

## Original

# Toll-like receptor 4-mediated signaling activated by lipopolysaccharide suppresses transforming growth factor-beta-induced nerve growth factor expression in periodontal ligament-derived fibroblasts

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**Abstract** : Sensory neurons in the periodontal ligament (PDL) transmit the impulses, which are generated by the mechanical stimulation of the tooth, into the trigeminal ganglion, resulting in the excitement of the nucleus ventralis posteromedialis of the thalamus. When sensory neurons are injured, the neurites become atrophied and degenerated. Nerve growth factor (NGF) belonging to neurotrophic factors plays important roles in neurite extension and regeneration of injured sensory neurons. We evaluated how the major component of the outer membrane of gram-negative bacteria, lipopolysaccharide (LPS), affected transforming growth factor-beta1 (TGF- $\beta$  1)-induced NGF expression in rat PDL-derived fibroblasts SCDC2 cells. qRT-PCR and ELISA analyses showed that LPS suppressed TGF- $\beta$  1-induced NGF synthesis through the activation of Toll-like receptor 4 (TLR4). In addition, inhibitor of  $\kappa$  B (I- $\kappa$  B) kinase-2 (IKK-2) inhibitor, TPCA-1, abrogated LPS-induced suppression of TGF- $\beta$  1-promoted NGF expression. Intriguingly, western blotting showed that LPS inhibited TGF- $\beta$  1-induced activation of p38 mitogen-activated protein kinase (MAPK) that mediated TGF- $\beta$  1-induced intra-cellular signal transduction for NGF expression. These results suggested that TLR4-mediated signaling activated by LPS suppresses TGF- $\beta$  1-induced NGF expression in PDL fibroblasts by inhibiting TGF- $\beta$  1-induced p38 MAPK activation in a nuclear

factor-  $\kappa$  B-dependent manner, possibly resulting in the suppression of the regeneration of injured PDL neurons.

(194 words)

**Key words :** Iipopolysaccharide, transforming growth factor-beta, nerve growth factor, p38 mitogen-activated protein kinase, periodontal ligament fibroblast

## Introduction

Mechanosensitive (MS) neurons in the periodontal ligament (PDL) that respond to the mechanical stimulation of teeth are known to transmit the output signals into the neurons of the trigeminal mesencephalic nucleus (Vmes)<sup>1) . 2)</sup>. Previous studies showed that the Ruffini ending, characterized as low threshold slowly adapting type II stretch receptors<sup>3)</sup>, was the primary mechanoreceptor of the MS neuron in the PDL<sup>4) . 5) . 6)</sup>. Histological analysis showed that collagenous encapsulation of the Ruffini ending was not observed in PDL, unlike in the cutaneous ligament<sup>7)</sup>. In addition, the large Ruffini ending was partially surrounded with lamellar Schwann cells, unlike the small Ruffini ending<sup>7)</sup>.

The neurites of MS neurons become atrophied and eventually degenerate, following physical invasion-induce crushing injury of the PDL tissue<sup>8)</sup>. Generally, regeneration of the injured peripheral nerve includes interaction between the nerve cells and extracellular matrix (ECM)<sup>9)</sup>. In addition, neurotrophic factors involving brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), and nerve growth factor (NGF) secreted from the mesenchymal stem cells (MSCs) in the PDL induced neurite extension of the human neuronal cells SHSY-5Y<sup>10)</sup>, suggesting that the mesenchymal cells play an important role in peripheral nerve regeneration in the PDL tissue. However, the

molecular mechanisms underlying the expression of neurotrophic factors in mesenchymal cells in the PDL tissue remain to be clarified.

Transforming growth factor-beta (TGF-  $\beta$ ) played an important role on the attenuation of the inflammation reaction *in vivo*<sup>11)</sup>. Macrophages, which homed into an inflammatory lesion, vigorously synthesized and secreted TGF-  $\beta$ <sup>12)</sup>. TGF-  $\beta$  binds to type I and type II receptors on the cell membrane. Then, the type II receptor phosphorylates the type I receptor during the formation of a tetramer consisting of two type I and two type II receptors. The phosphorylated and activated type I receptor sends intracellular signals by phosphorylating the receptor-regulated Smads (R-Smads)<sup>13) . 14) . 15)</sup>. The TGF-  $\beta$  superfamily induces the intracellular signals mediated by Smads, which are composed of three groups: 1) R-Smads (Smad1, Smad5, and Smad8) primarily activated by the bone morphogenetic protein (BMP) type I receptors, and Smad2 and Smad3 activated by TGF-  $\beta$  type I receptors, 2) the common mediator Smad (Co-Smad) such as Smad4, and 3) the inhibitory Smads (I-Smads) such as Smad6 and Smad7. Generally, activated R-Smads form complexes with the Co-Smad, which translocate to the nucleus and induce the transcription of specific target genes. I-Smads inhibit the phosphorylation of R-Smads through competition with R-Smads for interaction with type I receptor and through

recruitment of specific ubiquitin ligases, resulting in their proteasomal degradation. On the other hand, TGF- $\beta$  induces intra-cellular signals via non-Smad signaling: non-Smad TGF- $\beta$  signaling include the mitogen-activated kinase (MAPK) consisting of an extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38<sup>16)</sup>. Non-Smad TGF- $\beta$  signaling also involve phosphoinositide 3-kinase (PI3K) /Akt<sup>16)</sup>. Haas et al. reported that TGF- $\beta$  promoted NGF expression in pancreatic stellate cells through the activation of ALK5-mediated pathway<sup>17)</sup>. TGF- $\beta$  1 also promoted NGF expression in vascular smooth muscle cells in a Smad3-dependent manner<sup>18)</sup>. We had previously found that TGF- $\beta$  1 promoted NGF expression through Smad2/3- and p38 MAPK-mediated signal transduction in PDL fibroblasts (unpublished observation).

Toll-like receptor 4 (TLR4) belonging to the TLR family is activated by bacterial lipopolysaccharide (LPS), which is a major component of the outer membrane of gram-negative bacteria<sup>19)</sup>, <sup>20)</sup>. Dimerization of TLR4 is induced by the recognition of LPS by the TLR4 receptor system. The LPS-induced intracellular signals mediated by dimerized TLR4 leads to the expression of pro-inflammatory cytokines and interferons: TLR4 recruits MAL/TIRAP and MyD88 to their TIR domain for activating nuclear factor- $\kappa$  B (NF- $\kappa$  B), and MAPKs that regulate pro- and anti-inflammatory cytokine production. TLR4 also recruits TRAM and TRIF that activate IRF3 and delay the activation of MAPKs and NF- $\kappa$  B for the production of type I IFNs<sup>21)</sup>, <sup>22)</sup>. Borivar *et al.* demonstrated that LPS suppressed TGF- $\beta$  1-induced differentiation of cardiac fibroblasts into myofibroblasts through the inhibition of Smad3

activation<sup>23)</sup>. In contrast, LPS enhanced TGF- $\beta$  1-induced platelet-derived growth factor-B expression in the bile duct epithelial cells through the activation of NF- $\kappa$  B-mediated signaling, without affecting Smad2/3 activity<sup>24)</sup>. Therefore, the effect of LPS on TGF- $\beta$  1-induced intra-cellular signal transduction seems to be dependent on the cell type. It remained to be clarified whether LPS up-regulated or down-regulated TGF- $\beta$  1-induced signals in the cells derived from PDL tissue.

In this study, we investigated how LPS affected TGF- $\beta$  1-induced NGF expression in PDL-derived fibroblasts. In addition, we evaluated how LPS affected TGF- $\beta$  1-induced Smad2/3- and p38 MAPK-mediated signals that were known to positively regulate NGF expression in PDL-derived fibroblasts.

## Materials and Methods

### Reagents

Recombinant human TGF- $\beta$  1 was procured from PEPROTECH, Rocky Hill, NJ, USA. Recombinant rat NGF was obtained from Alomone Labs, Jerusalem, Israel. *Escherichia coli* (O26: B6) LPS was purchased from Difco Laboratories, Detroit, MN, USA. Adenosine 5' -O- (3-thio) triphosphate (ATP  $\gamma$  S; ab138911) was purchased from Abcam (Cambridge, UK). NGF receptor (Trk A) inhibitor GW441756 was obtained from Selleck Chemicals (Houston, TX, USA). TLR4 inhibitor TAK-242, MAPK/ERK kinase (MEK) inhibitor U0126, and PI3K/Akt inhibitor LY294002 were obtained from Calbiochem (La Jolla, CA, USA). Inhibitor of  $\kappa$  B (I- $\kappa$  B) kinase-2 (IKK-2) inhibitor TPCA-1 was purchased from R & D Systems, Inc. (Minneapolis, MN, USA).

### Cell culture

The isolation of rat PDL-derived fibroblasts

and establishment of single cell-derived cultures (SCDCs) were previously reported<sup>25)</sup>. SCDC2 cells were cultured in type I collagen-coated plastic dishes (Sumilon Celltight Plate; Sumitomo Bakelite Co., Tokyo, Japan) in Ham's F-12 medium (Sigma-Aldrich) supplemented with 2 mM glutamine (100 × solution; Gibco), 10% fetal bovine serum (FBS), penicillin-streptomycin (both from Invitrogen, Gaithersburg, MD, USA), 10 ng/mL fibroblast growth factor (FGF) -1 (R&D Systems Inc., Minneapolis, MN, USA), 15 µg/mL heparin (Sigma-Aldrich), and penicillin (Gibco, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. The rat pheochromocytoma cell PC12 was cultured in type I collagen tissue culture plastic dishes containing Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 2 mM glutamine (100 × solution; Gibco), 5% FBS, 10% horse serum, and penicillin (Gibco, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C.

#### *RNA isolation and quantitative reverse-transcription-polymerase chain reaction (qRT-PCR)*

Total RNA was isolated from SCDC2 cells by using ISOGEN reagent (Nippon Gene, Toyama, Japan), according to the manufacturer's protocol. First-strand complementary DNA (cDNA) was synthesized from total RNA using the PrimeScript RT reagent Kit (Takara-Bio, Shiga, Japan). PCR was subsequently performed on a Thermal Cycler Dice Real Time System (Takara-Bio) using SYBR Premix Ex Taq II (Takara-Bio) with specific oligonucleotide primers [rat NGF, 5' -TGCCAAGGACGCAGCTTTC-3' (forward) and 5' -TGAAGTTT TAGTCCAGTGGGCTTCAG-3' (reverse); and rat glyceraldehyde

3-phosphate dehydrogenase (GAPDH), 5' -GGCACAGTCAAGGCTGAGAATG-3' (forward) and 5' -ATGGTGGTGAAGACGCCAGTA-3' (reverse)]. The mRNA level of NGF was normalized to that of GAPDH, and the relative expression levels were shown as the fold increase or decrease relative to the control.

#### *Western blot analysis*

Cells were lysed in RIPA buffer [50 mM Tris-HCl (pH 7.2), 150 mM sodium chloride (NaCl), 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS; Sigma, St. Louis, MO)] or lysis buffer [20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1% Triton X-100] containing protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO). The protein content was measured using BCA reagent (Pierce, Rockford, IL). Samples containing equal amounts of protein were separated on 10% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking with 1% bovine serum albumin (BSA) or 1% skim milk in T-TBS [50 mM Tris-HCl (pH 7.2), 150 mM NaCl, and 0.05% Tween 20], the membranes were incubated with the appropriate primary antibody. Following antibodies were used: anti-Smad2/3 purified mouse monoclonal antibody (1:1000; 610842; BD Transduction Laboratories, Franklin Lakes, NJ), anti-phospho-Smad2/3, anti-p38 MAPK, anti-phospho-p38 MAPK, anti-MAPK-activated protein kinase 2 (MAPKAPK-2), anti-phospho-MAPKAPK-2 (Thr222), anti-Akt, anti-phospho-Akt, anti-p44/42 MAPK (Erk1/2), and anti-phospho-p44/42 (Erk1/2) (Thr202/Tyr204) rabbit antibodies (1:1000; #8828, #9212, #9211, #4691, #9271, #9102, and #9101,

respectively; Cell Signaling Technology, Beverly, MA, USA) . The blots were incubated with an appropriate alkaline phosphatase-conjugated secondary antibody, and the signals were detected using an alkaline phosphatase substrate kit (BCIP/NBT Substrate Kit; Vector Laboratories Inc., Burlingame, CA, USA) .

#### *Enzyme-linked immunosorbent assay (ELISA)*

SCDC2 cells were seeded into a type I collagen-coated 6-well plate at a cell density of  $1.4 \times 10^5$  cells/well containing the growth medium for SCDC2 cells and cultured for 24 h. The growth medium was replaced with Ham's F-12 without any supplementation. The cells were subsequently stimulated with or without TGF- $\beta$  1 (10 ng/mL) , LPS (0.5  $\mu$  g/mL) , and TAK-242 (4  $\mu$  M) for 6 days. The amount of NGF secreted into the medium was measured using Rat beta NGF ELISA Kit (ab193736; Abcam, Cambridge, UK) , according to the manufacturer's instructions.

#### *Immunofluorescence analysis of cultured cells*

For immunofluorescence analysis of the cultured cells, the cells were subcultured on non-coated 4-well glass culture slides (Thermo Fisher Scientific Inc., Waltham, MA, USA) at the indicated cell density and maintained in Ham's F-12 supplemented with 2 mM glutamine, penicillin-streptomycin, and 10% FBS. Then, the cells were incubated for the indicated times. Following incubation, the cells were fixed in 4% paraformaldehyde (Nacalai Tesque, Inc.) for 15 min and permeabilized with Triton X-y100 (Sigma, St. Louis, MO) . After background reduction with normal goat serum, the cells were incubated with anti-neurofilament H (RMdO 20) mouse monoclonal antibody (1:1000, #2836S; Cell

Signaling Technology, Inc.) at room temperature for 1 h. After washing with phosphate-buffered saline (PBS) to remove excess primary antibody, the cells were incubated with Alexa Fluor 568-conjugated goat anti-mouse IgG as appropriate (1:400; A-11031; Molecular Probes, Leiden, The Netherlands) . After washing with PBS to remove excess secondary antibody, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) -containing mounting medium, DAPI Fluoromount-G (Southern Biotech Inc., Birmingham, AL, USA) . The fluorescent signal was detected using an Olympus IX70 fluorescence microscope with the LCPianFI 20 objective lens (Olympus Co., Tokyo, Japan) .

#### *Evaluation of neurite extension from the PC12 cells co-cultured with SCDC2 cells*

SCDC2 cells ( $2 \times 10^4$  cells) and PC12 cells ( $1 \times 10^4$  cells) were mixed and seeded into each well of a non-coated 4-well glass culture slide (Thermo Fisher Scientific Inc., Waltham, MA, USA) containing the PC12 cell growth medium. The cells were subsequently incubated with or without TGF- $\beta$  1 (10 ng/mL) , LPS (0.5  $\mu$  g/mL) , SB-431542 (10  $\mu$  M) , and Trk A inhibitor GW441756 (2 nM) for 4 days. In addition, ATP  $\gamma$  S (100  $\mu$  M) was added to all co-cultures from the beginning of the culture. PC12 cells in the co-culture were specifically labeled with the anti-neurofilament H immunofluorescence antibody. For use as the positive control, PC12 cells alone were treated with NGF (50 ng/mL) . The status of the neurite extensions emerging from the PC12 cells was observed using the Olympus IX70 fluorescence microscope with the LCPianFI 20 objective lens (Olympus Co., Tokyo, Japan) . In addition, statistical

assessment of neurite extension was performed: cells bearing neurites longer than the length of the cell body were counted as cells with neurite extension. PC12 cells with and without neurite extensions were counted in eight different microscopic fields. The ratio of the number of neurite-extended cells to the total number of PC12 cells in the fluorescent microscopic field was statistically determined ( $n = 8$ ).

### Statistical analysis

Data are presented as the mean  $\pm$  standard deviation (SD;  $n = 6$  or  $8$ ) and statistically analyzed by Tukey's multiple comparison test.  $*P < 0.05$  was considered statistically significant. The results of all experiments are representative of a minimum of three separate experiments.

## Results

### *LPS suppressed TGF- $\beta$ 1-induced expression of NGF mRNA through TLR4 in an NF- $\kappa$ B-dependent manner.*

LPS (0.5–5.0  $\mu$ g/mL) significantly abrogated TGF- $\beta$  1 (10 ng/mL)-induced mRNA expression of NGF (Figure 1A). We also confirmed that TGF- $\beta$  1 type I receptor inhibitor SB-431542 (10  $\mu$ M) abrogated TGF- $\beta$  1-induced mRNA expression of NGF (data not shown). In addition, TLR4 inhibitor TAK-242 (4  $\mu$ M) clearly suppressed LPS-induced abrogation of TGF- $\beta$  1-induced mRNA expression of NGF (Figure 1B), indicating that the LPS-induced intra-cellular signal mediated by TLR4 inhibited TGF- $\beta$  1-induced mRNA expression of NGF. We also evaluated the protein level of NGF in the conditioned medium from SCDC2 cells by using ELISA. TGF- $\beta$  1 (10 ng/mL) increased the concentration of NGF from 2.2 to 9.2 ng/mL in

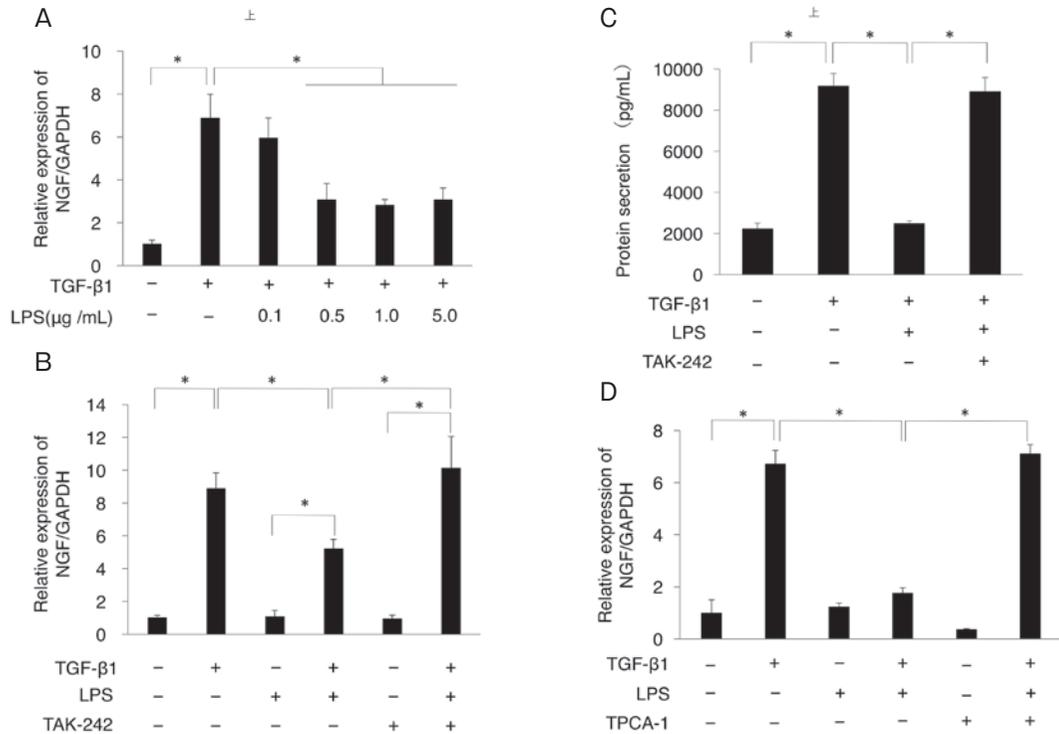
SCDC2-conditioned medium. LPS (0.5  $\mu$ g/mL) almost completely suppressed the TGF- $\beta$  1-mediated increase in NGF protein level in the conditioned medium (Figure 1C). In addition, TAK-242 (4  $\mu$ M) abrogated LPS-induced suppression of TGF- $\beta$  1-induced increase of NGF protein in the conditioned medium. Intriguingly, IKK-2 inhibitor TPCA-1 (10  $\mu$ M) clearly abrogated LPS-caused suppression of NGF expression promoted by TGF- $\beta$  1 (10 ng/mL) (Figure 1D).

### *LPS abrogated TGF- $\beta$ 1-induced activation of p38 MAPK, unlike Smad2/3.*

As described above, we previously found that TGF- $\beta$  1 promoted NGF expression through Smad2/3- and p38 MAPK-mediated signal transduction in PDL fibroblasts (unpublished observation). Here, we evaluated how LPS affected TGF- $\beta$  1-induced intra-cellular signal transduction mediated by Smad2/3- and p38 MAPK. As shown in Figure 2A, LPS (0.5  $\mu$ g/mL) clearly suppressed the phosphorylation of p38 MAPK at 60 min after TGF- $\beta$  1 (10 ng/mL) stimulation, unlike that observed in the case of Smad2/3 (Figure 2B).

### *Basal activity of ERK and PI3K/Akt plays an important role in TGF- $\beta$ 1-promoted NGF expression.*

We evaluated how TGF- $\beta$  1-activated intracellular signals other than Smad2/3- or p38 MAPK-mediated signals affected TGF- $\beta$  1-induced expression of NGF mRNA: we examined the phosphorylation status of ERK and Akt after TGF- $\beta$  1 stimulation. TGF- $\beta$  1 (10 ng/mL) did not affect basal phosphorylation levels of ERK and Akt at 30 and 60 min after stimulation (Figure 3A, upper and lower panels, respectively). Intriguingly, the MEK inhibitor U0126 (10  $\mu$ M) almost completely suppressed TGF- $\beta$  1-induced expression of



**Figure 1** : LPS suppressed TGF- $\beta$  1-promoted mRNA expression of NGF in SCDC2 cells through TLR4 in a NF- $\kappa$ B-dependent manner.

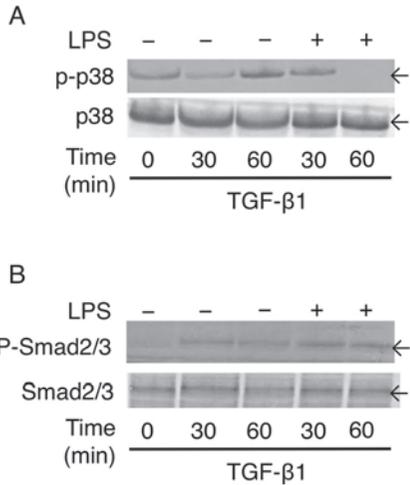
Cells were seeded into type I collagen-coated 12-well tissue culture plates at a density of  $7 \times 10^4$  cells/well in SCDC2 growth medium and maintained for 24 h. The growth medium was replaced with Ham's F-12 without FBS, FGF-1, and heparin, and the cells were cultured until starvation for 24 h. (A) The starved cells were treated with TGF- $\beta$  1 (10 ng/mL) with or without LPS at various concentrations for 24 h. (B) The starved cells were treated with or without TGF- $\beta$  1 (10 ng/mL) and LPS (0.5  $\mu$ g/mL) for 24 h. Some cells were pretreated with (B) TLR4 inhibitor TAK-242 (4  $\mu$ M) or (D) TPCA-1 (10  $\mu$ M) 30 min before LPS administration. The relative expression level of NGF was evaluated using qRT-PCR, as described in Materials and Methods. (C) The cells were seeded into type I collagen-coated tissue culture plates and maintained for 24 h. The cells were starved and subsequently cultured with or without TGF- $\beta$  1 (10 ng/mL) alone and with or without TGF- $\beta$  1 (10 ng/mL) plus LPS (0.5  $\mu$ g/mL) for 6 days. Some cells were pre-treated with TAK-242 (4  $\mu$ M), as described above. The concentration of NGF protein secreted into the culture medium was determined using ELISA. Data represent the mean  $\pm$  standard deviation (SD;  $n = 6$ ). \* $P < 0.05$  was considered significant. The cells not subjected to TAK-242 or TPCA-1 pretreatment were incubated with dimethyl sulfoxide (DMSO) as a vehicle control.

NGF mRNA (Figure 3B). In addition, the PI3K/Akt inhibitor LY294002 (10  $\mu$ M) partially and significantly suppressed TGF- $\beta$  1-induced expression of NGF mRNA (Figure 3B).

LPS suppressed neurite extension from the surface of PC12 cells that were stimulated by

NGF secreted from TGF- $\beta$  1-stimulated SCDC2 cells.

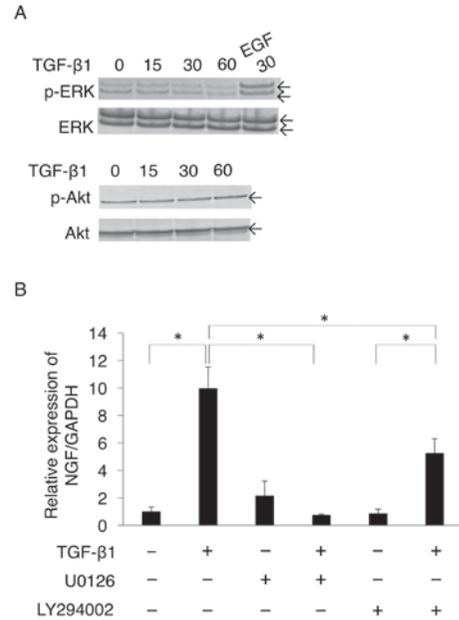
ATP  $\gamma$  S enhanced the sensitivity of PC12 cells to NGF stimulation by activating early response kinase 1/2 and p38 MAPK mediated through the P2Y<sub>2</sub> receptor. Then, NGF-induced neurite extension from the surface of PC12



**Figure 2 :** LPS suppressed TGF- β 1-induced activation of p38 MAPK.

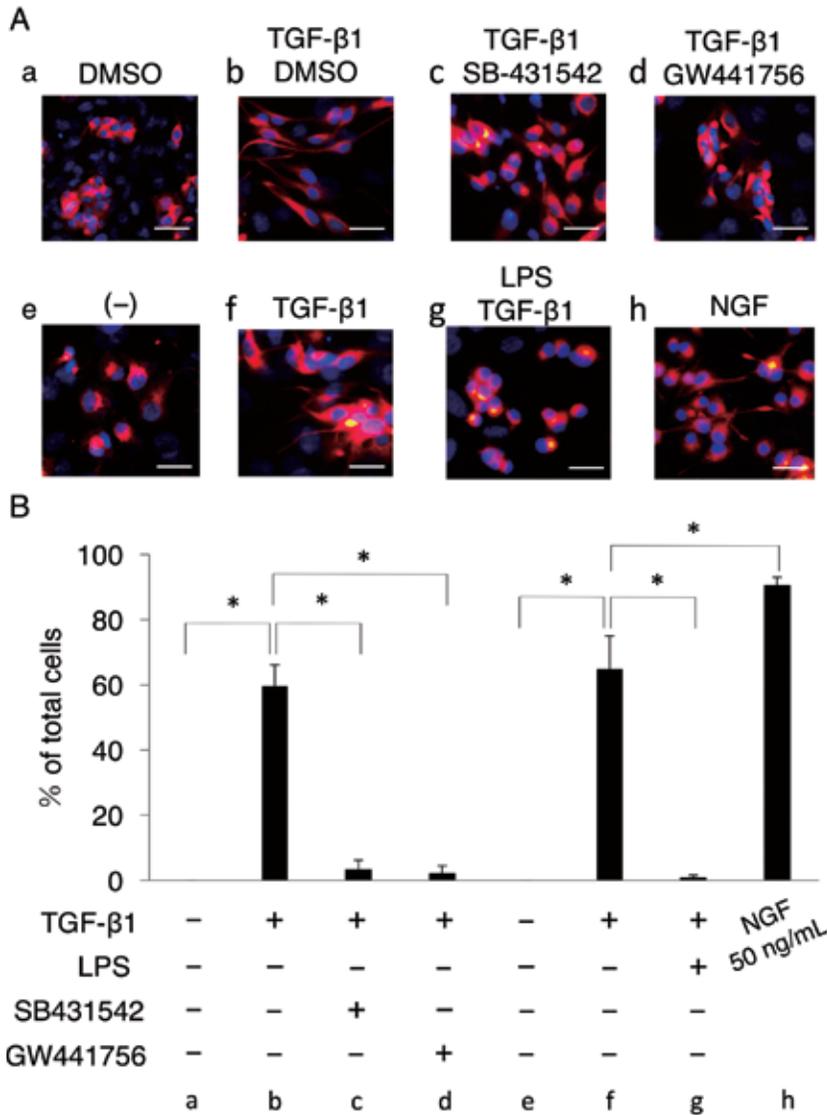
The cells were seeded into type I collagen-coated 6-well tissue culture plates at a density of  $1.4 \times 10^5$  cells/well in SCDC2 growth medium and maintained for 24 h. The cells were starved and treated with or without TGF- β 1 (10 ng/mL) alone, or TGF- β 1 (10 ng/mL) plus LPS (0.5 μ g/mL) for the indicated times. The phosphorylation status of (A) p38 MAPK and (B) Smad2/3 was evaluated by western blotting.

cells was accelerated<sup>26)</sup>. We previously demonstrated that the NGF secreted from TGF- β 1-stimulated SCDC2 cells promoted neurite extension from surfaces of PC12 cells primed with ATP γ S (unpublished observation). Here, we evaluated the effect of LPS on neurite extension from ATP γ S-primed PC12 cells co-cultured with TGF- β 1-stimulated SCDC2 cells. Co-culture of TGF- β 1 (10 ng/mL) -treated SCDC2 cells and PC12 cells showed that LPS (0.5 μ g/mL) suppressed neurite extension of PC12 cells (Figure 4A, panel g). We also confirmed that both TGF- β type I receptor inhibitor SB-431542 (10 μ M) (panel c) and NGF receptor (Trk A) inhibitor GW441756 (2 nM) (panel d) abrogated the neurite extension of PC12 cells



**Figure 3 :** Basal activity of ERK and PI3K/Akt seemed to play an important role in TGF- β 1-promoted NGF expression.

(A) The cells were seeded into type I collagen-coated 6-well tissue culture plates at a density of  $1.4 \times 10^5$  cells/well in the SCDC2 growth medium and maintained for 24 h. The cells were starved as described above, followed by stimulation with TGF- β 1 (10 ng/mL) for the indicated times. The phosphorylation status of ERK and Akt was evaluated by western blotting, as described in Materials and Methods. Because the basal level of ERK phosphorylation in the SCDC2 cells was low, the cells were stimulated with EGF (10 ng/mL) as a positive control to confirm the positive band position of phosphorylated ERK. (B) Cells were seeded into type I collagen-coated 12-well tissue culture plates at a density of  $7 \times 10^4$  cells/well in the SCDC2 growth medium and maintained for 24 h. The growth medium was replaced with Ham's F-12 without FBS, FGF-1, and heparin, and the cells cultured until starvation for 24 h. The starved cells were treated with or without TGF- β 1 (10 ng/mL) for 24 h. Some cells were pretreated with the ERK inhibitor U0126 (10 μ M) or the PI3K/Akt inhibitor LY294002 (10 μ M) 30 min before TGF- β 1 administration. The cells without U0126 and LY294002 pretreatment were incubated with dimethyl sulfoxide (DMSO) as a vehicle control. The relative expression level of NGF was evaluated by qRT-PCR, as described in Materials and Methods. Data represent the mean ± SD (n = 6). \*P < 0.05 was considered significant.



**Figure 4 :** LPS suppressed the neurite extension from the surface of PC12 cells which was stimulated by NGF secreted from TGF- $\beta$  1-stimulated SCDC2 cells.

(A) SCDC2 cells ( $2 \times 10^4$  cells) and rat pheochromocytoma cells PC12 ( $1 \times 10^4$  cells) were mixed and seeded into each well of non-coated 4-well glass culture slides (panels a to g). Cells were treated with (panels b, c, d, f, and g) or without (panels a and e) TGF- $\beta$  1 (10 ng/mL) and incubated for 4 days. In some cases, the cells were treated with the TGF- $\beta$  type I receptor inhibitor SB-431542 (10  $\mu$  M) (panel c), the NGF receptor (Trk A) inhibitor GW441756 (2 nM) (panel d), or LPS (0.5  $\mu$  g/mL) (panel g) from the start of the co-culture. In addition, ATP  $\gamma$  S (100  $\mu$  M) was added to all co-cultures (panels a to g). DMSO was used as a vehicle control for SB-431542 and GW441756 (panels a, and b). For the positive control experiment for neurite extension, PC12 cells alone were treated with NGF (50 ng/mL) (panel h). The neurite extension of PC12 cells was visualized by immunostaining with the anti-neurofilament H antibody (red). The nuclei were stained with DAPI (blue). Scale bar: 50  $\mu$  m. (B) Statistical assessment of neurite extension in the PC12 cells co-cultured with SCDC2 cells was performed. Data represent the mean  $\pm$  SD ( $n = 8$ ). \* $P < 0.05$  was considered significant.

in this co-culture system. In addition, neurite extension in the PC12 cells co-cultured with SCDC2 cells was statistically analyzed. As shown in Figure 4B, LPS (0.5  $\mu$  g/mL) clearly reduced the ratio of the number of neurite-extended PC12 cells to the total number of PC12 cells, which were activated by ATP  $\gamma$  S (100  $\mu$  M) treatment, and co-cultured with TGF- $\beta$  1-treated SCDC2 cells (64.8–0.8%) .

### Discussion

We had previously shown that 1) TGF- $\beta$  1 promoted NGF expression through Smad2/3- and p38 MAPK-mediated signal transductions in the PDL fibroblasts SCDC2, and that 2) NGF secreted from the TGF- $\beta$  1-treated SCDC2 cells promoted neurite extension from the surface of ATP  $\gamma$  S-primed PC12 cells through the NGF receptor Trk A (unpublished observation) . In this study, we evaluated how LPS affected NGF expression in SCDC2 cells. We found that the TLR4-mediated signal activated by LPS suppressed NGF synthesis in SCDC2 cells (Figure 1A, B, and C) . As described above, NF- $\kappa$  B is a major signal transduction molecule after TLR4 is activated by LPS. We found that IKK-2 inhibitor TPCA-1 (10  $\mu$  M) , which is known to inhibit the transcriptional activity of NF- $\kappa$  B by disturbing the translocation of NF- $\kappa$  B into the nucleus, reversed LPS-induced suppression of the TGF- $\beta$  1-promoted NGF expression in SCDC2 cells (Figure 1D) , suggesting that LPS suppressed TGF- $\beta$  1-promoted NGF expression through the activation of intra-cellular signals mediated by NF- $\kappa$  B. In addition, we showed that LPS abrogated TGF- $\beta$  1-induced activation of p38 MAPK, unlike Smad2/3 (Figure 2) . These results suggested that the TLR4-mediated signaling activated by LPS suppresses TGF- $\beta$

1-induced NGF expression in SCDC2 cells through the inhibition of TGF- $\beta$  1-induced p38 MAPK-mediated signal. We previously showed that the inflammatory cytokines IL-1  $\beta$  and TNF- $\alpha$  suppressed TGF- $\beta$  1-induced phosphorylation of both Smad2/3 and p38 MAPK (unpublished observation) , suggesting that TGF- $\beta$  1-induced intra-cellular signal transduction was differentially modified by inflammatory cytokines and LPS, respectively. On the other hand, TGF- $\beta$  1 did not up-regulate the phosphorylation status of ERK and Akt (Figure 3A) . However, the MEK inhibitor U0126 almost completely suppressed TGF- $\beta$  1-promoted NGF expression. In addition, the PI3K/Akt inhibitor LY294002 partially and significantly suppressed TGF- $\beta$  1-promoted NGF expression (Figure 3B) . These results suggested that the basal activities of ERK and PI3K/Akt play important roles in TGF- $\beta$  1-promoted NGF expression. In addition, we found that LPS suppressed neurite extension of PC12 cells co-cultured with TGF- $\beta$  1-treated SCDC2 cells (Figure 4) , suggesting that the LPS on the outer membrane of gram-negative bacteria in the oral cavity disturbs the regeneration of PDL neurons promoted by the NGF secreted from TGF- $\beta$  1-stimulated PDL fibroblasts.

Gram-negative bacteria such as *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitance*, *Prevotella intermedia*, *Fusobacterium nucleatum*, and *Tannerella forsythensis* are known to be the major pathogens involved in periodontitis<sup>27) , 28) , 29)</sup> . Some previous studies have shown that *P. gingivalis* LPS activated TLR2 rather than TLR4<sup>30) , 31) , 32)</sup> , while some other studies showed that *P. gingivalis* LPS activated TLR4<sup>33) , 34) , 35)</sup> . In addition, Kikuchi *et al.* demonstrated that *A. actinomycetemcomitance*

LPS induced interleukin (IL) -12 expression in human dendritic cells through the stimulation of TLR4<sup>36)</sup>. Kikkert *et al.* also showed that *A. actinomycetemcomitance* LPS promoted IL-8 expression in the human embryonic kidney cells through the stimulation of both TLR2 and TLR4<sup>37)</sup>. Thus, LPS on the outer membrane of the gram-negative bacteria in the oral cavity seemed to stimulate TLR2 and/or TLR4. Here, we studied how *E. coli* LPS affected TGF- $\beta$  1-induced NGF expression in PDL-derived fibroblasts. Actually, the chemical structure of *P. gingivalis* lipid A, which is the bioactive moiety of LPS, is remarkably different from that of *E. coli* lipid A<sup>38)</sup>. Therefore, *E. coli* LPS is known to activate TLR4, but not TLR2<sup>39)</sup>. Therefore, it remains to be clarified how LPS-induced TLR2-mediated signals affect TGF- $\beta$  1-promoted NGF expression in PDL-derived fibroblasts. Therefore, further studies are required to clarify this aspect.

As described above, neurotrophic factors including BDNF, GDNF, and NGF, secreted from the MSCs in PDL, induced neurite extension of human neuronal cells<sup>10)</sup>. In fact, the SCDC2 cells derived from rat PDL tissue expressed several MSC markers, including CD44, CD146, and vascular cell adhesion molecule (VCAM) -1<sup>25)</sup>, which may be related to the acquisition of the ability to express NGF in SCDC2 cells. In addition, SCDC2 cells such as MSCs differentiated into endothelial cell-like cells and constructed vessel-like structures in a three-dimensional culture with type I collagen gel<sup>25)</sup>. In addition, it remains to be clarified whether fully differentiated PDL fibroblasts, which did not show multipotency like MSCs, retain the ability to express NGF.

Thus, taken together, we showed that LPS-activated TLR4 suppressed TGF- $\beta$  1-promoted NGF synthesis through abrogation

of TGF- $\beta$  1-induced activation of p38 MAPK in a NF- $\kappa$ B-dependent manner. These results suggested that the gram-negative bacteria in the oral cavity are obstacles for the regeneration of PDL neurons promoted by the NGF secreted from PDL fibroblasts. Therefore, the findings of this study may help in identifying targets for treating damaged PDL neurons at the molecular level.

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### Conflict of interest statement

The authors declare no conflicts of interest regarding the publication of this article.

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