinoma

The study cohort included 176 patients with oral HNSCC. Baseline characteristics were not statistically significantly different

between patients with and without preoperative pain except for tumor location (Supplementary Table 2, available at <http://links.lww.com/PR9/A163>). Patients with preoperative pain more frequently had tongue tumors than patients without preoperative pain (Supplementary Table 2, available at <http://links.lww.com/PR9/A163>). Patients taking NSAIDs at the time of the preoperative visit had a lower rate of oral pain compared with the patients not taking NSAIDs (37.1% vs 58.2%; $P = 0.025$). A multivariate analysis showed that the odds of having oral pain preoperatively was 2.265 times (95% confidence interval: 1.021–5.024; $P = 0.044$) higher for the patients not taking NSAIDs after adjusting for pathological location (other vs tongue), PNI (yes vs no), and use of other analgesics (yes vs no) in the model. These results suggest that inflammation is an important component of pain in humans with oral cancers.

3.2. Human dorsal root ganglion and Fadu cell interaction promotes a proinflammatory microenvironment

Human head and neck squamous cell carcinoma can be highly inflammatory tumors. Among the signaling pathways that are upregulated in these tumors, there is clinical interest in the upregulation of IL-6 signaling because it has been identified as a significant predictor for poorer treatment response and reduced survival rates in esophageal SCC and it may be useful as a salivary biomarker for oral SCC.^{7,39} Using our novel experimental paradigm, we examined media from human DRG cultured in media only or in coculture with 1×10^5 Fadu cancer cells as well as Fadu cancer cells alone. Using the human array, we found by chemiluminescence ≥ 2 -fold increases in several cytokines, including IL-6, IL-10, leukemia inhibitory factor, macrophage inflammatory protein-1 beta, tumor necrosis factor alpha, and vascular endothelial growth factor A (VEGF-A) when coculture was normalized to media only and Fadu alone, >2 -fold increase in Fas when coculture was normalized to media only, and ≥ 2 -fold increases in brain-derived neurotrophic factor (BDNF) and beta nerve growth factor (B-NGF) when coculture was normalized to Fadu alone (Table 1). Median optical density for each cytokine is presented in Supplementary Figs. 1–3, available at <http://links.lww.com/PR9/A163>. It should be noted that the median optical density for IL-6 was not statistically different between media only and coculture, likely because of high variability among the coculture and Fadu samples. Using media collected from 5 experiments using patients' ($n = 3$) DRGs, ELISA showed that human IL-6 concentrations in coculture media were higher than that of media from DRG or Fadu cells cultured alone (Fig. 1). In addition, the level of human IL-6 expression in coculture was higher than the combined expression from media-only DRG and Fadu alone, suggesting that there may be a synergistic effect because of the interactions between cancer cells and DRG cells.

3.3. Cocultured small-diameter human dorsal root ganglion neurons exhibit sensitization after incubation with Fadu cancer cells

We examined response characteristics of small-diameter DRG neurons from patients' ($n = 4$) DRGs cultured in media only or in coculture with 1×10^5 Fadu cancer cells ($n = 10$ – 12 per group). Each patient represents a single experiment, with some patients contributing more than one DRG. We observed that human DRG become hyperexcitable in the presence of cancer cells. Coculture neurons demonstrated large depolarizing spontaneous fluctuations (DSFs) and a higher rate of SA, and current thresholds (Fig. 2A) were significantly lower among coculture neurons but there

Table 4**Characteristics of adult rat dorsal root ganglion neurons in media only or coculture with Fadu cancer cells.**

Characteristics	Media only	Coculture	Significance
Young adult male rats (3–5 mo)			
Percent with SA	0% (0/31)	25.8% (8/31)***	$P < 0.005$
Percent with large DSFs	16.1% (5/31)	38.7% (12/31)*	$P < 0.05$
AP amplitude (mV)	115.9 ± 2.0 (31)	109.3 ± 2.8 (30)	n.s. ($P = 0.09$)
AP rise time (ms)	3.2 ± 0.3 (31)	3.4 ± 0.3 (30)	n.s. ($P = 0.64$)
AP fall time (ms)	15.2 ± 2.4 (31)	19.2 ± 5.2 (30)	n.s. ($P = 0.48$)
Width at 0 mV (ms)	8.0 ± 1.7 (30)	6.7 ± 1.1 (30)	n.s. ($P = 0.52$)
AP overshoot (mV)	62.1 ± 2.5 (30)	59.2 ± 2.0 (30)	n.s. ($P = 0.37$)
AP afterhyperpolarization (mV)	11.3 ± 1.5 (15)	16.0 ± 1.9 (16)	n.s. ($P = 0.06$)
Threshold potential (mV)	-9.7 ± 2.2 (31)	-17.8 ± 1.4 (30)***	$P < 0.005$
Young adult female rats (3–5 mo)			
Percent with SA	7.4% (2/27)	15.4% (4/26)	n.s. ($P = 0.36$)
Percent with large DSFs	18.5% (5/27)	23.1% (6/26)	n.s. ($P = 0.68$)
AP amplitude (mV)	113.4 ± 2.9 (27)	105.3 ± 3.9 (28)	n.s. ($P = 0.10$)
AP rise time (ms)	3.6 ± 0.4 (27)	5.2 ± 1.0 (28)	n.s. ($P = 0.15$)
AP fall time (ms)	17.0 ± 1.8 (27)	21.4 ± 4.8 (28)	n.s. ($P = 0.40$)
Width at 0 mV (ms)	8.1 ± 0.8 (26)	12.1 ± 4.1 (27)	n.s. ($P = 0.35$)
AP overshoot (mV)	62.9 ± 1.4 (26)	56.9 ± 2.9 (27)	n.s. ($P = 0.07$)
AP afterhyperpolarization (mV)	10.3 ± 1.9 (13)	12.6 ± 2.9 (17)	n.s. ($P = 0.54$)
Threshold potential (mV)	-12.9 ± 1.6 (27)	-15.7 ± 1.7 (28)	n.s. ($P = 0.24$)
Older adult male rats (12–15 mo)			
Percent with SA	3.2% (1/31)	21.9% (7/32)*	$P < 0.05$
Percent with large DSFs	9.7% (3/31)	37.5% (12/32)**	$P < 0.01$
AP amplitude (mV)	120.6 ± 1.9 (31)	115.4 ± 3.2 (31)	n.s. ($P = 0.17$)
AP rise time (ms)	3.6 ± 0.6 (31)	4.4 ± 0.5 (31)	n.s. ($P = 0.31$)
AP fall time (ms)	13.0 ± 2.4 (31)	12.0 ± 1.5 (31)	n.s. ($P = 0.72$)
Width at 0 mV (ms)	7.7 ± 1.6 (29)	5.9 ± 1.0 (30)	n.s. ($P = 0.34$)
AP overshoot (mV)	66.7 ± 1.8 (29)	63.5 ± 2.5 (30)	n.s. ($P = 0.31$)
AP afterhyperpolarization (mV)	4.8 ± 0.9 (17)	10.6 ± 1.4 (24)***	$P < 0.005$
Threshold potential (mV)	-10.4 ± 1.8 (31)	-17.3 ± 2.2 (31)*	$P < 0.05$
Older adult female rats (12–15 mo)			
Percent with SA	0% (0/25)	23.1% (6/26)*	$P < 0.05$
Percent with large DSFs	8.0% (2/25)	46.2% (12/26)***	$P < 0.005$
AP amplitude (mV)	114.7 ± 2.3 (25)	107.3 ± 3.5 (26)	n.s. ($P = 0.09$)
AP rise time (ms)	2.2 ± 0.2 (25)	4.1 ± 0.5 (26)***	$P < 0.005$
AP fall time (ms)	13.0 ± 1.9 (25)	15.4 ± 1.6 (26)	n.s. ($P = 0.34$)
Width at 0 mV (ms)	7.0 ± 1.7 (23)	7.6 ± 1.1 (25)	n.s. ($P = 0.76$)
AP overshoot (mV)	61.9 ± 1.6 (23)	59.9 ± 2.2 (25)	n.s. ($P = 0.47$)
AP afterhyperpolarization (mV)	7.6 ± 1.3 (12)	12.4 ± 1.8 (16)	$P = 0.05$
Threshold potential (mV)	-9.6 ± 1.6 (25)	-16.0 ± 1.7 (26)**	$P < 0.01$

Data expressed as percentages or mean ± SEM with the number of neurons in parentheses.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

AP, action potential; DSF, depolarizing spontaneous fluctuations; SA, spontaneous activity.

was no difference in mean RMP (**Fig. 2B**). No differences in AP characteristics were found between the 2 groups (**Table 2**). Responses to current stimulation (the number of APs fired per second) were significantly higher among coculture neurons (**Fig. 2C**). These results suggest that DRG neurons become hyperexcitable in the presence of Fadu HNSCC.

3.4. Coculture media contains higher levels of interleukin 6 than rat dorsal root ganglion alone

Representative immunocytochemistry images of rat DRG coculture at ×2 and ×20 are shown in **Figure 3**. At ×2 magnification, the entire region occupied by DRG neurons can be viewed. The area inhabited by DRG neurons (green fluorescence, **Figs. 3A and B**) was not infiltrated by viable Fadu cells (far red fluorescence, **Figs. 3C and D**) during coculture. For reference, images of DRG cultured alone (Supplementary Fig. 4, available at <http://links.lww.com/PR9/A163>) and Fadu cells cultured without barriers (Supplementary Fig. 5, available at <http://links.lww.com/PR9/A163>) are also presented. To recapitulate our findings in human DRGs using rats, we evaluated the

levels of different inflammatory markers from rat DRG or Fadu cancer cells alone or in combination. Using the human array, we found by chemiluminescence ≥2-fold increases in the content of IL-6 in coculture and Fadu-only media as expected from our previous results, but we also found increases in IL-8 and tissue inhibitor of matrix metalloproteinase 1 (**Table 3**, Supplementary Figs. 6–8, available at <http://links.lww.com/PR9/A163>). We also performed chemiluminescence using the rat array (Supplementary Table 3, available at <http://links.lww.com/PR9/A163>). We observed >2-fold higher optical intensity for VEGF-A in coculture relative to media only in DRGs from older adult females, but no differences were observed between media only and coculture for any other group. Optical intensity was >2-fold higher in coculture relative to Fadu only for BDNF in young adult male rats, for Notch1, RAGE, and BDNF in young adult female rats, and for monocyte chemoattractant protein-1 in older adult male and female rats.

We turned our attention to IL-6 because it was also elevated in our human DRG experiments. Using media samples from 16 experiments, ELISA showed that coculture media had higher levels of rat IL-6 overall compared with media only (**Fig. 4**).

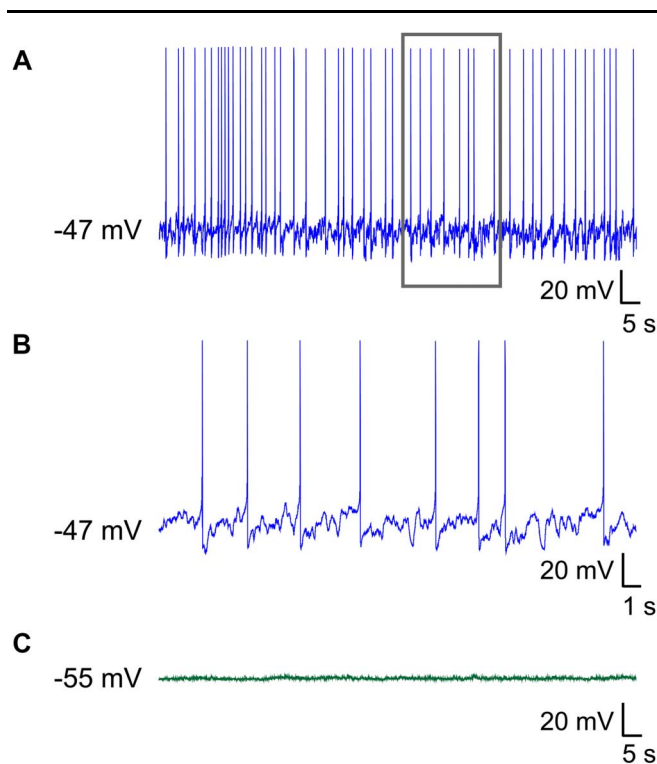


Figure 5. A significantly higher proportion of coculture dorsal root ganglion neurons demonstrated spontaneous activity and large depolarizing spontaneous fluctuations in young adult males, older adult males, and older adult females, but not young adult females. (A) Representative example of a small-diameter DRG neuron from an older adult male rat with SA after coculture with Fadu cancer cells. The area indicated by the grey box is enhanced in (B) to show DSFs between action potentials. (C) Representative example of a small-diameter DRG neuron from an older adult male rat treated with media only shows normal low-level fluctuations in resting membrane potential. Although the SA rate was higher in DRG neurons from older adult male rats after coculture with Fadu cancer cells (25.3 ± 14.4 spikes/min) compared with young adult rats (12.6 ± 4.2 spikes/min), this difference was not significant ($t_{12} = 0.8$, $P = 0.414$). DRG, dorsal root ganglion; DSF, depolarizing spontaneous fluctuation; SA, spontaneous activity.

3.5. Small-diameter rat dorsal root ganglion neurons exhibit sensitization after incubation with Fadu cells or Fadu conditioned media

To determine whether we could replicate our coculture results using rat DRGs, we examined the response characteristics of small-diameter DRG neurons from young adult male rats ($n = 10$), young adult female rats ($n = 7$), older adult male rats ($n = 10$), and older adult female rats ($n = 5$) cultured in media only or coculture with 1×10^5 Fadu cancer cells ($n = 25$ – 31 neurons per group). Each rat represents a single experiment.

In young adult male rats, more coculture neurons demonstrated SA and large DSFs⁴⁵ in RMP (Table 4). In contrast, there were no differences in the proportion of neurons with SA or DSFs in young adult female rats (Table 4). Coculture neurons from both male and female older adult rats demonstrated SA and DSFs more frequently than media-only control neurons (Table 4). A representative example of SA in a coculture neuron from an older adult male rat DRG is presented in Figures 5A and B. In young adult male rats, mean AP threshold potential was lower for coculture neurons relative to media-only neurons (Table 4). In older adult males, APs in coculture DRG neurons had greater afterhyperpolarization and higher threshold potential (Table 4). In older adult females, AP rise time was significantly higher and threshold potential was significantly lower in coculture neurons (Table 4).

In young adult male rats, current thresholds tended to be lower after coculture with Fadu cancer cells compared with media only, but this difference was not significant (Fig. 6A). Current thresholds were significantly lower in coculture neurons with SA compared with those without SA (Table 5). Current thresholds were not significantly lower in coculture neurons from young adult female rats (Fig. 6B) but were significantly lower in coculture neurons from both male and female older adult rats (Figs. 6C and D) and significantly lower in coculture neurons with SA compared with those without SA (Table 5). Among coculture neurons from young adult males, mean AP amplitude was lower for those with SA compared with those without SA (Table 5). In older adult male coculture neurons, AP amplitude and overshoot were lower for those with SA compared with those without SA (Table 5). Coculture neurons with SA from older adult females also had significantly lower AP amplitude and higher AP rise time (Table 5).

Resting membrane potential was significantly higher (more depolarized) in coculture neurons in young adult males (Fig. 6E). Among coculture neurons, those with SA also had higher RMP than those without SA (Table 5). Resting membrane potential was not significantly higher in coculture neurons from young adult females (Fig. 6F) or older adult males (Fig. 6G). In older adult females, cocultured neurons had higher RMP relative to media-only neurons (Fig. 6H). Further, among cocultured neurons, those with SA had higher RMP compared with those without SA (Table 5).

Responses to current stimulation at $1\times$, $2\times$, and $3\times$ rheobase were expressed in APs/sec. In young adult male rats, responses to current stimulation (Fig. 7A) were higher among coculture DRG neurons but did not differ between the treatment groups in young adult females (Fig. 7B). For older adult male (Fig. 7C) and female (Fig. 7D) rats, responses were significantly higher in coculture DRG neurons.

To assess whether media conditioned by Fadu cancer cells would also induce sensitization, we treated DRG neurons from older adult female rats ($n = 2$) with Fadu CCM or nonconditioned media (control) for 24 hours. Neurons incubated in Fadu CCM had significantly lower rheobase (Fig. 8A) and higher RMP (Fig. 8B) as well as increased responses to current stimulation (Fig. 8C), which is consistent with what we observed in coculture DRG neurons.

3.6. Interleukin 6 induces sensitization in dorsal root ganglion neurons and blocking interleukin 6 receptors prevent sensitization by Fadu cells

To assess whether the elevated IL-6 levels contributed to sensitization in cocultured neurons, we treated naïve rat and human DRG neurons with 60 ng of rIL-6 or hIL-6, respectively. In DRG neurons from older adult male rats ($n = 5$), treatment with rIL-6 depolarized RMP (Fig. 9A) and induced SA. In DRG neurons from young adult females ($n = 3$), no changes in RMP occurred (Fig. 9B), but SA was induced in 1 of 10 neurons. In neurons from patients' ($n = 6$) DRG neurons, application of hIL-6 depolarized RMP (Fig. 9C) but did not induce SA. A representative example of a neuron from an older adult male DRG demonstrating SA after application of rIL-6 is shown in Figure 9D. A 1-hour incubation period with rIL-6 also induced SA in older adult male rat DRG neurons but not young adult female DRG neurons. These data suggest that IL-6 is a likely contributor of the increased excitability observed in coculture conditions. To determine whether inhibition of hIL-6R would prevent neuronal sensitization, we treated media-only and coculture wells of DRG collected from older adult males ($n = 3$) with 5 μ g/mL of tocilizumab (a monoclonal antibody against hIL-6R) or vehicle (PBS) for 24 hours before recording. Vehicle-treated coculture neurons had significantly lower rheobase relative

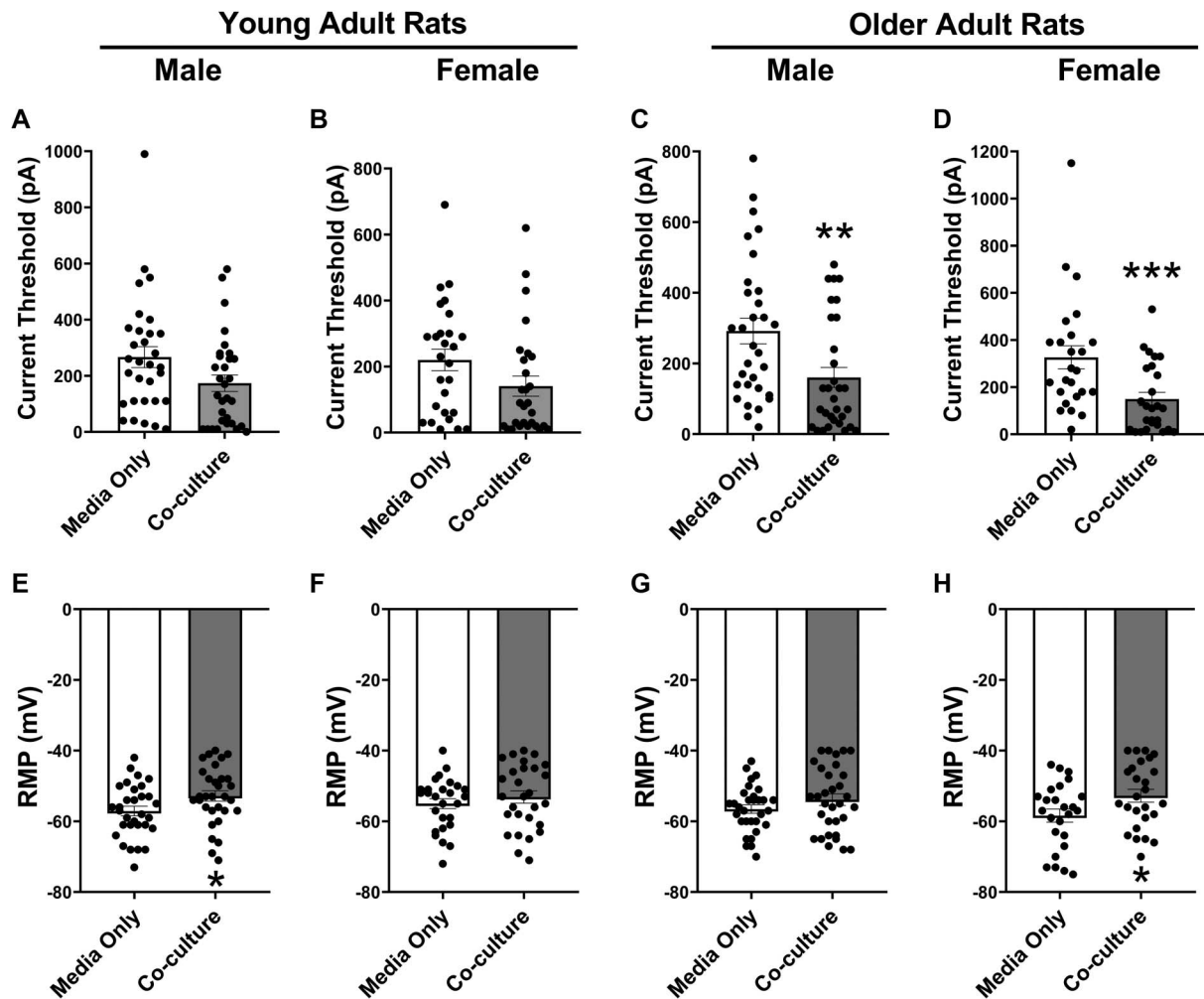


Figure 6. Coculture with Fadu cancer cells led to changes in excitability of rat dorsal root ganglion neurons from young adult males, older adult males, and older adult females but not young adult females. Dorsal root ganglion neurons ($n = 25\text{--}31$ per group) in coculture for 24 hours with Fadu cancer cells had lower rheobase in young adult males, but this difference was not significant ($t_{60} = 1.97$, $P = 0.053$; A). Rheobase did not differ between media-only and coculture DRG neurons in young adult females ($t_{51} = 1.8$, $P = 0.071$; B). Rheobase was significantly lower for coculture neurons from older adult males ($t_{60} = 2.9$, $P = 0.006$; C) and older adult females ($t_{49} = 3.2$, $P = 0.003$; D). Resting membrane potential was significantly higher in coculture neurons from young adult male rats ($t_{60} = 2.2$, $P = 0.034$; E) but not from young adult female rats ($t_{53} = 0.9$, $P = 0.394$; F). In contrast, resting membrane potential was not significantly higher in coculture neurons from older adult male rats ($t_{61} = 1.3$, $P = 0.201$, G) but was higher in older adult female rats ($t_{49} = 2.1$, $P = 0.037$, H). Data are expressed as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ vs media only. DRG, dorsal root ganglion.

to tocilizumab-treated coculture neurons, which did not differ from either media-only treatment groups (Fig. 9E). Further, RMP in vehicle-treated coculture neurons was significantly more depolarized compared with tocilizumab-treated coculture neurons, tocilizumab-treated media-only neurons, and vehicle-treated media-only neurons (Fig. 9F). This suggests that inhibiting hIL-6R activity attenuated sensitization induced by Fadu cells.

4. Discussion

Inflammation seems to play an important role in oral cancer pain as evidenced by our data from retrospective patient studies and human DRG experiments. Incubation with Fadu cancer cells resulted in high levels of the pro-inflammatory cytokine IL-6 in coculture media and triggered changes in the excitability of cultured human DRG neurons. We also observed higher expression of IL-6 in patient DRG coculture. All the human DRG tissues came from cancer patients undergoing various types of cancer treatment and surgery, which could lead to an upregulation in basal expression of IL-6. Our patient population had a

median age of 63 [57–72] years, and a previous study showed that basal IL-6 levels in the plasma of healthy subjects increased with age in men but not in women.⁶² It should be noted that in analyzing media samples, we are unable to normalize to a total protein count when using ELISA and therefore cannot account for possible differences in total cell numbers across samples. Although additional cytokines were found to be upregulated in coculture with human DRGs, these were not altered in rat DRG coculture, except for VEGF-A in older adult female coculture only. Our results are in agreement with previous research indicating that IL-6 sensitized DRG neurons and intraplantar injection of IL-6 produced mechanical hyperalgesia in rats.^{43,44} We also showed that inhibition of human IL-6 receptors blocked the development of SA and changes in current thresholds and RMP in cocultured rat neurons, providing further support for the role of IL-6 activity in neuronal sensitization because of Fadu cancer cells. Tocilizumab was first used clinically for the treatment of moderate-to-severe rheumatoid arthritis.⁵⁴ More recently, it has been approved for the treatment of cytokine release syndrome and is currently under emergency use authorization for treatment in COVID-19.^{33,58} In

Table 5**Characteristics of Coculture dorsal root ganglion neurons from adult rats with or without spontaneous activity.**

Characteristic	Non-SA	SA	Significance
Cocultured DRG neurons—young adult male rats (3–5 mo)			
Current threshold (pA)	219.6 ± 32.6 (23)	41.3 ± 31.3 (8)**	<i>P</i> < 0.01
RMP (mV)	−54.6 ± 1.7 (23)	−47.8 ± 2.2 (8)*	<i>P</i> < 0.05
AP amplitude (mV)	112.7 ± 2.6 (22)	100.1 ± 6.5 (8)*	<i>P</i> < 0.05
AP rise time (ms)	3.2 ± 0.3 (22)	4.0 ± 0.6 (8)	n.s. (<i>P</i> = 0.18)
AP fall time (ms)	17.9 ± 6.8 (22)	22.9 ± 6.0 (8)	n.s. (<i>P</i> = 0.79)
Width at 0 mV (ms)	5.8 ± 0.9 (22)	9.1 ± 3.6 (8)	n.s. (<i>P</i> = 0.21)
AP overshoot (mV)	58.8 ± 1.8 (22)	60.0 ± 5.6 (8)	n.s. (<i>P</i> = 0.24)
AP afterhyperpolarization (mV)	13.9 ± 2.5 (9)	18.6 ± 2.9 (7)	n.s. (<i>P</i> = 0.41)
Threshold potential (mV)	−17.1 ± 1.6 (22)	−19.5 ± 3.1 (8)	n.s. (<i>P</i> = 0.48)
Cocultured DRG neurons—older adult male rats			
Current threshold (pA)	195.6 ± 31.8 (25)	13.3 ± 2.1 (6)**	<i>P</i> < 0.01
RMP (mV)	−54.2 ± 1.8 (25)	−52.6 ± 4.1 (7)	n.s. (<i>P</i> = 0.69)
AP amplitude (mV)	116.9 ± 2.6 (25)	96.2 ± 13.5 (6)*	<i>P</i> < 0.05
AP rise time (ms)	4.4 ± 0.6 (25)	3.8 ± 1.0 (6)	n.s. (<i>P</i> = 0.65)
AP fall time (ms)	10.5 ± 1.4 (25)	15.4 ± 4.7 (6)	n.s. (<i>P</i> = 0.19)
Width at 0 mV (ms)	5.5 ± 1.1 (24)	7.7 ± 2.9 (6)	n.s. (<i>P</i> = 0.41)
AP overshoot (mV)	66.5 ± 1.8 (24)	48.8 ± 9.3 (6)***	<i>P</i> < 0.005
AP afterhyperpolarization (mV)	9.7 ± 1.8 (18)	12.6 ± 2.5 (6)	n.s. (<i>P</i> = 0.41)
Threshold potential (mV)	−14.6 ± 1.6 (25)	−19.3 ± 7.2 (6)	n.s. (<i>P</i> = 0.34)
Cocultured DRG neurons—older adult female rats			
Current threshold (pA)	187.5 ± 32.3 (20)	21.7 ± 7.9 (6)*	<i>P</i> < 0.05
RMP (mV)	−55.0 ± 2.0 (20)	−45.5 ± 2.4 (6)*	<i>P</i> < 0.05
AP amplitude (mV)	112.3 ± 3.3 (20)	90.9 ± 7.2 (6)**	<i>P</i> < 0.01
AP rise time (ms)	3.4 ± 0.3 (20)	6.6 ± 1.6 (6)***	<i>P</i> < 0.005
AP fall time (ms)	14.1 ± 1.4 (20)	19.6 ± 5.3 (6)	n.s. (<i>P</i> = 0.16)
Width at 0 mV (ms)	6.6 ± 0.6 (19)	10.6 ± 4.2 (6)	n.s. (<i>P</i> = 0.12)
AP overshoot (mV)	61.8 ± 2.5 (19)	53.6 ± 4.3 (6)	n.s. (<i>P</i> = 0.12)
AP afterhyperpolarization (mV)	10.2 ± 2.6 (10)	12.4 ± 1.8 (6)*	<i>P</i> < 0.05
Threshold potential (mV)	−14.3 ± 1.9 (20)	−21.7 ± 7.9 (6)*	<i>P</i> < 0.05

Data are expressed as percentages or mean ± SEM with the number of neurons in parentheses.

P* < 0.05, *P* < 0.01, ****P* < 0.005.

AP, action potential; DRG, dorsal root ganglion; RMP, resting membrane potential; SA, spontaneous activity.

addition to these anti-inflammatory effects, anticancer properties have also been identified, suggesting that it could also be a candidate for treatment of cancer pain.^{1,15,30} Thus, we elected to use this drug to assess whether inhibition of IL-6R signaling in Fadu cells would attenuate their effects on DRG neurons.

Incubation with Fadu cancer cells led to higher frequency of abnormal SA and large DSFs, decreased current thresholds, and enhanced responses to current stimulation in DRG neurons. Our results are in line with previous studies in which neurons were cocultured with cancer cells, immune cells, or other cell types that have been stimulated to release pro-inflammatory factors or other neuropeptides. DRG neurons cocultured with polymorphonuclear leukocytes demonstrated increased excitability,⁵⁵ and transient receptor potential vanilloid 1 (TRPV1) currents in DRG neurons were enhanced after coculture with pancreatic stellate cells stimulated with sonic hedgehog.²² Glutamatergic neurons derived from human-induced pluripotent stem cells cocultured with glioma cells also showed increased excitability.¹⁸ Mouse trigeminal ganglia neurons cocultured with HNSCC had increased expression of P2X2 and P2X3, and medium-sized neurons showed enhanced sustained current responses to adenosine triphosphate (ATP), although other electrophysiological properties were not affected.⁶³ Coculture of rat hippocampal neurons with macrophages led to enhanced outward K⁺ currents.^{24,25} Conversely, rat cortical neurons were hyperpolarized after coculture with macrophages.⁶¹

Evidence of neuronal sensitization was observed in coculture DRG neurons from older adult male and female rats and young adult male rats but not young adult female rats. This effect was more robust in older adult animals overall, and although coculture DRG neurons from young adult males had more SA and DSFs,

higher RMP, and elevated responses to current stimulation, the characteristics and responses of coculture DRG neurons from young adult females did not differ from media-only DRG neurons. Furthermore, no differences were found when comparing current thresholds, RMP, AP characteristics, or responses to current stimulation among media-only neurons from young adult male and female rats (Supplementary Table 4, available at <http://links.lww.com/PR9/A163>). This suggests that neurons from young female rats are resistant to the sensitizing effects of Fadu cells. Expression of IL-6 in DRG media from young female rats was lower overall compared with young males, which may explain some of the differences seen in the current study. Another possibility is that IL-6R expression differs in young adult females compared with young male or older adult rats of either sex, but we did not assess receptor expression in the current study.

Age-related changes in electrical membrane properties have been identified in mice, but these changes tended toward lesser electrical excitability.⁵³ In the current study, DRG neurons from older adult male rats did have lower AP afterhyperpolarization compared with young adult male rats ($t_{30} = 3.8$, *P* = 0.0006), and neurons from older adult female rats had lower AP rise time compared with young adult female rats ($t_{50} = 3.1$, *P* = 0.004), but no other differences in electrical properties were observed between the 2 age groups. Aged rats have previously demonstrated hyperexcitability of nociceptive dorsal horn neurons and enhanced responses to complete-Freund's adjuvant-induced inflammation.^{27,31} Changes in immune function of aged animals (termed *immunosenesence*) promote a pro-inflammatory profile that could enhance nociceptor hyperexcitability when exposed to inflammatory tumor microenvironments.^{20,40} Our coculture system does not allow for the recruitment and activation of circulating

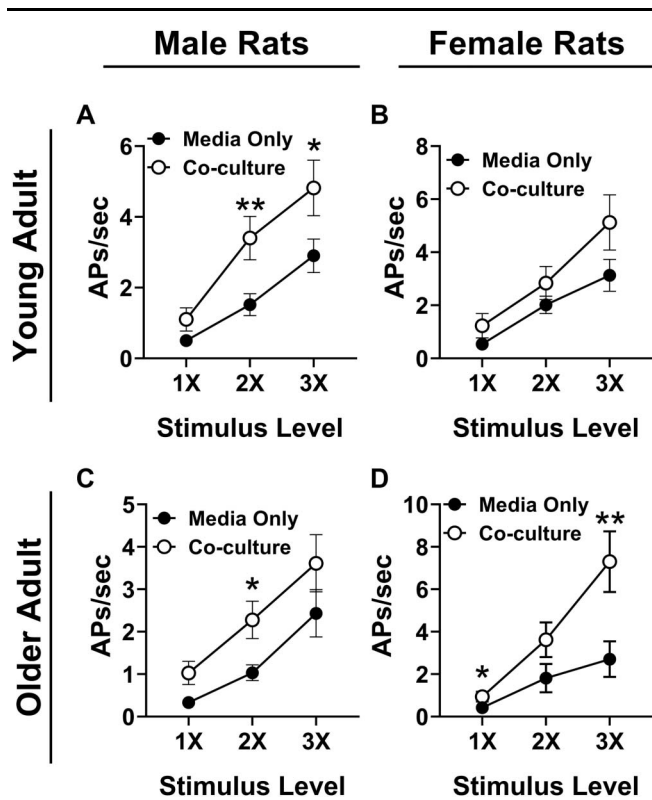


Figure 7. Coculture with Fadu cancer cells increased responses to current stimulation in rat dorsal root ganglion neurons from young adult males, older adult males, and older adult females, but not young adult females. Responses to current stimulation at 1×, 2×, and 3× rheobase were significantly higher in coculture DRG neurons from young adult male rats, and post-hoc tests (Bonferroni) showed significantly higher response rates at 2× and 3× rheobase ($F_{1,57} = 7.4$, $P = 0.009$; A). Responses were not different in young adult female rats ($F_{1,53} = 2.8$, $P = 0.098$; B). Responses were higher in coculture neurons from older adult male rats, and post-hoc tests (Bonferroni) showed that this difference was significant at 2× rheobase ($F_{1,33} = 4.3$, $P = 0.047$; C). Responses were also higher in older adult female rats, and although the main effect for group was not significant ($F_{1,30} = 3.4$, $P = 0.076$), there was a significant group × stimulus interaction ($F_{2,94} = 4.8$, $P = 0.011$). Post-hoc tests indicated that responses were higher at 1× and 3× rheobase ($F_{2,94} = 4.8$, $P = 0.011$; D). Data are expressed as mean ± SEM. * $P < 0.05$, ** $P < 0.01$ vs media only. DRG, dorsal root ganglion.

immune cells, which could limit the extent of immune-mediated changes that rely on cells that are not typically found in DRG tissue under naïve conditions, such as macrophages.^{29,64} Targeting signaling pathways for pro-inflammatory cytokines released by cancer cells could attenuate nociceptor hyperexcitability because of PNI and provide more adequate pain relief for cancer patients, although more investigation is needed to determine whether targeting cytokine function would affect tumor growth.

The changes in cocultured DRG neurons that we identified in human, male rats, and older adult female rats reiterate those found in other chronic pain models and studies on human nociceptors.^{36,42,45} Cocultured DRG neurons with SA had more depolarized RMP and lower rheobase when compared with those without SA. Spontaneous activity in primary nociceptors has been linked to spontaneous pain-like behavior in rodent models.^{2,16,37,45} Patients with cancer frequently report spontaneous pain, and this aspect of cancer pain is particularly resistant to analgesic treatment.^{13,32,46} Our results are also in agreement with studies where DRGs cocultured with cells or tissue harvested from chronically inflamed joints induced upregulation of genes (including IL-6/interferon β 2) or receptors involved in nociception (including TRPV1 and bradykinin 2).^{34,60} Our findings are relevant given that the median age of patients with HNSCC is 55 to 65 years, and diagnoses in males far outweigh those in females, with ratios ranging from 2:1 to 15:1 based on tumor site.^{41,59} In addition, the perception of pain is strongly affected by age, and the majority (~70%) of older adult patients with cancer included in a recent study reported severe functional limitations associated with pain.^{23,47}

A study in patients with cancer with pain resulting from bone metastasis failed to find sex differences in pain characteristics or analgesic prescriptions in patients whose median age was >55 years. Women with metastatic cancer have reported inadequate pain management more frequently than men, although patient age being ≥ 70 years was a stronger predictor than sex.⁹ Pain resulting from oral cancer has been found to favor men, women, or neither in different studies. No gender differences were identified in the presence or absence of oral pain in patients from one study, whereas age was a significant predictor for the presence of pain.⁴⁹ Among patients with oral cancer for whom oral pain was the initial complaint, men outnumbered women (9:1).¹¹

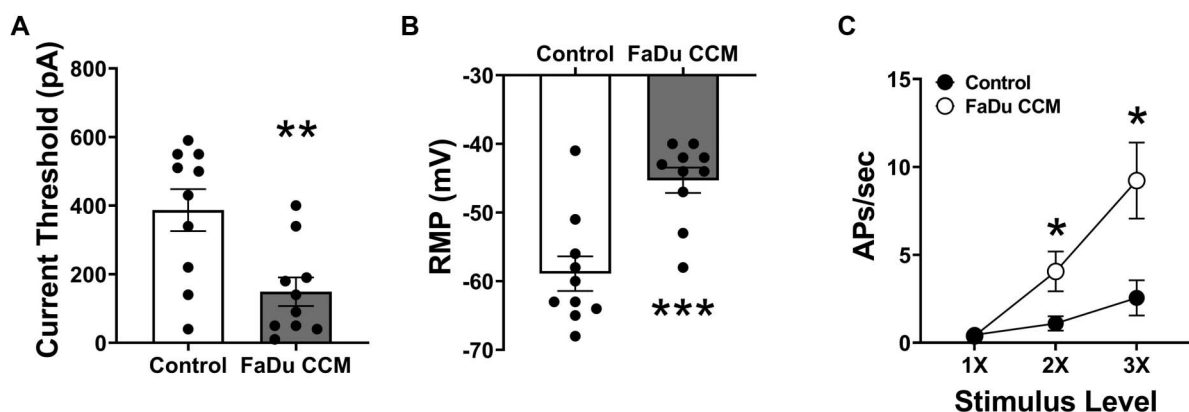


Figure 8. Fadu cancer conditioned media increases excitability of adult female rat dorsal root ganglion neurons. DRG neurons from older adult females ($n = 10$ per group) incubated in Fadu CCM had significantly lower rheobase ($t_{18} = 3.22$, $P = 0.005$; A) and higher RMP ($t_{18} = 4.34$, $P = 0.0004$; B) compared with control neurons. Fadu CCM-treated neurons also demonstrated increased responses to current stimulation ($F_{1,17} = 10.08$, $P = 0.006$; C), and post-hoc tests (Bonferroni) showed that this effect was significant at 2× and 3× rheobase. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. CCM, cancer conditioned media; DRG, dorsal root ganglion; RMP, resting membrane potential.

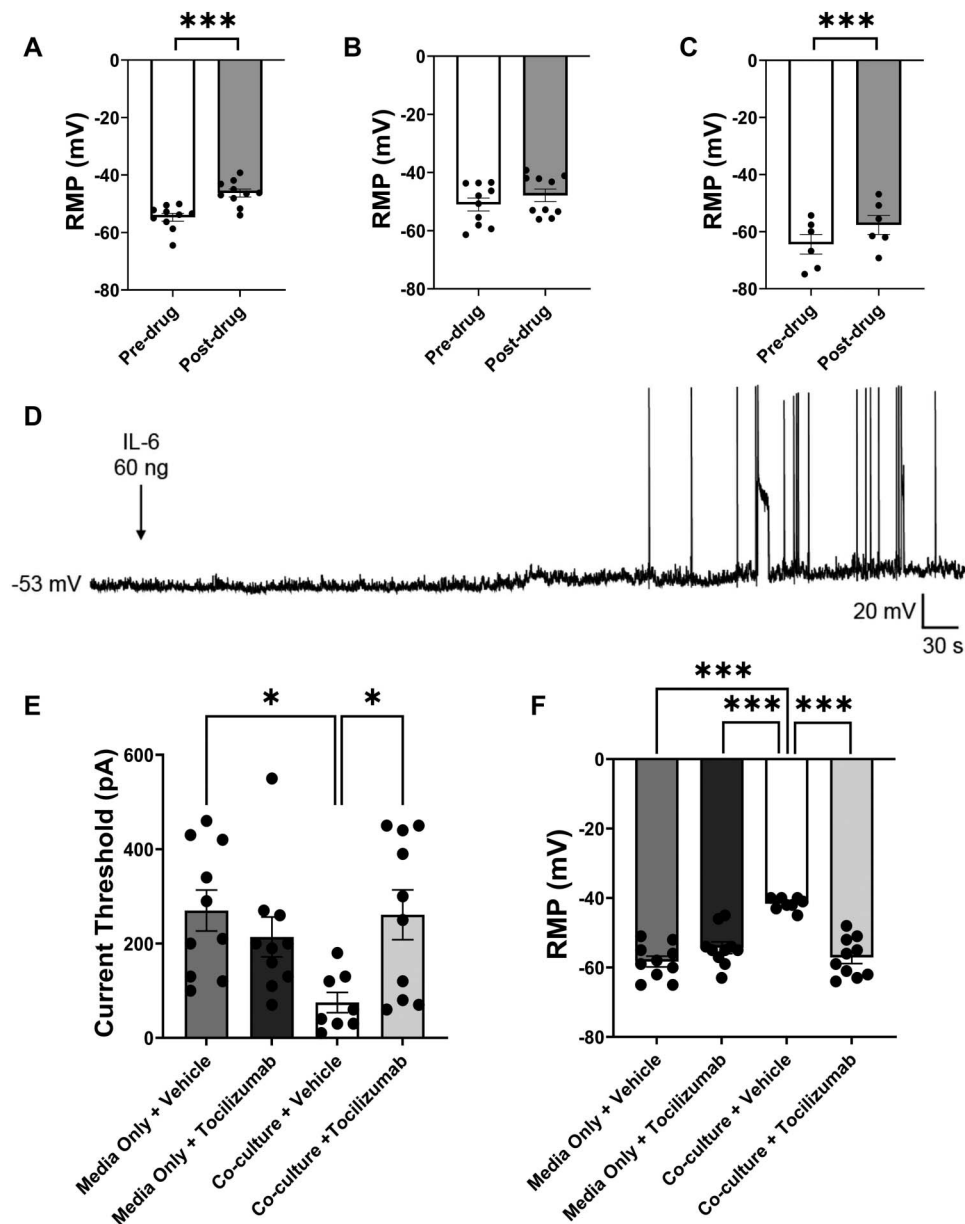


Figure 9. Recombinant interleukin 6 increased excitability in dorsal root ganglion neurons and blocking human interleukin 6 receptors decreased sensitization in cocultured rat dorsal root ganglion neurons. Naïve rat neurons were treated with rIL-6 (60 ng), which is comparable to the expression level found in Fadu conditioned media after 24 hours. (A) Dorsal root ganglion neurons from older adult males ($n = 10$) showed increased (more depolarized) RMP post-drug ($t_9 = 4.3$, $P = 0.002$), with 20% (2 of 10) firing action potentials after treatment. (B) In contrast, naïve rat neurons from young adult female rats ($n = 10$) treated with rIL-6 (60 ng) did not show significant depolarization in RMP on average ($t_9 = 1.62$, $P = 0.140$), but 10% (1 of 10) of neurons fired action potentials after treatment. (C) Naïve human DRG neurons ($n = 6$) treated with human recombinant IL-6 (hrIL-6, 60 ng) also showed increased RMP post-drug ($t_5 = 5.0$, $P = 0.004$), but none fired action potentials. A representative older adult male rat DRG neuron with SA after rIL-6 application is shown in (D). In addition, after a 1 hour incubation period with rIL-6 (60 ng), 3 out of 7 (42.9%) DRG neurons from older adult male rats demonstrated SA, while none (0 of 10) of the neurons from young adult female rats had SA. Further, treatment with tocilizumab, a human IL-6 receptor antagonist, attenuated sensitization in cocultured rat DRG neurons. Media only or coculture DRG neurons ($n = 9-10$ per group) from young adult male rats were treated with vehicle (PBS) or tocilizumab (5 $\mu\text{g}/\text{mL}$) for 24 hours before recording. Vehicle-treated coculture neurons had lower rheobase ($F_{3,34} = 3.88$, $P = 0.017$; E) and more depolarized RMP ($F_{3,34} = 21.8$, $P < 0.0001$; F) compared to tocilizumab-treated coculture neurons. In coculture, 22.2% (2 of 9) of vehicle-treated neurons had large DSFs and 11.1% (1 of 9) had SA, while none of the coculture tocilizumab-treated neurons, vehicle-treated media only neurons, or tocilizumab-treated media only neurons had DSFs or SA. * $P < 0.05$, *** $P < 0.001$. DRG, dorsal root ganglion; IL-6, interleukin 6; DSF, depolarizing spontaneous fluctuation; rIL-6, recombinant rat IL-6; SA, spontaneous activity; RMP, resting membrane potential.

Men with oral cancer were also found to have higher function-related pain and sharpness.¹⁰ This contrasts with studies where women with oral SCC had higher pain intensity, and women with HNSCC reported severe pain more frequently.^{48,50} Thus, no clear sex-based bias has been shown in oral cancer pain, and the median age of this patient population (~55–65 years at diagnosis) makes meaningful comparisons across age groups and between sexes difficult. In rodents, sex differences have been identified in

the types of immune cells that contribute to nociception.^{56,57} In female mice, infiltration of T cells contributed to hypersensitivity in oral cancer.⁵¹ In vivo, these differences result in distinct patterns of pain sensitivity, but the extent of their effect in an in vitro set up is unknown. Further, the protective effect conferred to young adult female rats in the current study was not maintained in older adults. Whether this was because of differences in sex hormones or changes inherent with aging is unclear. A previous study showed

enhanced pain sensitivity in female mice with oral tumors, an effect mediated by differences in neutrophil infiltration and activation in the tumor.⁵⁰ Further studies are required to determine what differences are present in young adult female rats that confer protection against sensitization. Determining whether this phenomenon translates to the patient population is important, especially given that pain is often the initial symptom that leads patients with cancer to seek medical treatment and the growing number of younger patients and women being diagnosed with head and neck cancer.²⁶

One limitation in the current study is the use of an HNSCC line for coculture with human DRG neurons when it would be more logical to coculture with trigeminal ganglion (TG) neurons. We are currently limited to human DRG tissue for cell culture, but acquisition of TG from cadaver donors will be useful for future oral cancer studies. Related to this, we used rat DRG neurons instead of TG neurons for better comparison with our human data. Future studies should incorporate rodent TG neuron data to allow for broad comparison between these ganglion types and for different oral cancer lines. Our patient data also focused on preoperative pain in cancers of the oral cavity, and cancer lines from the oral cavity would be more ideal for comparison than the hypopharynx because there is great heterogeneity among cancer cell lines in HNSCC.

In conclusion, we have shown that exposure to Fadu cancer cells is sufficient to induce SA in DRG neurons and that upregulated IL-6 expression is likely to contribute to changes in naïve DRG neurons that promote AP firing. Aging and sex seem to play a significant role in our findings. We consider our finding clinically relevant given that the median age of patients with HNSCC and that head and neck cancer diagnoses in men far outweigh those in women.⁵⁹ Future studies should explore whether sensitization is because of changes in ion channel activity, how enhanced cytokine levels affect satellite glial cells in coculture conditions, and the impact of cell-to-cell communication not dependent on cytokine signaling.

Disclosures

The authors have no conflicts of interest to declare.

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Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at <http://links.lww.com/PR9/A163>.

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