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Lipopolysaccharides affect compressed periodontal ligament cells via Eph–ephrin signaling

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Abstract

Objectives: The aim of this study is to investigate the underlying mechanism of the recovery of periodontal ligament cells (PDLCs) sequentially exposed to inflammation and mechanical loading.

Materials and Methods: We divided PDLCs into four groups: control; compressive force (CF) alone (2.0 g/cm^2); lipopolysaccharides (LPS) pretreatment (0.1 μg/ml) followed by simultaneous LPS and CF stimulation, simulating uncontrolled periodontitis; and LPS pretreatment followed by CF exposure, simulating controlled periodontitis. The expression of EphB4-ephrinB2 and EphA2-ephrinA2, and the level of osteoclastogenesis and osteogenesis were evaluated.

Results: Simultaneous stimulation by LPS and CF, compared with CF alone and sequential LPS and CF exposure, significantly suppressed EphB4 and enhanced ephrinA2 expression. Similarly, the most intense osteoclastic differentiation was observed under simultaneous LPS and CF stimulation, while sequential exposure to LPS and CF only slightly increased osteoclastic cell numbers. Both the activation of EphB4 signaling and ephrinA2 silencing lowered osteoclastic differentiation, which had previously been upregulated by simultaneous LPS and CF stimulation. These treatments also increased osteogenic differentiation.

Conclusions: Simultaneous LPS and CF stimulation critically enhances osteoclastogenesis in PDLCs through the suppression of EphB4 and the induction of ephrinA2 signaling. Sequential LPS and CF exposure partially abolishes the osteolytic effects of simultaneous stimulation.

KEYWORDS

compressive force, Eph–ephrin, inflammation, periodontal ligament cells

1 | INTRODUCTION

Orthodontic tooth movement is achieved by coordinated alveolar bone remodeling. Bone resorption on the compression side is accompanied by bone formation on the tension side. Tooth movement toward the area of pressure is the result of mechanical force-induced alveolar bone remodeling mediated by the periodontal ligament (PDL). The PDL is a unique tissue between the teeth and

the alveolar bone, and its cells are the first recipient of orthodontic force loading (Lekic & McCulloch, 1996). Healthy PDL cells (PDLCs) that reside in the compression area tend to express elevated levels of osteoclastogenic genes, such as the receptor activator of nuclear factor κB ligand (RANKL), macrophage colony-stimulating factor (M-CSF) and tumor necrosis factor (TNF)-α (Kim et al., 2013; Yoshino et al., 2014), and reduced levels of osteogenic genes, including those for alkaline phosphatase (ALP) and Runt-related transcription factor 2 (Runx2) (Y. H. Lee et al., 2007). Thus, compression leads to "physiological" bone resorption. However, the periodontitis-induced pathological destruction of tooth-supporting alveolar bone by lipopolysaccharide (LPS), a crucial pathogen-associated molecule, is potentially an irreversible process that ultimately leads to tooth loss (Darveau, 2009). If periodontal inflammation is not kept under control, orthodontic treatment may aggravate the periodontal problem (Boas Nogueira et al., 2013). The prevalence of destructive periodontal inflammatory diseases is higher in adult patients than in young patients who seek orthodontic treatment (Gkantidis et al., 2010). In patients with active periodontitis, a further breakdown of alveolar bone may occur as a consequence of the concurrent application of orthodontic force. Therefore, orthodontic therapy should be performed only after proper treatment for active periodontitis and successful infection control. However, whether PDLCs behave differently in response to orthodontic force with and without inflammation control is still unclear. Furthermore, even if inflammation has been well controlled, PDLCs may not behave in the same way as healthy cells without previous exposure to inflammatory conditions; this also remains to be determined.

Erythropoietin-producing human hepatocellular receptor (Eph) tyrosine kinases are a large family of transmembrane proteins with an extracellular globular domain that enables them to bind with the N-terminal sites of ephrin ligands (Egea & Klein, 2007; Kullander & Klein, 2002). The combination of Eph and ephrin activates the single cytoplasmic kinase region of the Eph and subsequently transduces bidirectional signals into both receptor- and ligand-expressing cells. Forward signal transduction through Ephs and reverse signaling via ephrins triggers a number of downstream cascades that regulate bioprocesses in cells and tissues. The EphA2-ephrinA2 and EphB4-ephrinB2 systems have been reported to be important mediators of bone cell communication and skeletal development, and their role in the coupling of osteoclasts and osteoblasts has been well studied (Matsuo & Otaki, 2012). The interaction between ephrinA2 and EphA2 generally promotes osteoclastic differentiation and suppresses osteoblastic differentiation, whereas that between ephrinB2 and EphB4 does the opposite, and thus benefits bone formation. Applying compressive force (CF) to PDLCs has been shown to enhance EphA2-ephrinA2 signaling but suppress EphB4-ephrinB2 signaling, which suggests that these two pairs of molecules participate in orthodontic bone modeling (Diercke et al., 2011).

Although administering periodontal inflammation control before orthodontic tooth movement is strongly recommended, the underlying mechanism of PDLC recovery after previous exposure to inflammatory stimuli and its reaction to mechanical loading are still uncertain. In addition, the involvement of Eph–ephrin signaling in inflammation-associated mechanically loaded PDLCs has received little attention. Therefore, this study aimed to elucidate the influence of the differential effects of simultaneous versus sequential LPS and CF exposure on the expression of Eph–ephrin signaling in PDLCs.

2 | MATERIALS AND METHODS

Please see the Appendix S1 for details of the materials and methods.

2.1 | Experimental design

The cells were divided into the following four subgroups:

1. Single application of CF: after reaching 80% confluence, the PDLCs were grown for another 24 hr in α-Minimum Essential Medium, and then exposed to CF (2.0 g/cm^2).
2. Simultaneous LPS and CF stimulation: the PDLCs were treated with *Porphyromonas gingivalis* (*P. gingivalis*) LPS (0.1 μg/ml) for 24 hr, followed by simultaneous stimulation of CF and *P. gingivalis* LPS, which simulated the uncontrolled inflammation.
3. Sequential LPS and CF exposure: the PDLCs were treated with *P. gingivalis* LPS (0.1 μg/ml) for 24 hr followed by treatment with CF in the absence of *P. gingivalis* LPS, which simulated the cell status after inflammation control.
4. No intervention control group.

3 | RESULTS

3.1 | Effects of LPS and CF on Eph–ephrin signaling

To obtain the optimal duration of mechanical compression for the induction of Eph–ephrin expression, we compressed the PDLCs for 1, 4, 8, and 24 hr, and the results of a subsequent experiment indicated that the most appropriate force-loading duration was 4 hr (Figure S1) (Appendix S1).

The mRNA level of ephrinB2 decreased significantly with all three treatments, and its values showed no significant inter-group differences (Figure 1a; Table 1). But the expression of ephrinB2 protein only decreased in simultaneous and sequential exposure to LPS and CF (Figure 1e; Table 2). In contrast, the mRNA and protein levels of EphB4 were lowest under simultaneous LPS and mechanical CF stimulation, compared with CF loading alone and sequential LPS and CF exposure (Figure 1b,e; Table 1,2). These data suggest that EphB4-ephrinB2 signaling is severely hampered especially by simultaneous LPS and CF stimulation, but significantly restored under sequential exposure to LPS and CF.

The mRNA level of ephrinA2 was only marginally elevated after treatment with CF for 4 hr, but its protein level was slightly enhanced

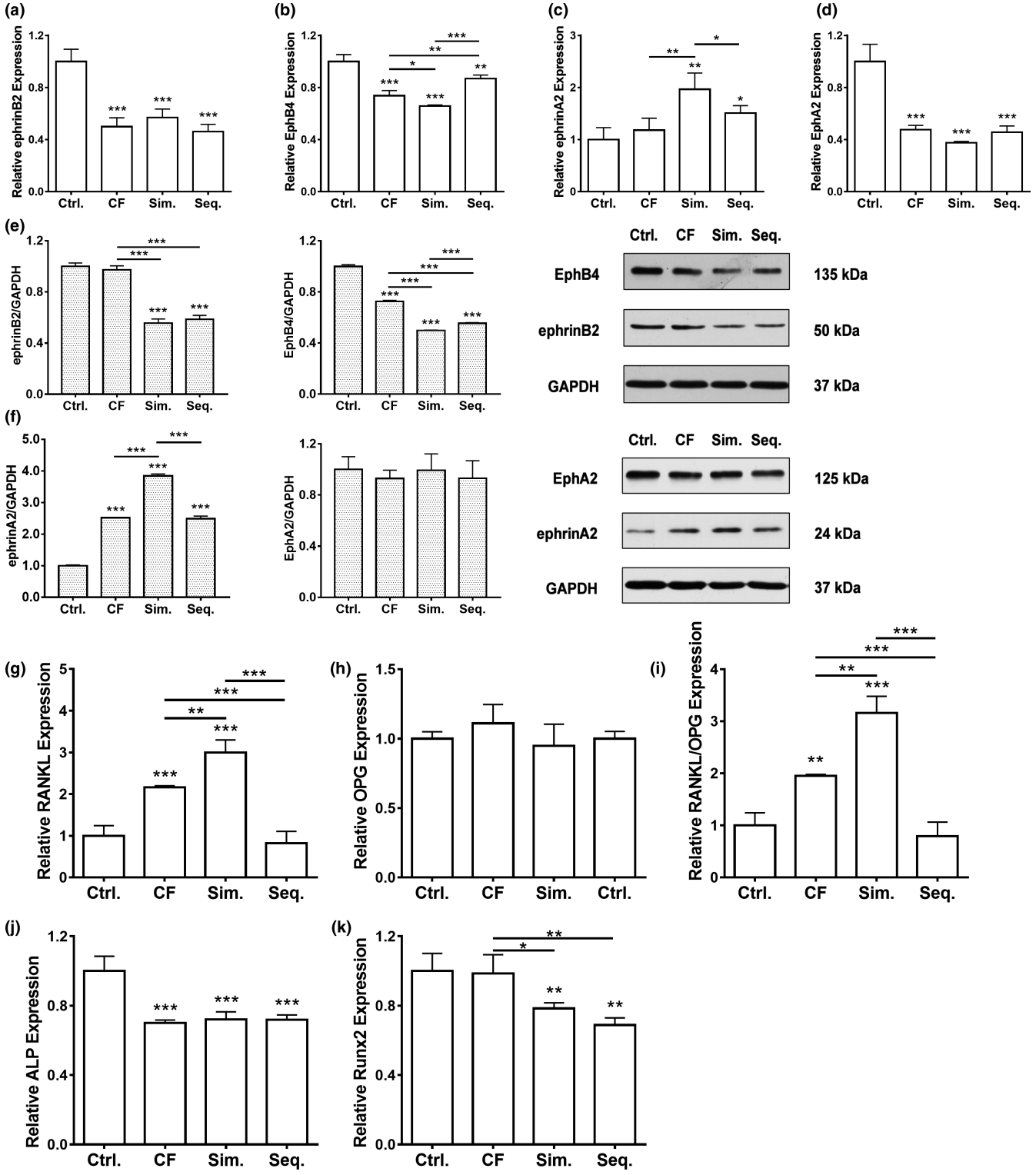

FIGURE 1 Effects of CF and LPS on expression of Eph–ephrin and osteoclastogenic and osteogenic genes. PDLCs were subjected to CF, simultaneous LPS and CF stimulation (Sim.), and sequential LPS and CF exposure (Seq.), respectively. The expression of EphB4, ephrinB2, EphA2, and ephrinA2 was determined at the mRNA level (a, b, c, d) by qRT-PCR and at the protein level (E, F) by Western blot. The mRNA expression of RANKL, OPG (g–i), ALP (j), and Runx2 (k) was also evaluated. Data are presented as means ± SDs. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ indicate a significant difference

(Figure 1c,f; Table 1,2). The expression of ephrinA2 was further up-regulated by simultaneous treatment with LPS and CF, but was only partly elevated after sequential LPS and CF exposure. In contrast, EphA2 mRNA expression decreased significantly with all three treatments, although the changes in protein level were not significant (Figure 1d,f; Table 1,2).

TABLE 1 Effects of CF and LPS on mRNA expression of Eph–ephrin and osteoclastogenic and osteogenic genes

	Control ($n = 3$)	CF ($n = 3$)	Sim. LPS and CF ($n = 3$)	Seq. LPS and CF ($n = 3$)
Ephrinb2	1.0 ± 0.09	0.5 ± 0.07	0.57 ± 0.07	0.46 ± 0.06
EphB4	1.0 ± 0.05	0.75 ± 0.05	0.66 ± 0.01	0.87 ± 0.03
Ephrina2	1.0 ± 0.23	1.18 ± 0.23	1.96 ± 0.31	1.51 ± 0.14
EphA2	1.0 ± 0.13	0.48 ± 0.03	0.38 ± 0.01	0.46 ± 0.05
RANKL	1.0 ± 0.24	2.16 ± 0.03	2.96 ± 0.31	0.82 ± 0.27
OPG	1.0 ± 0.05	1.11 ± 0.14	0.95 ± 0.16	1.04 ± 0.15
RANKL/OPG	1.0 ± 0.24	1.95 ± 0.03	3.16 ± 0.32	0.79 ± 0.27
ALP	1.0 ± 0.08	0.71 ± 0.02	0.72 ± 0.04	0.72 ± 0.03
Runx2	1.0 ± 0.1	0.99 ± 0.11	0.78 ± 0.03	0.7 ± 0.05

Note: Values are means ± *SD* (relative expression). Statistical significance was analyzed by ANOVA with Bonferroni correction.

TABLE 2 Effects of CF and LPS on protein expression of Eph–ephrin signaling

	Control ($n = 3$)	CF ($n = 3$)	Sim. LPS and CF ($n = 3$)	Seq. LPS and CF ($n = 3$)
Ephrinb2	1.0 ± 0.03	0.97 ± 0.03	0.56 ± 0.03	0.59 ± 0.03
EphB4	1.0 ± 0.01	0.73 ± 0.01	0.5 ± 0.003	0.55 ± 0.01
Ephrina2	1.0 ± 0.02	2.52 ± 0.001	3.84 ± 0.06	2.49 ± 0.08
EphA2	1.0 ± 0.1	0.93 ± 0.06	0.99 ± 0.13	0.93 ± 0.14

Note: Values are means ± *SD* (relative expression). Statistical significance was analyzed by ANOVA with Bonferroni correction.

3.2 | Effects of LPS and CF on the expression of osteoclastogenic and osteogenic genes

The RANKL/osteoprotegerin (OPG) system, a decisive osteoclastogenesis-regulating signaling system, was evaluated in the PDLCs. As expected, the RANKL/OPG mRNA ratio was elevated by CF and further increased under simultaneous stimulation with LPS and CF (Figure 1g–l; Table 1). Remarkably, the RANKL/OPG mRNA ratio remained at its basal level under sequential LPS and CF exposure.

We also measured the mRNA expression of two osteogenic genes and discovered that ALP expression decreased under all circumstances (Figure 1j; Table 1), while Runx2 expression was suppressed only with the involvement of LPS, whether simultaneously or sequentially applied, with no significant difference between the two groups (Figure 1k; Table 1).

These results suggest that bone metabolism related molecules are differentially expressed under simultaneous versus sequential exposure to LPS and CF.

3.3 | Effects of CF and LPS on the differentiation of tartrate-resistant acid phosphatase (TRAP) positive cells in co-culture with PDLCs

Compared with the control treatment, co-culturing RAW264.7 cells with mechanically compressed PDLCs resulted in the more pronounced formation of osteoclast-like TRAP-positive cells. In addition, simultaneous LPS and CF stimulation amplified the formation of multinuclear cells compared with that under mechanical treatment alone. The number of TRAP-positive cells was slightly lower in the sequential LPS and CF exposure group than in the simultaneous LPS and CF group (Figure 2; Table 3). These data suggest that compared with simultaneous treatment of LPS and CF, the sequential application of LPS and CF to PDLCs, which simulates well-controlled inflammation, decreases the formation of osteoclastic cells.

3.4 | EphrinB2-Fc-mediated gene expression and osteoblastic differentiation in PDLCs under simultaneous LPS and CF stimulation

To explore the role of EphB4 in PDLCs under concomitant LPS and CF stimulation, we used recombinant ephrinB2-Fc to activate EphB4 signaling in PDLCs. Simultaneous LPS and CF stimulation upregulated RANKL expression in PDLCs, but this was partially inhibited by the preaddition of 4 μg/ml ephrinB2-Fc chimeras (Figure 3a; Table 4). As no obvious differences in OPG level were observed among the groups (Figure 3b; Table 4), the trend in the RANKL/OPG ratio was similar to that of RANKL (Figure 3c; Table 4). The mRNA levels of the osteogenic genes ALP and Runx2 were repressed by challenges from inflammatory and mechanical factors. Under ephrinB2-Fc stimulation, ALP expression returned to the control level (Figure 3d; Table 4). However, ephrinB2-Fc did not significantly change Runx2 expression compared with that under simultaneous LPS and CF stimulation (Figure 3e; Table 4).

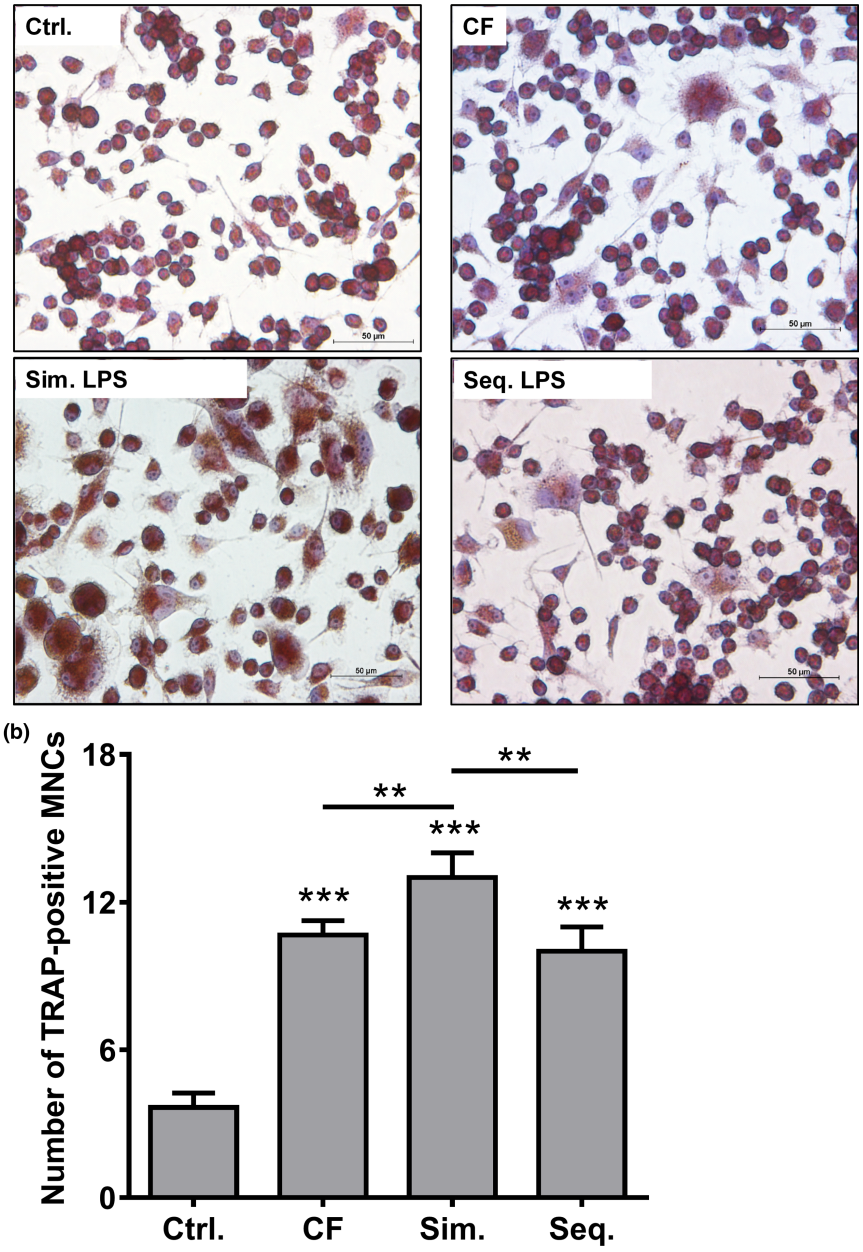

FIGURE 2 (a) Formation of TRAP-positive multinucleated cells in a co-culture of RAW264.7 cells with PDLCs for 5 d (magnification, × 40; bar = 50 μm). The PDLCs were pretreated with LPS and/or CF, then co-cultured with RAW264.7 cells. The differentiation of TRAP-positive osteoclastic cells was slightly lower under sequential LPS and CF exposure (Seq.) than under simultaneous LPS and CF stimulation (Sim.). (b) Multinucleated osteoclast-like cells (with three or more nuclei) in six random areas in each well were counted under a microscope. Data are presented as means ± SDs. $^{**}p < .01$ and $^{***}p < .001$ indicate a significant difference

	Control ($n = 3$)	CF ($n = 3$)	Sim. LPS and CF ($n = 3$)	Seq. LPS and CF ($n = 3$)
TRAP-positive MNCs	3.67 ± 0.58	10.67 ± 0.58	13.0 ± 1.0	10.0 ± 1.0.0

Note: Values are means ± *SD* (number of the cells). Statistical significance was analyzed by ANOVA with Bonferroni correction.

TABLE 3 Effects of CF and LPS on formation of TRAP-positive multinucleated cells in a co-culture of RAW264.7 cells with PDLCs

Based on our studies of the effects of ephrinB2-Fc on the key genes responsible for bone remodeling, we next sought to determine the function of EphB4-dependent signaling on the mineralization of PDLCs. After 14 d of treatment (2 and 4 μg/ml ephrinB2-Fc), Alizarin Red S staining revealed that the activation of EphB4 signaling by ephrinB2 protein significantly increased mineralized nodules. In addition, 4 μg/ml compared with 2 μg/ml of ephrinB2-Fc caused more profound osteoblastic differentiation in PDLCs (Figure 3f; Table 5). These data suggest that the activation of EphB4 signaling restores the osteogenic activities inhibited by simultaneous LPS and CF stimulation.

3.5 | Effects of ephrinA2 knockdown on PDLCs under simultaneous LPS and CF stimulation

To test the capacity of small interfering RNA (siRNA) to knock down ephrinA2 expression in PDLCs, we used lipofectamine to transfect

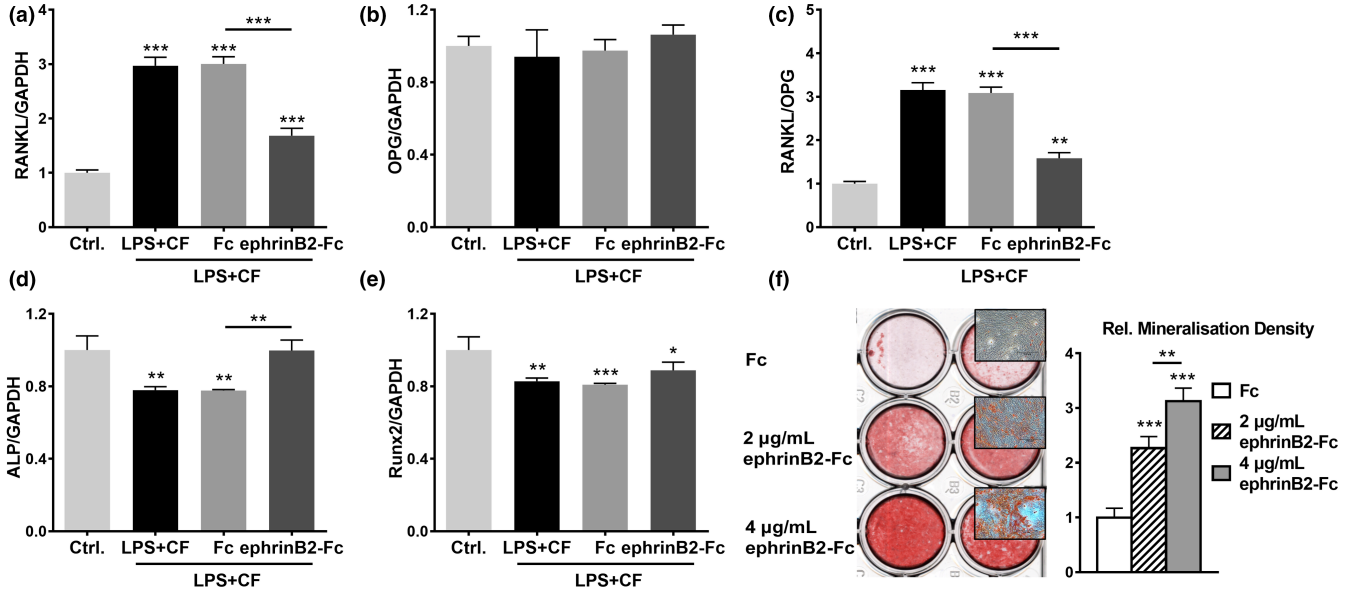

FIGURE 3 (a–e) The ephrinB2-Fc-mediated mRNA expression of osteoclastogenic and osteogenic genes in PDLCs under simultaneous LPS and CF stimulation. The expression of RANKL/OPG, ALP, and Runx2 was measured by qRT-PCR. (f) Osteoblastogenesis of PDLCs. The formation of mineralized nodules in the cell cultures was analyzed after 14 d of treatment with ephrinB2-Fc. The relative intensity of Alizarin Red S staining was measured using ImageJ software. Magnified views of the mineralized nodules are shown in the upper right of the lower images (magnification, × 4; bar = 500 μm). Data are presented as means ± SDs. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ indicate a significant difference

TABLE 4 The ephrinB2-Fc-mediated mRNA expression of osteoclastogenic and osteogenic genes in PDLCs under simultaneous LPS and CF stimulation

	Control ($n = 3$)	LPS+CF ($n = 3$)	Fc ($n = 3$)	ephrinB2-Fc ($n = 3$)
RANKL	1.0 ± 0.05	2.97 ± 0.16	3.0 ± 0.13	1.68 ± 0.14
OPG	1.0 ± 0.05	0.94 ± 0.15	0.97 ± 0.06	1.06 ± 0.05
RANKL/OPG	1.0 ± 0.05	3.16 ± 0.17	3.08 ± 0.14	1.58 ± 0.13
ALP	1.0 ± 0.08	0.78 ± 0.02	0.78 ± 0.01	1.0 ± 0.06
Runx2	1.0 ± 0.07	0.83 ± 0.02	0.81 ± 0.01	0.89 ± 0.04

Note: Values are means ± *SD* (relative expression). Statistical significance was analyzed by ANOVA with Bonferroni correction.

TABLE 5 Effects of ephrinB2-Fc on formation of mineralized nodules in PDLCs

	Control ($n = 3$)	2 μg/ml ephrinB2-Fc ($n = 3$)	4 μg/ml ephrinB2-Fc ($n = 3$)
Relative Mineralization Density	1.0 ± 0.17	2.27 ± 0.21	3.13 ± 0.24

Note: Values are means ± *SD*. Statistical significance was analyzed by ANOVA with Bonferroni correction.

siRNA to the cells and monitored ephrinA2 attenuation at the transcriptional level using the quantitative real-time polymerase chain reaction (qRT-PCR). Compared with a scrambled siRNA negative control, siRNA-ephrinA2 administration led to weakened ephrinA2 expression after 8 hr, and this effect remained significant up to 72 hr (Figure 4a; Table 6).

The cell viability of PDLCs treated with siRNA-scrambled and siRNA-ephrinA2 was measured using Cell Counting Kit-8 (CCK-8) assay. Figure 4b indicated that cell viability was unaltered after 8-hr treatment with siRNAs, which certified that siRNAs did not produce cytotoxic effects to PDLCs and that the osteogenesis of PDLCs were not affected by siRNAs in the designated time of treatment.

The qRT-PCR also revealed that RANKL synthesis was induced in PDLCs treated simultaneously with LPS and CF and then completely downregulated by the knockdown of ephrinA2 expression (Figure 4c; Table 6). OPG expression was unaffected by both co-treatment and siRNA transfection (Figure 4d; Table 6), and the RANKL/OPG ratio was decreased by ephrinA2 silencing (Figure 4e; Table 6). The CF- and LPS-dependent reduction of ALP and Runx2 was partly promoted by siRNA-mediated ephrinA2

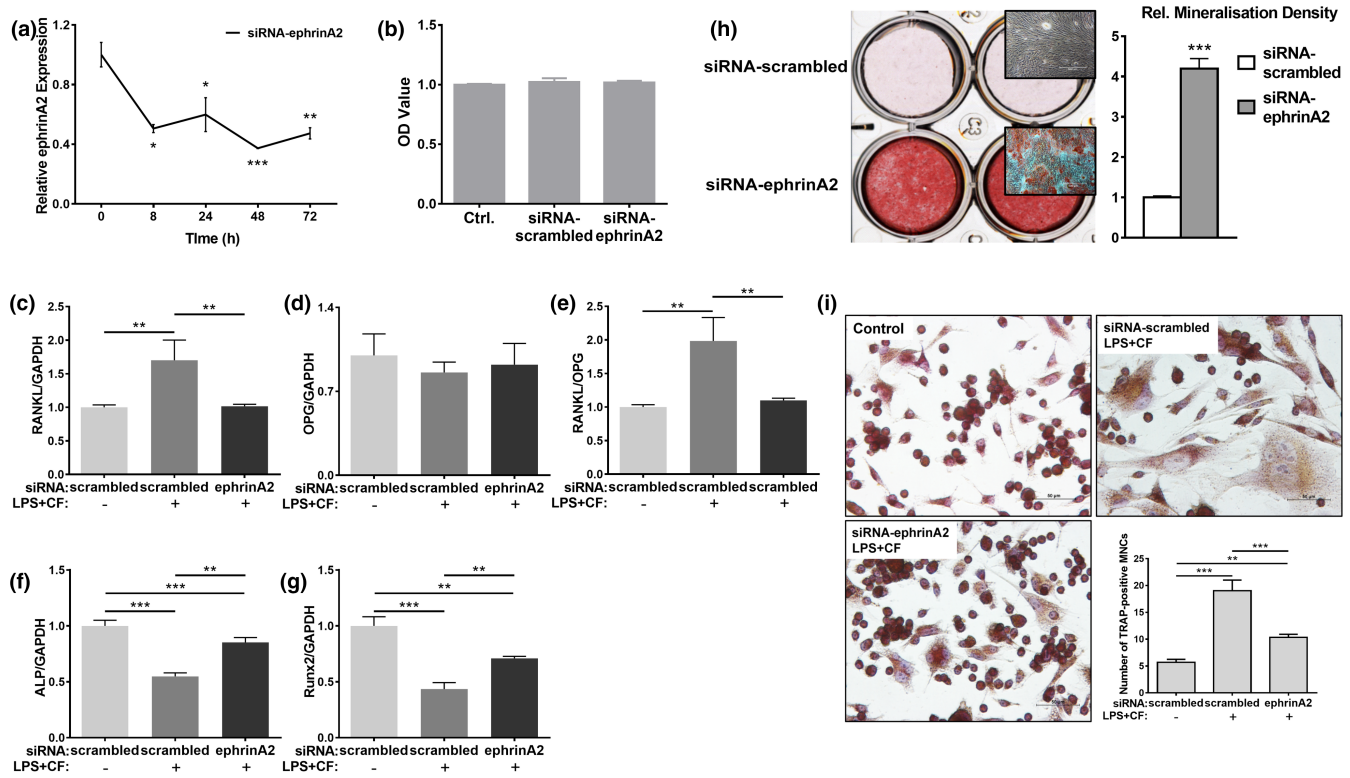

FIGURE 4 (a) PDLCs transfected with siRNA-mediated ephrinA2 knockdown. The mRNA expression of ephrinA2 was attenuated between 8 and 72 hr. (B) Effects of siRNAs on cell viability of PDLCs detected by CCK-8. (c–g) siRNA-ephrinA2 transfection-mediated mRNA expression of osteoclastogenic and osteogenic genes in PDLCs stimulated simultaneously by LPS and CF (LPS + CF). The expression of RANKL, OPG, ALP, and Runx2 was measured by qRT-PCR. (h) Osteoblastogenesis of PDLCs. The formation of mineralized nodules in the cell cultures was analyzed after 14 d of treatment with siRNA-ephrinA2. The relative intensity of Alizarin Red S staining was measured using ImageJ software. Magnified views of the mineralized nodules are shown in the upper right of the lower images (magnification, × 4; bar = 500 μm). (i) Differentiation of TRAP-positive osteoclast-like cells in co-culture of RAW264.7 cells and PDLCs subjected to simultaneous LPS and CF stimulation (magnification, × 40; bar = 50 μm). The number of multinucleated cells with three or more nuclei in six random areas per well was counted under a microscope. Data are presented as means ± SDs. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ indicate a significant difference

	siRNA-scrambled ($n = 3$)	siRNA-scrambled + LPS + CF ($n = 3$)	siRNA-ephrinA2 + LPS + CF ($n = 3$)
RANKL	1.0 ± 0.04	1.7 ± 0.29	1.02 ± 0.03
OPG	1.0 ± 0.18	0.86 ± 0.09	0.92 ± 0.18
RANKL/OPG	1.0 ± 0.04	1.98 ± 0.35	1.1 ± 0.03
ALP	1.0 ± 0.05	0.55 ± 0.03	0.85 ± 0.04
Runx2	1.0 ± 0.08	0.44 ± 0.06	0.71 ± 0.02

Note: Values are means ± *SD* (relative expression). Statistical significance was analyzed by ANOVA with Bonferroni correction.

TABLE 6 siRNA-ephrinA2 transfection-mediated mRNA expression of osteoclastogenic and osteogenic genes in PDLCs stimulated simultaneously by LPS and CF

silencing (Figure 4f,G; Table 6). In addition, the density of mineralization was higher in siRNA-ephrinA2 transfected than in non-transfected PDLCs (Figure 4h; Table 7). These data suggest that ephrinA2 attenuation improved the uncontrolled inflammation-weakened osteoinduction of PDLCs.

We analyzed the ephrinA2 signaling in more detail to obtain further insight into its regulation of osteoclast differentiation under LPS and CF environment. The formation of osteoclastic multinucleated cells was observed in the co-culture of RAW264.7 cells and PDLCs stimulated simultaneously with LPS and CF. Silencing ephrinA2 expression in stimulated PDLCs co-cultivated with RAW264.7 cells resulted in significantly reduced differentiation of osteoclasts compared with that in scrambled siRNA-transfected cells (Figure 4i; Table 8). These data suggest that the upregulated formation of TRAP-positive cells resulting from uncontrolled inflammation can be reduced by attenuating of ephrinA2 signaling.

4 | DISCUSSION

Periodontitis is an inflammatory disease with its most common oral manifestation seen as the alveolar bone destruction. Among the most crucial periodontopathogens are LPS from most gram-negative species including *P. gingivalis*. LPS, as the principal component of gram-negative bacteria, can activates the innate immune system by triggering toll-like receptors (TLRs).(Silva et al., 2015) A significantly immune and inflammatory response to the pathogens leads to the destruction of periodontium and alveolar bone.(Silva et al., 2015) From the micro-level speaking, previous study(Kato et al., 2014) has shown that *P. gingivalis* LPS promotes the production of inflammatory cytokines including interleukin (IL)-1b, IL-6 and IL-8 and inhibits osteoblastic differentiation of periodontal ligament stem cells. When stimulated with LPS and IL-1b, PDLCs express RANKL that is related to the regulation of bone resorption.(Wada et al., 2004) Therefore, LPS is regarded to be able to effectively mimic the in vitro microenvironment of periodontitis (Krajewski et al., 2009; Thammasitboon et al., 2006) and thus is used in our study which aimed to investigate the regulation of osteoclastogenesis in inflammation environment.

Force applied to the tooth is transmitted to the alveolar bone by PDL tissue, within which the dominant cells are PDLCs. In this study, we first confirmed that EphB4-ephrinB2 expression in PDLCs in a time-dependent manner decreased in response to CF (Figure S1b). As it is well-documented that CF induces bone resorption, these findings suggest that EphB4-ephrinB2 signaling has an anti-osteoclastogenic role in this process (see appendix). The increase in ephrinA2 mRNA expression, contributes to CF-dependent osteoclastogenesis, at an early stage (1 hr) (Figure S1a) was verified at the protein level, which showed an upward trend at 4 hr. Diercke et al. (Diercke, Kohl, et al., 2011) reported that the expression of EphB4-ephrinB2 and EphA2-ephrinA2 signaling was altered in response to CF loading from 2 to 4 hr. Another study showed that CF suppresses both EphB4 and ephrinB2 expression in osteoblast precursors up to 2 hr. (Hou et al., 2014) These results with short-term loading are coincident with our findings. The longest duration of CF application in previous research explored Eph–ephrin signaling is 6 hr that presented an increase of EphA2-ephrinA2 and decrease of EphB4-ephrinB2 expression, which further attenuated osteogenesis (Diercke, Kohl, et al., 2011). The RANKL/OPG ratio was elevated under CF after 4 hr (Figure S1c), which is consistent with the earlier finding that CF enhances bone destruction through the modulation of RANKL/OPG ratio (Jin et al., 2015; Nettelhoff et al., 2016). Collectively, the PDLCs that underwent CF for 4 hr demonstrated the most dynamic changes in target gene expression; therefore, we selected a 4 hr duration for the subsequent experiment. The RANKL/OPG ratio declined dramatically after 24 hr (Figure S1c), perhaps because the PDLCs became insensitive to the continuing mechanical stimulus or because the durable force decreased cell viability (Goga et al., 2006). More intriguingly, under long-term mechanical stimulation (24 hr), as the expression of EphA2-ephrinA2 was decreased, the major factor responsible for the suppression of bone-forming activities may be the reduction in EphB4-ephrinB2 signaling (Figure S1a,b). It is reported that the co-stimulation of LPS and long-term CF (5 days) to PDLCs displayed an additional upregulation of RANKL expression compared to compression alone (Jia et al., 2020), which is similar to our results using 4 hr mechanical loading. Though the force-loading models are different, it sheds light on the enhancement of osteoclastogenesis in LPS and CF exposure. The effects of Eph–ephrin signaling on PDLCs with longer period of CF loading are worth researching closer in the future.

Although substantial clinical evidence of the microbiological response to periodontitis treatment has been obtained (Hung & Douglass, 2002; Deas & Mealey, 2010), and periodontal therapy has been shown to significantly reduce the number of inflammatory cytokines in gingival crevicular fluid (GCF) (Kinney et al., 2014; de Lima Oliveira et al., 2012), cellular and molecular modifications occurring after inflammation control remain largely unknown. To clarify this mechanism, our study established PDLC cultures under simultaneous or sequential LPS and CF exposure, simulating different environments of periodontal inflammation. Our data showed that the RANKL/OPG ratio was higher under co-stimulation by CF and LPS than under mechanical stimulation alone, consistent with the results of other studies (Nokhbehsaim et al., 2010; Romer et al., 2013; Yamamoto et al., 2011). In our earlier research (Li et al., 2017), we observed upregulated ephrinA2 and downregulated EphB4 expression in PDLCs treated with LPS, suggestive of the inflammation-related dysfunction of bone remodeling via ephrinA2 and EphB4 signaling. In the current study, the compressed PDLCs with a simultaneous inflammatory stressor showed a reduction in EphB4 expression and an upregulation of ephrinA2 expression stronger than those produced by CF alone. Interestingly, however, the partial recovery of these changes to the control level was observed in the non-inflammatory environment of the sequential LPS and CF exposure group. In

TABLE 7 Effects of siRNA-ephrinA2 on formation of mineralized nodules in PDLCs

	siRNA-scrambles ($n = 3$)	siRNA-ephrinA2 ($n = 3$)
Relative Mineralization Density	1.0 ± 0.04	4.2 ± 0.25

Note: Values are means ± *SD*. Statistical significance was analyzed by student's *t* test.

TABLE 8 siRNA-ephrinA2 transfection-mediated formation of TRAP-positive multinucleated cells in a co-culture of RAW264.7 cells with PDLCs stimulated simultaneously by LPS and CF

	siRNA-scrambled ($n = 3$)	siRNA-scrambled + LPS + CF ($n = 3$)	siRNA-ephrinA2 + LPS + CF ($n = 3$)
TRAP-positive MNCs	5.67 ± 0.58	19.0 ± 2.0	10.33 ± 0.58

Note: Values are means ± *SD* (number of the cells). Statistical significance was analyzed by ANOVA with Bonferroni correction.

addition, the mRNA expression of EphB4 was even higher under sequential LPS and CF exposure than under the single stimulus of CF. Similarly, the RANKL/OPG ratio was even lower under sequential treatment than under CF application alone. These discoveries indicate that although sequential LPS and CF exposure may balance osteogenesis and osteoclastogenesis, PDLCs previously exposed to LPS might exhibit a dulled reaction to CF loading.

The mRNA is formed by means of transcription, while the process by which mRNA directs protein synthesis is called translation. RANKL, a ligand for the receptor RANK, directly stimulates osteoclast maturation. (Theoleyre et al., 2004) Functional assessment is needed to examine the behavior of corresponding protein even though the protein expression of RANKL/OPG was not specifically evaluated. Therefore, co-culture of PDLCs and RAW264.7 cells was performed to assess the formation of multinuclear osteoclastic cells. The co-culture system used in the present study is based on previous research.(Jin et al., 2015; Mayahara et al., 2012; Romer et al., 2013) Human PDLCs express RANKL that is able to stimulate RAW264.7 cells from murine origin differentiate into osteoclasts, and it might be because human and mouse RANKL share 85% in their amino acid sequences.(Ono et al., 2020) Besides, Lossdörfer et al. (Lossdorfer et al., 2011) had proved that RAW264.7 responded robustly to RANKL from human PDLCs in the co-culture system. In our research, osteoclastogenesis in a co-culture of RAW264.7 cells and pretreated PDLCs had revealed the difference in gene expression between the simultaneous and sequential LPS and CF exposure groups. Indeed, prominent osteoclast formation under simultaneous LPS and CF stimulation was partially suppressed under sequential inflammatory and CF exposure. The results were mostly in accordance with the expression of EphB4 and ephrinA2 signaling and osteoclastogenic genes, with slightly inter-group difference, possibly due to the lag between transcription and protein translation. Consequently, it appears that both forward signaling via EphB4 and reverse signaling via ephrinA2 contribute not only to the synergistic detrimental effect of CF and LPS on osteoclastogenesis, but also to cellular recovery from inflammatory stimuli. These findings are in line with the clinical observation that the combination of orthodontic force and uncontrolled periodontitis exacerbates pathological bone loss. (Boas Nogueira et al., 2013) It is also coincident with the fact that periodontal therapy (inflammation control) reduces the expression of osteoclastic factors in GCF in patients with periodontitis (Romano et al., 2018). The inflammatory influence of LPS enhances osteoclastic responses and weakens osteogenic reactions beyond those induced by CF alone. Therefore, periodontal therapy before orthodontic treatment is necessary to bring active inflammation under control. But it is interesting that the molecular expressions in sequential exposure to LPS and CF are different from that in CF application alone, and the mechanism behind inflammation control deserves further investigation.

However, there was no significant difference in the expression of osteogenic genes (ALP and Runx2) between simultaneous and sequential exposure to LPS and CF. It might be because the osteoclastogenic differentiation plays a dominant role during inflammation control. Notably, under single CF loading, the ALP expression was declined, while the Runx2 expression remained unchanged. The inconsistency of the expression of ALP and Runx2 affected by CF was also reported by Lee et al. (S. Y. Lee et al., 2015) ALP is an early marker of cell differentiation, implying that compression may involve PDLCs differentiation. Besides, ALP has been reported as a putative downstream regulator target gene of Runx2, and Runx2 controls ALP activity in osteoblasts. (Jo et al., 2019) A hypothesis might be that reciprocal control of ALP and Runx2 in osteogenic differentiation might lead to the different expression between ALP and Runx2 under CF loading. Further experiments are needed to clarify this issue.

EphB4-ephrinB2 bidirectional communication has been shown to be required for bone remodeling. The blockage of this interaction by a peptide antagonist or recombinant extracellular domain of EphB4 (sEphB4) reduces the number of late-phase osteoblast differentiation markers in murine stromal cells and osteoblasts, but increases the presence of osteoclastogenic cytokines such as RANKL and IL-6 (Allan et al., 2008; Takyar et al., 2013). Therefore, EphB4-ephrinB2 interaction functions as an osteoclastogenesis inhibitor. In this study, simultaneous LPS and CF stimulation downregulated EphB4 in PDLCs, and EphB4 expression was increased by sequential LPS and CF exposure, suggesting that EphB4 protects the above-stated process of stimulation-induced bone resorption. Soluble EphB4-Fc has been reported to induce the phosphorylation of ephrinB2 ligands in PDL stem cells (Zhu et al., 2017). To determine whether EphB4 was causal for osteogenic activities in PDLCs, we used ephrinB2-Fc to activate forward signaling in a CF plus LPS environment. ALP expression downregulated by simultaneous LPS and CF stimulation recovered to baseline, providing evidence that ALP is a downstream effector of EphB4 signaling in PDLCs subjected to CF and LPS. The enhanced mineralization of PDLCs treated with ephrinB2-Fc further confirmed the induction of osteogenic marker gene expression. These findings are in parallel with the observation of ephrinB2-Fc-increased ALP activity in osteoblasts (Zhao et al., 2006) and ephrinB2-Fc-upregulated expression of osteogenic genes in static PDLCs (Diercke et al., 2011). The study thus verifies EphB4 forward signaling as a putative trigger of osteogenic activities in compressed PDLCs even under LPS-induced inflammation.

Unexpectedly, the RANKL/OPG ratio was also reduced by ephrinB2-Fc. According to previous studies (Vrahnas & Sims, 2015), ephrinB2 reverse signaling contributes to impaired osteoclast differentiation. The ephrinB2 ligand is expressed by both osteoblasts and osteoclasts; however, osteoclasts do not express the corresponding EphB4 receptor (Zhao et al., 2006). Apart from directly inducing ephrinB2-dependent suppression of osteoclastogenesis through reverse signaling, the findings of this study suggest that another mechanism of EphB4 forward signaling is to downregulate RANKL/OPG ratio in non-osteoclasts such as PDLCs and osteoblasts. Further research is needed to determine the relative importance of RANKL/OPG downregulation via EphB4 forward signaling in PDLCs and direct suppression via ephrinB2 reverse signaling in osteoclasts.

The communication mediated by ephrinA2 and EphA2 during bone remodeling is less well understood than EphB4-ephrinB2

communication. The first work to explain the role of EphA2-ephrinA2 bidirectional pathway in osteoblast–osteoclast interaction was published by Irie et al. (2009), which showed that ephrinA2 reverse signaling prompts osteoclast differentiation, while EphA2 forward signaling can both inhibit osteoblasts and stimulate osteoclast formation. We have demonstrated that simultaneous LPS and CF stimulation upregulates ephrinA2 expression and modifies the expression of various genes involved in bone homeostasis. Therefore, to elucidate the involvement of ephrinA2 reverse signaling in gene regulation during LPS stimulation in compressed PDLCs, we performed ephrinA2 gene silencing. The suppression of ephrinA2 mRNA in inflamed and compressed PDLCs reduced the RANKL/OPG ratio to basal level, suggesting that ephrinA2 signaling promotes LPS- and mechano-induced osteoclastogenesis through RANKL expression. A recent experiment in rats (Yan & Ye, 2015) showed that bone abnormalities induced by ovariectomy were accompanied by the markedly enhanced expression of RANKL/OPG and EphA2-ephrinA2, indicating that the mechanism underlying bone deficiency is modulated by EphA2-ephrinA2 signaling. Additionally, the increased number of osteoclast-like cells in the co-culture system was partially downregulated by the knockdown of ephrinA2 expression, which further suggests that ephrinA2 reverse signaling in PDLCs contributes to osteoclastogenesis induced by simultaneous CF and LPS stimulation. In addition, ephrinA2 silencing re-upregulated ALP and Runx2 expression, which had previously decreased under simultaneous inflammation and compression. This was presumably due to the reduction of EphA2 receptor signaling, as EphA2 signaling was confirmed to suppress osteoblastic differentiation (Irie et al., 2009). A similar result in ephrinA2-knockdown PDLCs revealed that calcified nodule formation had been significantly reinforced.

It is shown that *P. gingivalis* LPS regulates the expression of proinflammatory cytokines via stimulating both TLR-2 and TLR-4. (Herath et al., 2013) However, some studies ascribe the activation of TLR-2 to a contamination of lipoprotein in LPS preparations.(Ogawa et al., 2007) The *P. gingivalis* LPS used in the present study was a commercial preparation that underwent enzymatic treatment to remove lipoproteins by the supplier, and according to the manufacturer's instructions, it activates only TLR-4. Further investigation is needed to clarify whether or not TLR-4 is dependent on the regulation of Eph–ephrin signaling. The current studies were mainly done in vitro investigating the cellular and molecular biology under the compression-loaded and LPS-affected situation. The experimental condition imitated the orthodontic force and periodontal inflammation, while no in-depth in vivo experiments were carried out, therefore limiting the conclusion of the current research based on only in vitro findings. An in vivo animal model should be adopted in future research to provide corresponding changes of Eph–ephrin expression, as well as to evaluate the temporal-spatial distribution of Eph–ephrin signaling in PDL tissue during inflammation control.

In conclusion, LPS exacerbates CF-induced osteoclastogenesis mainly by reducing EphB4 forward signaling and by enhancing ephrinA2 reverse signaling. If LPS treatment is followed by CF exposure in the absence of LPS, osteoclastogenesis will be reduced through the modulation of Eph–ephrin bidirectional communication. These findings may help to explain the molecular mechanism underlying LPS-affected and compressed PDLCs.

5 | SUPPORTING MATERIAL

The Appendix S1 is a separated file including "Methods and Materials," a small part of "Results," "Table 1. Primer sequences of the target genes for qRT-PCR," and "Figure 1. Time-dependent effects of CF (2.0 g/cm^2) on mRNA expression of Eph–ephrin and RANKL/OPG in PDLCs."

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Minjie Li: Data curation; Formal analysis; Investigation; Methodology; Writing-original draft. **Zhongyuan Tang:** Formal analysis; Investigation. **Chengfei Zhang:** Conceptualization; Methodology; Resources. **Lijian Jin:** Methodology; Resources; Writing-review & editing. **Koichi Matsuo:** Conceptualization; Writing-review & editing. **Yanqi Yang:** Conceptualization; Funding acquisition; Project administration; Supervision; Validation.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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