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Inhibition of lysophosphatidic acid receptor 1 signaling in periodontal ligament stem cells reduces inflammatory paracrine effect in primary astrocyte cells

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Lysophosphatidic acid (LPA) is a bioactive lipid messenger involved in the pathogenesis of chronic inflammation and various diseases. Recent studies have shown an association between periodontitis and neuroinflammatory diseases such as Alzheimer's disease, stroke, and multiple sclerosis. However, the mechanistic relationship between periodontitis and neuroinflammatory diseases remains unclear. The current study found that lysophosphatidic acid receptors 1 (LPAR1) and 6 (LPAR6) exhibited increased expression in primary microglia and astrocytes. The primary astrocytes were then treated using medium conditioned to mimic periodontitis through addition of *Porphyromonas gingivalis* lipopolysaccharides, and an increased nitric oxide (NO) production was observed. Application of conditioned medium from human periodontal ligament stem cells with or without LPAR1 knockdown showed a decrease in the production of NO and expression of inducible nitric oxide synthase and interleukin 1 beta. These findings may contribute to our understanding of the mechanistic link between periodontitis and neuroinflammatory diseases.

Keywords: Lysophosphatidic acid receptor 1, Periodontitis, Neuroinflammatory diseases, Periodontal ligament stem cell, Astrocytes

Introduction

Periodontitis is one of the major causes of chronic inflammation which may lead to the development of the systemic diseases including cardiovascular diseases, diabetes, and rheumatoid arthritis [1–3]. The systemic inflammation induced by periodontitis is associated with activation of brain immune cells such as microglia and astrocytes [4,5]. Glial cells, including microglia and astrocytes, are in change of inflammatory responses in the central nervous system. The neuroinflammation is associated with neurodegenerative diseases, such as Alzheimer's diseases, multiple sclerosis, ischemic stroke, and Parkinson's diseases [6,7]. Recent reports started to show the relationship between periodontitis and the neurodegenerative disease though the mechanistic link remains unclear [8,9].

In the previous study, we identified that the inhibition of lysophosphatidic acid receptor 1 (LPAR1) decreased the production of pro-inflammatory cytokines and promoted the osteogenic differentiation of periodontal ligament stem cells (PDLSCs) in the inflammatory condition induced by *Porphy-*

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romonas gingivalis lipopolysaccharide (Pg-LPS) treatment [10]. LPA signaling is involved in the regulation of central nervous system, including glial cell activation, neuronal cell death, axonal outgrowth, and the development of neuropathic disease [11,12]. LPAR1 is also an important regulator in the survival and function of peripheral glial cells [13].

In this study, we investigated the involvement of LPAR1 in relaying the astrocytes and human PDLSCs through the conditioned medium (CM). In the previous study, CM from PDLSCs promoted the tissue regeneration [14]. In the current study, CM from LPAR1-inhibited or -knockdown PDLSCs showed a significant decrease of inflammatory responses induced by Pg-LPS in the astrocytes. In comparison with the control CM, low-LPAR1 CM reduced the production of nitric oxide (NO) and interleukin 1 beta (IL-1β). These results suggest the inhibition of LPAR1 may augment the utility of PDLSC CM in the future application.

Materials and Methods

1. Cell culture and preparation of conditioned medium

Human PDLSCs were obtained from Lonza (Cat No. CC-7049; Basel, Switzerland) and cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS, Hyclone), 100 g/mL streptomycin and 100 U/mL of penicillin (Hyclone) at 37℃ in a humidified atmosphere of 5% CO_2. The cells were used between passage numbers 5 and 10. To obtain CM, when the cells had grown to 70% confluence, PDLSCs were transiently transfected with 1 g of LPAR1-specific shRNA (#TG313300; Origene, Rockville, MD, USA) or non-target control shRNA (pGFP-V-RS) using jetPRIME reagent (Polyplus, New York, NY, USA) according to the manufacturer's instructions. LPAR1 mRNA expression by quantitative real time polymerase chain reaction (PCR). Transfected cells were cultured in 20 mL serum free DMEM containing 100 g/mL streptomycin and 100 U/mL of penicillin with Pg-LPS (InvivoGen, San Diego, CA, USA) or vehicle and the cells were incubated for 1 hour. After stimulation, the cells were washed with phosphate-buffered saline and replaced with fresh medium. CM was collected 24 hours after incubation. And then, CM was centrifuged at 1,000 rpm for 10 minutes and filtered through a 0.22 m syringe filter to remove the cells and debris.

2. Isolation of primary astrocyte and microglia cells

Primary astrocytes and microglial cells were prepared form whole brain of neonatal (postnatal day 2 day) C57BL/6 mice. Mouse whole brains were dissociated and grinded in the plate involving DMEM high glucose (Hyclone) supplemented with 10% FBS (Hyclone) on ice. Dissociated cells were passed through mesh cell strainer and collected by centrifugation at 500 rpm for 3 minutes and seeded on in T75 flasks, and cultured in DMEM containing 10% FBS, and the medium was replaced every 2–3 days. After 14 days, microglia and astrocyte cells were isolated from mixed glial cells via shaking method. Mixed glial cells were replaced on shaker at 240 rpm for 4 hours. The supernatant containing the detacted microglial cells were collected and reseeded. And astrocytes were obtained by shaking mixed glial cells at 240 rpm for 16 hours, and then culture media were discarded. Astrocytes were dissociated using trypsin-EDTA (Gibco, Grand Island, NY, USA) and then collected by centrifugation at 200 g for 3 minutes. Purity of the cells, as determined by immature neuronal (Nestin), astrocyte (GFAP), and microglia (Iba-1) marker using quantitative real time PCR.

3. RNA isolation and quantitative real-time PCR

Total RNA from primary astrocyte treated with PDLSCs-CM for 24 hours was extracted using the Sensi-TriJol™ reagent (Cat No. LGR-1117; Lugen Sci Co., Ltd, Bucheon, Korea) according to the manufacturer's instructions. cDNA was synthesized using MMLV reverse transcriptase (Cat No. #3201; Beams Bio, Seongnam, Korea). After reverse transcription, real-time PCR analyses were performed with Bright-Green 2X qPCR MasterMix-ROX (ABM, Vancouver, BC, Canada) using a StepOnePlus Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) for ~40 cycles. The target DNA species were amplified by real-time PCR using the following primer sets: GFAP sense primer, 5'-CAG ACT TTC TCC AAC CTC CAG-3'; GFAP antisense primer, 5'-AAT CTG GTG AGC CTG TAT TGG-3'; Iba-1 sense primer, 5'-TCT GCC GTC CAA ACT TGA AG-3'; Iba-1 anti-sense primer, 5'-TCT AGG TGG GTC TTG GGA AC-3'; IL-1β sense primer, 5'-AGG GCT GCT TCC AAA CCT TTG AC-3'; IL-1β antisense primer, 5'-ATA CTG CCT GCC TGA AGC TCT TGT-3'; TNF-α sense primer, 5'-TGG GAC AGT GAC CTG GAC TGT-3'; TNF-α antisense primer, 5'-TTC GGA AAG CCC ATT TGA GT-3'; LPAR1 sense primer, 5'-CCA TGA ACG AAC AAC AGT GC-3';

LPAR1 antisense primer, 5'-CTG TGT TCC ATT CTG TGG CT-3'; LPAR2 sense primer, 5'-CTG TGA CTT GGA CAG TTG CT-3'; LPAR2 antisense primer, 5'-GAG TAG GAA GAC AAG CAG GC-3'; LPAR3 sense primer, 5'-GGT GAT CAC AAA CCG GAA GT-3'; LPAR3 antisense primer, 5'-CAT CAG GAA CAC GTA AGC GA-3'; LPAR4 sense primer, 5'-GCC ATT GTC TAT CCC TTC CG-3'; LPAR4 antisense primer, 5'-ACT AGG ATC CAG ACT CCA GC-3'; LPAR5 sense primer, 5'-GCC AAT TCT TCA GCC AAC AC-3'; LPAR5 antisense primer, 5'-GCC AAT ACC AGG CTG TAG AC-3'; LPAR6 sense primer, 5'-CAG TGC CCT TAT GAC GAC TC-3'; LPAR6 antisense primer, 5'-GAT ATC AGC CCA AGC ACG AA-3'; β-actin sense primer, 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3'; β-actin antisense primer, 5'-TAA AAC GCA GCT CAG TAA CAG TCC G -3'. Data were normalized for gene expression using β-actin as an internal control. The expression pattern of β-actin in our experimental sets did not differ between groups. The $2^{-\triangle\triangle CT}$ method was used to analyze the relative quantification of gene expression.

4. Immunoblotting

Primary astrocytes were treated with PDLSCs-CM for 24 hours and were homogenized with RIPA lysis buffer (Cat No. LGB-1243; Lugen Sci Co., Ltd) containing protease inhibitor cocktail (Roche, Rotkreuz, Switzerland). Briefly, protein concentration was measured by the Bradford assay (#5000006; Bio-Rad, Hercules, CA, USA), and 25 g of proteins from each sample were separated by SDS-PAGE and transferred onto PVDF membranes by electrophoretic transfer. The membrane was blocked with 5% non-fat skim milk in TBS-Tween and incubated with antibodies against inducible nitric oxide synthase (iNOS) (1:500, #sc-650; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membrane was incubated with HRP-conjugated rabbit secondary antibody (1:3,000, #7074; Cell Signaling, Danvers, MA, USA), and the immunoreactive signals were detected by Chemiluminescent detection reagent (#34095; Thermo Fisher Scientific). Protein density was normalized using β-actin (1:4,000, #A5441; Sigma-Aldrich, St. Louis, MO, USA).

5. Measurement of NO production

NO production was assayed by measuring the nitrite concentration in the supernatant of cultured primary astrocyte cells. The cells were seeded in 96 well plate and were changed to the various PDLSCs-CM. After 24 hours or 48 hours, supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 2.5% H_3PO_4) and was incubated at room temperature for 5 minutes. Nitrite concentrations were determined by measuring the absorbance of the supernatant at 570 nm. Sodium nitrite ($NaNO_2$) was used to generate a standard curve.

6. Statistical analyses

Statistical analyses were performed in GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA). All data are expressed as the mean ± SEM. Data were analyzed with a one-way or two-way analysis of variance followed by the Tukey's multiple comparison test for unequal replications. A Student's t-test was used to compare the two groups.

Results

1. Expression of LPAR in primary glial cells

To determine the expression pattern of LPARs on the primary glial cells, astrocytes and microglia were isolated from postnatal day 2 mouse pups. The cellular identities were confirmed by microglial-specific marker Iba-1 (Fig. 1A) and astrocyte-specific marker GFAP (Fig. 1C) by real-time PCR. The expression of the immature neuronal marker Nestin was not detected in either microglia or astrocytes. The expression of LPAR1-6 on microglia and astrocytes were determined by real-time PCR (Fig. 1B and 1D). The results indicate that LPAR1 and LPAR6 are highly expressed in primary microglia and astrocytes.

2. Preparation of CM from shLPAR1 transfected PDLSCs

To investigate whether the inhibition of LPAR1 in human PDLSCs has differential effect on CM activity, shRNA of LPAR1 was introduced during the culture. Transfected cells showed about 50% decrease in LPAR1 mRNA expression (Fig. 2A). shLPAR1 transfected PDLSCs were pretreated with Pg-LPS or vehicle to evaluate the alteration in the effect of PDLSCs in inflammation condition on primary astrocyte (Fig. 2B). In previous study, we identified that the LPAR1 decreased the inflammatory cytokine of PDLSCs in inflammatory condition induced by Pg-LPS. Therefore, we sought to examine the effects of shLPAR1 transfected PDLSCs on primary astrocyte using periodontitis-mimicking inflammatory CM. In addition, our previous study showed that the treatment of LPAR1 antagonist

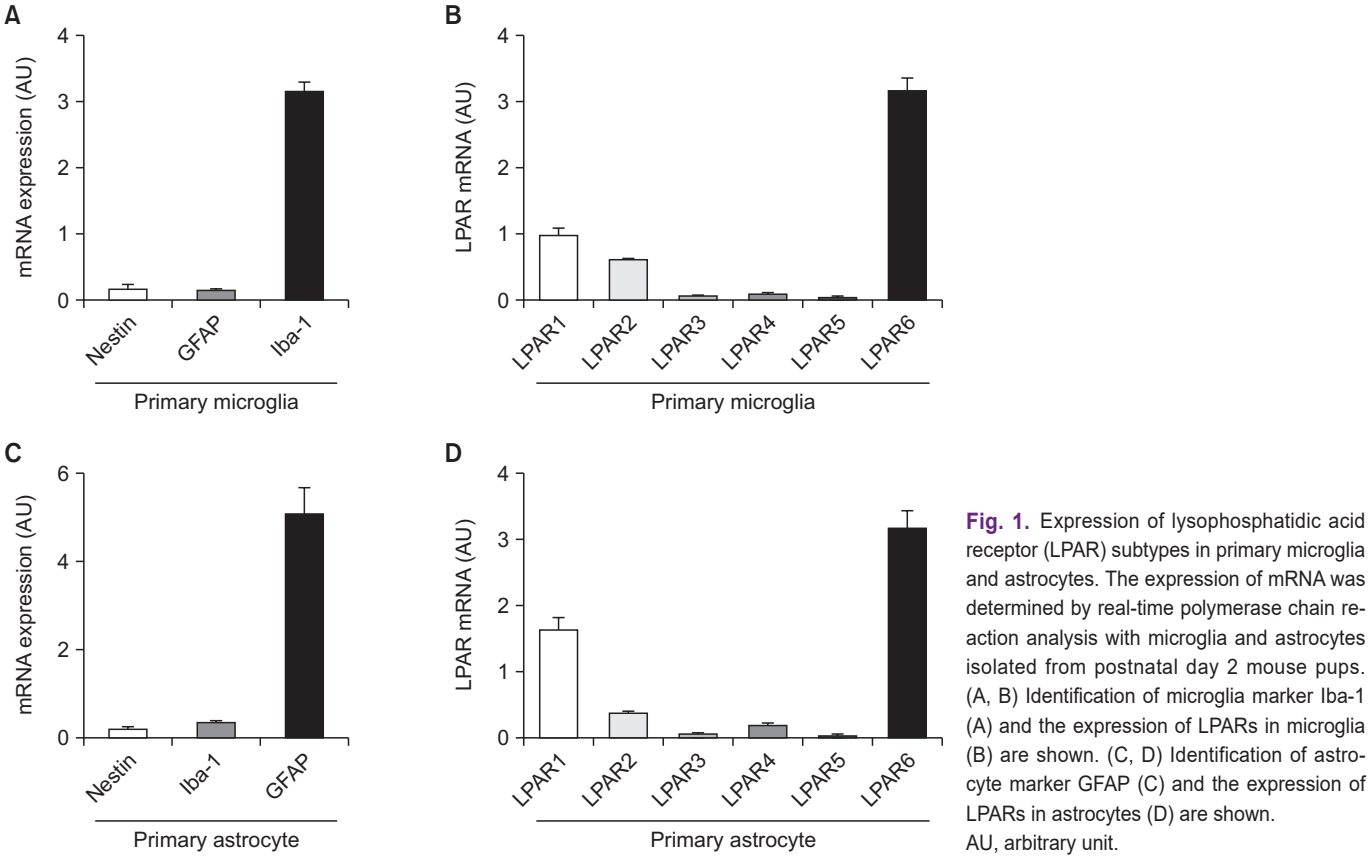

Fig. 1. Expression of lysophosphatidic acid receptor (LPAR) subtypes in primary microglia and astrocytes. The expression of mRNA was determined by real-time polymerase chain reaction analysis with microglia and astrocytes isolated from postnatal day 2 mouse pups. (A, B) Identification of microglia marker Iba-1 (A) and the expression of LPARs in microglia (B) are shown. (C, D) Identification of astrocyte marker GFAP (C) and the expression of LPARs in astrocytes (D) are shown.
AU, arbitrary unit.

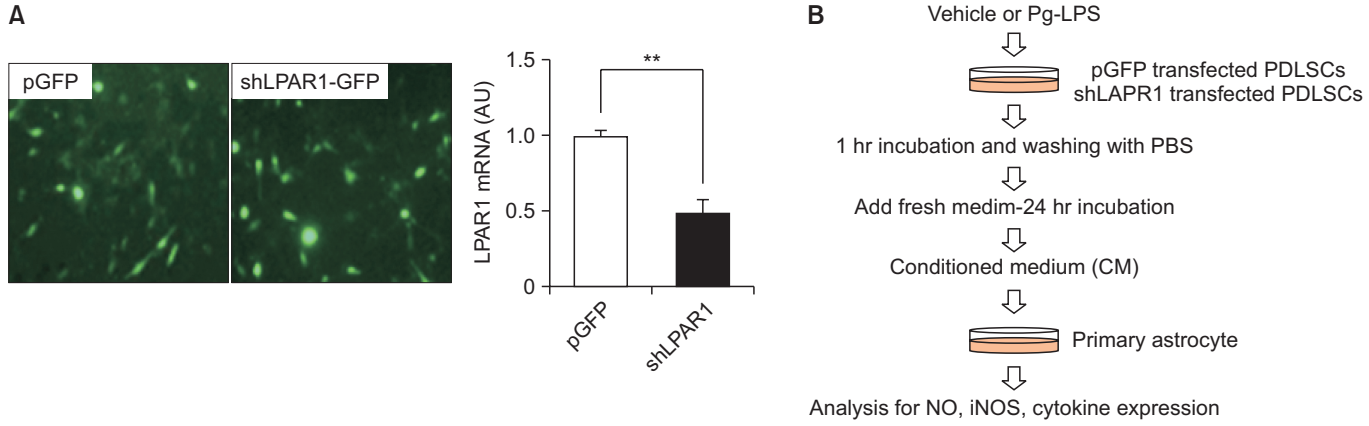

Fig. 2. Preparation of conditioned medium (CM) from periodontal ligament stem cells (PDLSCs) transfected with lysophosphatidic acid receptor 1 (LPAR1) shRNA. (A) PDLSCs were transfected with GFP-labelled negative control (pGFP) or GFP-labelled LPAR1 shRNA (shLPAR1-GFP). Cells were monitored for GFP expression at 48 hours after transfection and LPAR1 mRNA level determined by real-time polymerase chain reaction. Data is presented as means ± SEM. ×10 magnification. (B) Schematic representation of the experimental process for CM preparation is shown.
AU, arbitrary unit; Pg-LPS, *Porphyromonas gingivalis* lipopolysaccharide; PBS, phosphate-buffered saline; NO, nitric oxide; iNOS, inducible nitric oxide synthase.
$^{**}p < 0.01$.

AM095 in PDLSCs resulted in the reduction of inflammation and induction of osteogenic differentiation in Pg-LPS induced periodontitis mimicking condition [10].

3. Effect of Pg-LPS-induced CM from LPAR1-inhibited PDLSCs on the inflammatory response of primary astrocyte

We evaluated whether PDLSCs could communicate with

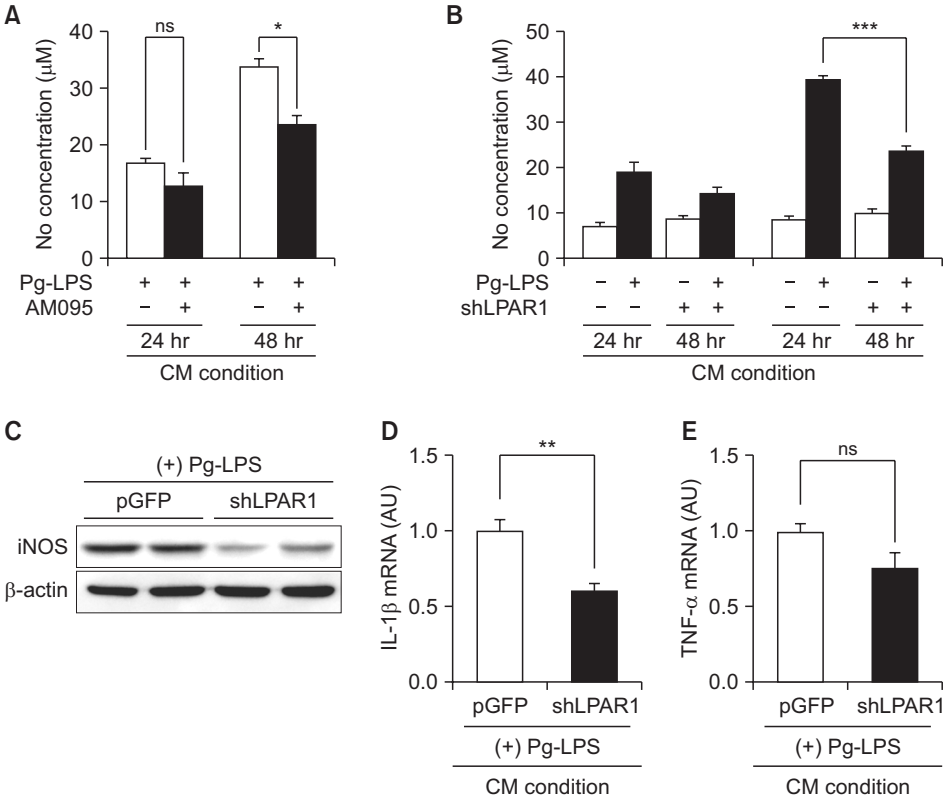

Fig. 3. Effect of lysophosphatidic acid receptor 1 (LPAR1) inhibition in linking periodontitis and neuroinflammation. (A, B) Nitric oxide (NO) production in primary astrocytes after treatment of conditioned medium (CM) from periodontal ligament stem cells (PDLSCs) with LPAR1 inhibition by AM095 (A) or LPAR1 shRNA (B) is shown. (C) Inducible nitric oxide synthase (iNOS) expression in astrocytes was analyzed by immunoblotting. β-actin expression is shown as the control. (D, E) The relative expression of pro-inflammatory cytokines was analyzed by quantitative real-time polymerase chain reaction. The results shown are the mean ± SEM.
Pg-LPS, *Porphyromonas gingivalis* lipopolysaccharide; IL-1β, interleukin 1 beta; TNF-α, tumor necrosis factor-α; AU, arbitrary unit; ns, non-significant.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

astrocyte through CM. PDLSCs pre-treated with Pg-LPS or vehicle control were subjected to LPAR1 inhibition by AM095 inhibitor or shLPAR1 knockdown as shown in Fig. 2. When CMs were applied to primary astrocytes, Pg-LPS-pre-treated CM showed the elevated NO production. LPAR1-inhibited CM showed the meaningful reduction in NO production at 48 hours after CM application in comparison with control CM (Fig. 3A and 3B). Western blot analysis of astrocytes showed the decreased expression of iNOS in LPAR1-knockdown CM in comparison with the control CM (Fig. 3C). Real-time PCR analysis of astrocytes showed a significant decrease in the expression of IL-1β whereas the reduction in TNF-α expression was insignificant (Fig. 3D and 3E). These results suggest that LPAR1 inhibition in PDLSCs can decrease the inflammatory responses of astrocytes in Pg-LPS-exposed condition.

Discussion

LPA receptors (LPAR1-6) has been reported to be expressed in various tissues including lung, heart, testis, spleen, and brain. Several studies have suggested that LPA and LPARs are involved in various diseases, including cancer, fibrosis, arthritis and neuropathic pain [15-18]. The involvement of LPA in neurogenesis suggests the active LPA signaling in neural progenitors, neurons, astrocytes, microglia, and oligodendrocytes in the brain [19-21]. The expression of LPAR1-3 were detected in the human brain, in which LPAR1 in astrocytes and LPAR2 in the ependymal cells of the lateral ventricle [22]. Recent reports are revealing the function of LPAR in stem cells including PDLSCs, which are neural crest derivatives and hold the potential as therapeutic targets in dental disease including periodontitis [23].

In the previous study, we reported that LPAR1 regulated the osteogenic differentiation of PDLSCs in periodontitis-mimicking inflammatory condition [10], and in the current study, we showed the role of LPAR1 in relaying periodontitis and neuroinflammation by utilizing Pg-LPS-pre-treated PDLSC CM. The intervention of LPA1 activity on PDLSC remarkably reduced the inflammatory responses in primary astrocyte. These results suggested that LPAR1 is an important factor linking periodontitis and neuroinflammation. Recently studies suggested that the neuroinflammation could be contributed from peripheral infections such as periodontitis. Among periodontitis-inducing pathogens, *P. gingivalis* and its secreted substance LPS was shown to change the permeability of blood brain barrier [24,25].

Taken together, our findings suggest a novel contribution of LPAR1 in the progression of neuroinflammation initiated by periodontitis-mimicking condition. These results indicate that

LPA1 in PDLSCs can be a target for the therapeutic intervention in breaking the link between periodontitis and neurodegenerative disease.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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