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C5L2 Silencing in Human Pulp Fibroblasts Enhances Nerve Outgrowth Under Lipoteichoic Acid Stimulation

Fanny Chmilewsky, PhD,* Imad About, PhD,[†] Lyndon F. Cooper, DDS, PhD,* and Seung H. Chung, DVM, PhD*

Abstract

Introduction: We recently reported that caries-associated C5a receptor (C5aR) expression and activation result in up-regulation of brain-derived neurotrophic factor secretion by pulp fibroblasts inducing prominent neurite outgrowth toward the carious site. Our data further showed a negative regulation of this brain-derived neurotrophic factor secretion by C5L2, another C5aR. C5L2 was considered a nonfunctional receptor and thus has received much less attention than C5aR. The aim of this study was to identify the role of C5L2 in pulp fibroblast-mediated neurite outgrowth.

Methods: In this study, lipoteichoic acid (LTA) was used to mimic dental caries-like inflammation. To evaluate the role of C5L2 in pulp neurite outgrowth, human pulp fibroblasts were C5L2 small interfering RNA silenced and cocultured with human neurons in a nerve growth assay system. **Results:** C5L2 silencing drastically increased the neurite outgrowth toward the LTA-stimulated pulp fibroblasts. The number of neurites detected was increased in the LTA-treated pulp fibroblasts.

Conclusions: Our results show that C5L2 constitutes a negative regulator of the neurite outgrowth under LTA stimulation. Of the events occurring during dentin-pulp regeneration, nerve regeneration is the key factor for maintaining tooth viability after infection or injury. Our study provides a foundation for creating therapeutic tools that target pulp fibroblasts during pulp/nerve regeneration. (*J Endod* 2018; ■:1–6)

Key Words

C5a receptor, C5L2, nerve outgrowth, pulp fibroblast

Tooth nerve damage is a common feature of tooth decay, and proper dental pulp innervation is required for maintaining pulp viability and integrity. Supporting this concept,

pulp denervation in rat

molars significantly reduced the survival and regenerating rate of exposed dental pulp tissue and caused a significant reduction in immunocompetent cell recruitment after dentin injury (1, 2). Further *in vivo* studies using rodent models have shown that pulp denervation resulted in severe morphologic tooth deformation including dentoalveolar ankyloses, reduced periodontal ligament space and Malassez epithelium, and decreased population of the recruited osteoclasts (3–5). Although the dental nerve appears to have critical importance in maintaining tooth viability, very little is known about the molecular mechanism that regulates dental pulp innervation and regeneration. Enhanced nerve regeneration may promote the morphologic and functional recovery in damaged pulp.

Complement C5a release from human pulp fibroblasts has been suggested to provide a link between pulp inflammation and regeneration ability (6). We showed for the first time that human pulp fibroblasts express C5a receptor (C5aR) in carious tooth decay *in vivo* and after lipoteichoic acid (LTA) stimulation *in vitro* (7, 8). C5aR signaling results in brain-derived neurotrophic factor (BDNF) secretion by pulp fibroblasts that induces prominent neurite outgrowth toward the site of carious injury (7). These first steps in response to carious injury offer new opportunities for incipient/early interventions to promote dentinal repair.

The second receptor, C5L2, shows a similar homology with C5aR but produces no down-signaling pathway after binding with C5a (9, 10). However, recent studies provided evidence that C5L2 physically interacts with C5aR and negatively regulates its activity (11). In support of this observation, our previous study showed that BDNF secretion by pulp fibroblasts is oppositely regulated by these 2 receptors. Pulp fibroblast BDNF secretion was negatively regulated by C5L2 (12). Because we believe that BDNF is a key factor in controlling nerve regeneration in damaged pulp, we now hypothesize that the inhibition of C5L2 increases nerve outgrowth under LTA-induced modeled *in vitro* caries injury. Because C5a is involved in many biological processes, identifying an alternative path to modify C5a signaling will provide new or complimentary avenues to investigate and translate mechanisms of dentin-pulp regeneration or repair under caries injury. The alternative approach of targeting C5L2 could provide an innovative therapeutic strategy (ie, the possibility to enhance the positive

Significance

It has long been established that the dentin-pulp complex has high regeneration capacity. Of the events occurring during dentin-pulp regeneration, nerve regeneration is the key factor for maintaining tooth viability after infection or injury.

action of C5aR in dental pulp regeneration by blocking the “inactive/inhibitory” C5L2 receptor).

Materials and Methods

Cell Cultures

Human immature third molars, freshly extracted for orthodontics purposes, were obtained in compliance with French legislation (informed patients’ consent and institutional review board approval of the protocol used at Aix-Marseille Université, Marseille, France). Human pulp fibroblasts were prepared from immature third molars at the two-thirds root formation stage by the explant outgrowth method and routinely cultured as previously described (7, 13).

Silencing of C5L2 Expression by Small Interfering RNA

Human pulp fibroblasts were grown in a 24-well plate culture chamber in 500 μ L free-antibiotic medium up to 70% confluence, and then transient transfection with small interfering RNA (siRNA) was performed using the siRNA Reagent System (sc-45064; Santa Cruz Biotechnology, Inc, Dallas, TX) according to the manufacturer’s protocol. Cells were incubated at 37°C in a CO₂ incubator in 250 μ L free-antibiotic and free-serum transfection solution containing a mixture of transfection reagent and 40 pmol/mL/well C5L2 siRNA (sc-105165; Santa Cruz Biotechnology, Inc, Dallas, TX) or control siRNA, which is a nontargeting siRNA designed as a negative control (sc-37007; Santa Cruz Biotechnology, Inc). After an incubation period of 6 hours, 250 μ L of medium containing 2 times the normal serum and antibiotic concentration was added in each well without removing the transfection mixture. After 24 hours, the medium was aspirated and replaced with fresh normal growth medium (Dulbecco modified Eagle medium + 4.5 g/L glucose, L-glutamine, sodium, pyruvate + 10% heat-inactivated fetal bovine serum + 100 μ g/mL streptomycin, and 100 U/mL penicillin). Assays using siRNA silenced cells were performed within 72 hours after the addition of fresh medium. Real-time polymerase chain reaction was performed to confirm the efficiency of C5L2 silencing in pulp fibroblasts.

Immunofluorescence Staining

Human pulp fibroblasts were seeded in 1×10^4 cells per 8-well glass culture chamber overnight before stimulation with LTA (10 μ g/mL). After 24 hours of treatment, cells were fixed and permeabilized as previously described (7). Then, cells were incubated for 1 hour with mouse anti-C5L2 (5 μ g/mL; BioLegend, San Diego, CA), rabbit anti-fibroblast surface protein (FSP) (20 μ g/mL; Abcam, Cambridge, MA), or their respective control isotypes. Finally, cells were incubated for 40 minutes with a mix of Alexa Fluor-594 antimouse immunoglobulin G (Jackson ImmunoResearch Inc, West Grove, PA) (2 μ g/mL) and 4’,6-diamidino-2-phenylindole (1 μ g/mL). The coverslips were sealed, and photographs were taken using a Leica DMI6000 B microscope (Leica Biosystems, Wetzlar, Germany).

Neurite Outgrowth Assays

Neurite outgrowth experiments were performed using an assay kit based on the use of 24-well plates (lower chambers) equipped with cell culture inserts (upper chambers) containing a permeable membrane with 3- μ m pores (EMD Millipore, Burlington, MA). Briefly, the underside of the insert membrane surface was coated with poly-L-lysine by placing the inserts into a well containing 400 μ L of a 0.1 mg/mL poly-L-lysine solution (Sigma-Aldrich, St Louis, MO) for 2 hours at 37°C (Fig. 1Aa). After coating, the inserts were placed into a new well containing human pulp fibroblasts silenced or not for C5L2 (as described previously) and treated with or without LTA (10 μ g/mL) (Fig. 1Aa). Then, 100,000 primary human neuronal cells (Sciencell, Carlsbad, CA) were seeded onto the top of the membrane (Fig. 1Aa). The system was incubated for 48 hours at 37°C to allow neurites to extend to the underside of the membrane. After 48 hours, the insert’s membranes were fixed for 20 minutes in cold methanol and then placed into 400 μ L Neurite Stain Solution (EMD Millipore) for 30 minutes at room temperature (Fig. 1Ab). Finally, the neuron cell bodies on the upper side of the insert membranes were wiped off using a cotton bud (Fig. 1Ab). For each experiment, the total neurite length detected on

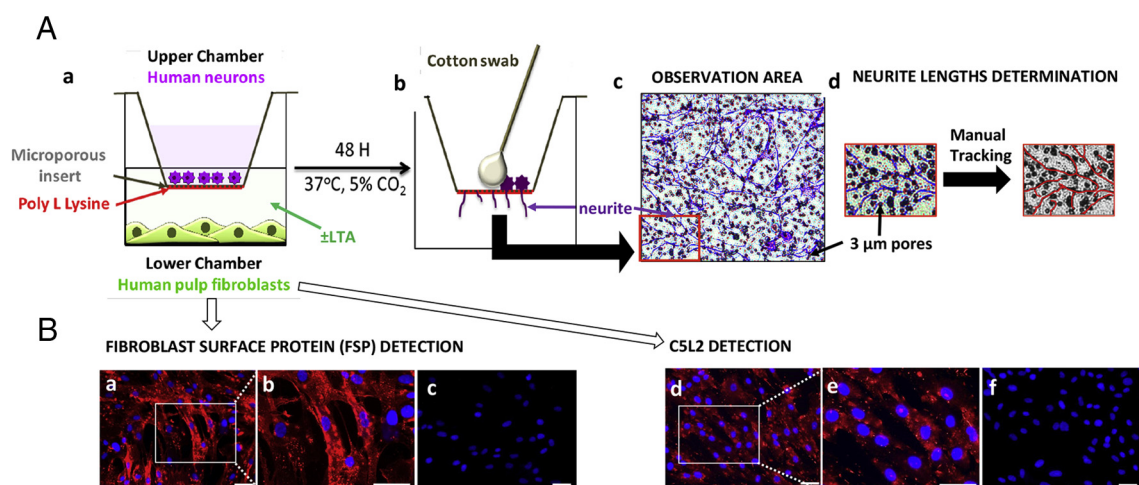


Figure 1. A) A schematic drawing of the neurite outgrowth protocol. (a) Neurite outgrowth was assayed in 24-well plates equipped with transwell inserts with a 3- μ m pore-size poly-L-lysine-coated membrane. Human neurons were seeded in the upper chamber, and human pulp fibroblasts silenced for C5L2 were incubated with or without LTA in the lower chamber. The neurite outgrowth was realized from the upper to the lower chamber through the porous insert. (b) After a 48-hour neurite extension period, neurons were fixed with cold methanol and incubated in a neurite stain solution, and neuron cell bodies were swabbed from the top inserts. (c) Pictures of the lower surface of the porous membrane were realized under a light microscope. (d) A reduced surface of the observation area was chosen to illustrate the determination of the neurite lengths by manual tracking. (B) (a and b) Immunofluorescent staining was used to confirm the expression of FSP by human pulp cells used in this study. (c) No FSP labeling was detected in the negative controls. (d and e) Moreover, we confirmed by immunofluorescent staining that human pulp fibroblasts express the C5L2 receptor. (f) No C5L2 labeling was detected in the negative controls. Nuclei were counterstained with 4’,6-diamidino-2-phenylindole (blue). b and e are magnified from the box in a and d, respectively. Scale bar = 50 μ m.

the lower surface of the membrane of each insert (Fig. 1Ac) was tracked in 5 random fields using ImageJ 1.43 software (National Institutes of Health, Bethesda, MD), and the results were expressed as mm neurite/mm² (Fig. 1Ad).

Statistical Analysis

All experiments were repeated at least 3 times, and statistical significance was determined using the Student *t* test to compare the different treatments and their respective controls. Data were expressed as means \pm standard deviation and considered significant at $P < .05$.

Results

Isolated Human Pulp Fibroblasts in a Nerve Outgrowth Assay Chamber Express C5L2

Previously, we used the Axon Investigation System device (Sigma-Aldrich), which allowed us to investigate the interaction between the 2 different cell types: pulp fibroblasts and neurons (7, 8). In this study, we established a simple and efficient coculture method to measure C5L2-mediated nerve outgrowth. Human pulp fibroblasts were placed in the lower chamber, and cortical neurons were housed in the upper chamber (Fig. 1A). We first checked the cell viability in these chambers under normal and LTA stimulation. Because gram-positive bacteria constitute the predominant cariogenic bacteria (14), we decided to use LTA, which is a complex component of gram-positive bacteria cell walls, to simulate the presence of these bacteria in the pulp.

FSP and C5L2 expression was validated using anti-FSP/C5L2 immunofluorescence staining (Fig. 1B). FSP expression is restricted to the cytoplasm of the pulp fibroblasts (Fig. 1Ba and Bb). C5L2 labeling is

also strongly detected in these cells (Fig. 1Bd and Be). Higher-magnification images combined with 4',6-diamidino-2-phenylindole staining show that all cells in the lower chamber express both FSP (Fig. 1Bb) and C5L2 (Fig. 1Be). No FSP and C5L2 expression is detected in negative (no primary antibodies) control isotypes (Fig. 1Bc and Bf).

C5L2 siRNA-silenced Pulp Fibroblasts Express Significantly Less C5L2

To identify the role of C5L2 in our nerve outgrowth assay kit, an siRNA-mediated gene silencing approach was used. The C5L2 siRNA-silencing efficiency was checked on pulp fibroblasts transfected either with control (Fig. 2A–E) or C5L2-siRNAs (Fig. 2F–J). Immunostaining with anti-C5L2 antibody showed that C5L2 was strongly expressed in all fibroblasts transfected with control siRNA (Fig. 2B). When the pulp fibroblasts were transfected with C5L2 siRNA, C5L2 expression was significantly decreased (Fig. 2G), confirming our successful gene silencing efficiency. Furthermore, real-time polymerase chain reaction analysis has been performed to confirm the efficiency of C5L2 silencing in pulp fibroblasts. The C5L2 messenger RNA expression was significantly decreased in the silencing pulp fibroblasts (Fig. 2K).

C5L2 Inhibition Increased Neurite Outgrowth under LTA Stimulation

Because C5L2 in LTA-treated human pulp fibroblasts seems to be implicated in the modulation of BDNF secretion (7), a neurite outgrowth assay was performed using human cortical neurons and pulp fibroblasts to identify the role of C5L2 in the nerve outgrowth process (Fig. 1A).

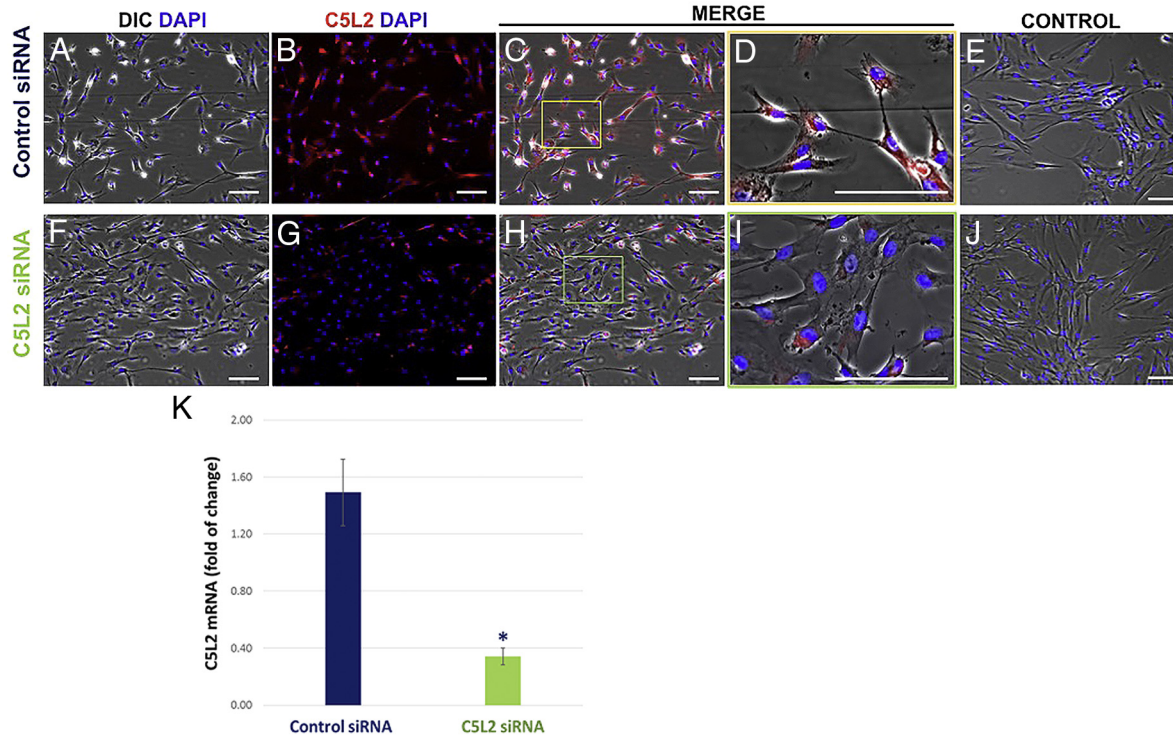


Figure 2. To confirm siRNA silencing efficiency, fluorescent staining was performed on pulp fibroblasts transfected with either (A–E) control or (F–J) C5L2 siRNAs. C5L2 was expressed in all fibroblasts transfected with (B and C) control siRNA and is clearly visible at (D) a higher magnification. However, C5L2 expression was reduced in pulp fibroblasts transfected with (G–I) C5L2 siRNA. (E and J) No C5L2 labeling was detected in the negative controls. D and I are magnified views from the boxes in C and H, respectively. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue). (K) Real-time polymerase chain reaction analysis further confirms the efficiency of C5L2 silencing in pulp fibroblasts. DIC, differential interference contrast. Scale bar = 50 μ m.

Fewer neurite outgrowth was detected on the underside of the insert's membrane when pulp fibroblasts were transfected with control siRNA in the lower chamber (Fig. 3A and B). The number of visualized neurites appeared to be increased in the LTA treatment group (Fig. 3C and D). The number of neurites detected was increased when the pulp fibroblasts of the lower chamber were silenced with C5L2 siRNA (Fig. 3E and F). Moreover, LTA stimulation of these C5L2-silenced fibroblasts significantly increased neurite outgrowth to the underside of the insert's membrane (Fig. 3G and H).

We performed quantitative assessment of actual nerve growth (Fig. 1Ad). Consistent with previous publications (7, 8), neurite outgrowth was significantly higher in LTA stimulation than in the unstimulated condition (Fig. 4: *blue bar untreated* = 825.13 ± 98.38 mm/mm² vs *blue bar LTA* = 1411.99 ± 146.66 mm/mm²). This increase in neurite length was more significant when the pulp fibroblasts placed in the lower chamber were silenced for C5L2 (Fig. 4: *green bar untreated* = 1093.22 ± 60.77 mm/mm² vs *green bar LTA* = 2206.66 ± 258.03 mm/mm²). Interestingly, we were able to confirm that C5L2 silencing also significantly increased neurite

outgrowth without LTA stimulation (Fig. 4: *blue bar untreated* vs *green bar untreated*), suggesting its capacity to regenerate injured nerves in the normal condition. Taken together, these data show that C5L2 inhibition drastically increased neurite outgrowth, constituting C5L2 as a negative regulator of neurite outgrowth under LTA stimulation.

Discussion

Our study revealed a novel role of the previously less explored C5a receptor C5L2 in controlling nerve outgrowth by pulp fibroblasts under an LTA-induced carieslike inflammatory context, constituting C5L2 as a negative regulator of neurite outgrowth. Dental caries is one of the most common human infectious diseases (15). Its progression into dental pulp leads to multiple responses including inflammation, necrosis, and regeneration. Nerve fiber/ending sprouting is observed in regenerating pulp (16, 17), and significant differences of nerve fiber innervation pattern and density were reported in carious teeth compared with healthy teeth, suggesting nerve regeneration's critical role(s) in the caries

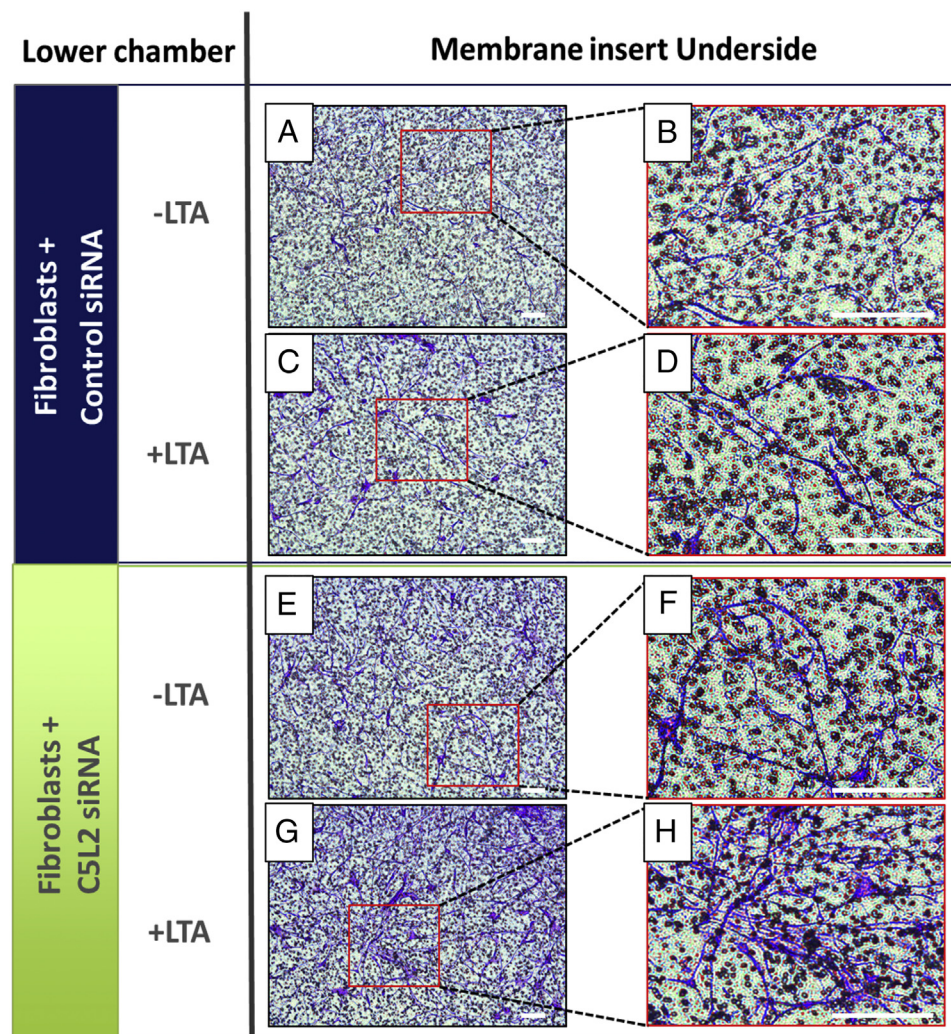


Figure 3. Representative images of neurite outgrowth on the lower surface of the porous membrane. (A and B) Few neurites were observed when pulp fibroblasts transfected with control siRNA were plated in the lower chambers of the system. (C and D) The number of neurites detected seemed to be increased by the incubation of these fibroblasts with LTA. (E and F) The number of neurites detected was increased when the pulp fibroblasts of the lower chamber were silenced for C5L2 expression. (G and H) Moreover, LTA stimulation of these C5L2-silenced fibroblasts drastically increased the number of neurites detected. B, D, F, and H are magnified from the box in A, C, E, and G, respectively. Scale bars: A–H = 50 μ m.

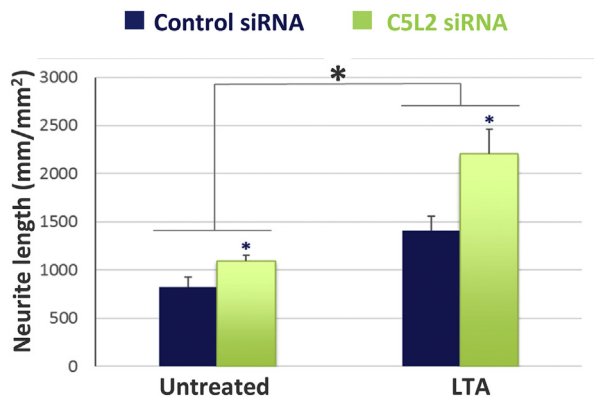


Figure 4. Neurite outgrowth experiments were shown in a histogram as the neurite length in $\text{mm}/\text{mm}^2 \pm$ standard error of the mean. When pulp fibroblasts were stimulated with LTA, the length of the neurite outgrowth toward the lower chamber was significantly higher than in unstimulated conditions. This increase was significantly higher when the pulp fibroblasts plated in the lower chamber were silenced for C5L2 expression. Interestingly, we observed that C5L2 expression silencing also significantly increased neurite outgrowth even in the unstimulated condition. * $P < .05$. $n = 3$ per group.

process (18). Despite the nociceptive role of dental nerve cells, nerves may influence regeneration. Indeed, they affect regeneration by the induction of immune cell recruitment and vascular permeability enhancement (18–21). Evidently, a mutual interaction between the immune and nervous systems is necessary in the defense against bacterial invasion (22, 23).

Gram-positive bacteria constitute the predominant cariogenic bacteria (14). LTA from gram-positive *Staphylococcus aureus* was used to simulate the presence of these bacteria in the pulp. We are aware that LTA treatment from *Staphylococcus aureus* cannot mimic all facets of dental caries *in vivo* given the possible role of gram-negative bacteria with lipopolysaccharide, but it is generally accepted that LTA is a major component of oral pathogens in carious decays (24, 25). Indeed, our Brown and Brenn coloration staining confirmed a predominant part on carious tooth sections was gram-positive bacteria (12).

Despite earlier studies (21, 26, 27) underscoring the relationship between innervation and wound healing in other tissues, there remain significant gaps in understanding innervation during pulp regeneration. The biological and molecular events orchestrating this process are still unknown. Pulp fibroblasts have been established as a source of regeneration signals in traumatic injury (28). However, their ability to secrete regeneration signals in response to caries has not been given much attention (6). Our previous study showed a critical role of C5aR, expressed by pulp fibroblasts, as a positive regulator in nerve regeneration of carious teeth (7). We have also shown that pulp fibroblasts co-express both C5aR and C5L2 under LTA stimulation, demonstrating that C5a fixation on C5aR induced its phosphorylation and mediated local BDNF secretion. Additionally, silencing C5L2 increased BDNF secretion (12). Thus, our work provides a confirmation of these data by showing that C5L2 acts as a negative regulator of BDNF secretion by pulp fibroblasts and nerve outgrowth under carious injuries.

This was performed using a simplified Boyden chamber specifically adapted to investigate neuron-fibroblast interactions and nerve sprouting. This simple model can further be used to study the interactions between neurons and other cell types in pulp inflammation and regeneration processes. We used human cortical neurons in the neurite growth assay instead of human trigeminal neurons because we were not able to acquire human trigeminal neurons.

Different types of nerve endings have been known to possess different biochemical properties because they differ in functionality such that the cortical neurons receive multiple inputs from nearby neurons and interneurons, whereas the sensory neurons mostly contribute 1-way signal conduction. There may be specific interactions required between the trigeminal neuron fiber endings with pulp fibroblasts in the context of dental sensory innervation. However, our study confirms that pulp fibroblasts can interact with nerve fibers of cortical neurons, thus raising the possibility of using these cells in other peripheral nerve injury models.

The results from this study further reveal C5a/C5L2 as a key initial signal in dental pulp nerve sprouting under carious injuries by providing direct evidence of C5L2's critical role in pulp fibroblast implication in inducing significant neurite outgrowth toward the injured site. A new mechanism of the complement system and the novel receptor identified in this study may provide a useful future therapeutic tool in targeting the pulp fibroblasts in the dentin-pulp regeneration process.

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The authors deny any conflicts of interest related to this study.

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