Osteogenesis, Osteoclastogenesis and their Crosstalk in Lipopolysaccharide-induced Periodontitis in Mice

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Objective: To determine the crosstalk of osteogenesis and osteoclastogenesis of alveolar bone in lipopolysaccharide (LPS)-induced periodontitis in mice.

Methods: A representative periodontitis model was established by treating mice with LPS, and osteoblasts and osteoclasts were cultured. Osteoblasts and osteoclasts were cocultured to determine the effects of LPS on the crosstalk of osteogenesis and osteoclastogenesis. Quantitative polymerase chain reaction (qPCR) was performed to determine the expression of osteoclastogenesis makers underlying the potential mechanisms.

Results: The morphological and pathological changes in alveolar bone were observed in LPS-induced mice and LPS dose-dependently suppressed osteogenesis. The mRNA expression of cathepsin K, as a marker of osteoclasts, was accordingly downregulated in the coculture. The mRNA expression of osteoprotegerin was increased, while that of receptor activator of nuclear factor-κB ligand (RANKL) was decreased with an increased concentration of LPS. Moreover, the mRNA expression of toll-like receptor 4 (TLR4) was upregulated by LPS, whereas TLR4 knockout partially recovered osteoclast differentiation in the upper layer of the coculture.

Conclusion: LPS dose-dependently suppressed osteogenesis but had a bidirectional effect on osteoclastogenesis. The combined effects of LPS on osteogenesis, osteoclastogenesis and their crosstalk via TLR4 account for alveolar bone loss in periodontitis.

Key words: coculture, lipopolysaccharide, osteoclastogenesis, osteogenesis, periodontitis

Periodontitis is one of the most common oral diseases¹. It is characterised by destruction of the alveolar bone and ultimately leads to tooth loss. It is usually caused by the accumulation of bacteria in periodontal pockets. Thus, much research has focused on the relationship between bacteria and bone resorption via inflammatory mechanisms²-⁴. In this study, we focused on bone homeostasis in periodontitis.

Bone homeostasis depends on the balance between osteogenesis and osteoclastogenesis⁵. It is therefore not sufficient to study osteogenesis or osteoclastogenesis separately to determine the mechanism of bone loss in periodontitis. Osteoclastogenesis requires differentiation factors secreted by osteoblasts, such as macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG)⁶. To the best of our knowledge, the underlying mechanism that leads to accelerated bone loss in periodontitis has not been elucidated. In this study, we investigated this mechanism from the perspective of bone homeostasis, including osteogenesis, osteoclastogenesis and their crosstalk.

Lipopolysaccharide (LPS) is a key macromolecule that comprises the outer surface of gram-negative bacteria⁷. It is recognised by the host as a foreign molecule.
and elicits an immune response that is designed to eliminate the bacterial intruder. In periodontitis, it was thought that exposure to LPS initiated inflammatory bone loss through induction of host-derived inflammatory cytokines. Toll-like receptor 4 (TLR4), an important member of the TLR family, is considered a major receptor for identifying LPS. The LPS-induced TLR4 signaling pathway plays a key role in periodontitis by eliciting proinflammatory gene expression. TLR4 knockout mice have been reported to be hyporesponsive to LPS. In this study, we established a representative periodontitis model via LPS treatment and examined the morphological and pathological changes of alveolar bone in periodontitis in vivo. To study both bone formation and resorption, we conducted cell culture/coculture in vitro and induced osteogenesis and osteoclastogenesis separately. Overall, we sought to determine the role of the LPS-TLR4 axis in osteogenesis and osteoclastogenesis and their crosstalk in the alveolar bone in periodontitis.

Materials and methods

Mice

All animal experiments were performed in line with the guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of West China Hospital, Sichuan University. Male C57BL/6J mice were obtained from the Ensiweier Institute of Biotechnology (Chongqing, China), and 3.6GFP-Col1a1 and TLR4−/− mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Four-week-old mice were randomly divided into two groups of five animals each: the control group and LPS-treated periodontitis group. The mice were subjected to ligature-induced periodontitis and received LPS (1 mg/kg) around the first maxillary molars at days 3, 5, and 7 postsurgery. An E. coli strain (O55:B5) LPS was procured from Sigma-Aldrich (St Louis, MO, USA). The mice were sacrificed after 8 weeks, and the samples were collected.

Histology

For the microCT analysis, the samples were scanned using a microCT scanner (µCT50, Scanco, Zurich, Switzerland), operated at 70 kV, 180 μA, 450 ms exposure time and 12-μm resolution. We used standardized nomenclature for the bone parameters measured according to the microCT scanner protocol. Radiographs were taken using an Xtreme In-Vivo Imaging System (Bruker, Billerica, MA, USA). Samples were fixed in 4% paraformaldehyde (PFA), then made into paraffin sections and cut into 5-μm thick sections using a microtome (Leica, Wetzlar, Germany). The sections were processed with Masson trichrome staining and tartrate-resistant acid phosphatase (TRAP) staining according to the respective manufacturers’ protocol. Masson trichrome staining kits were purchased from Solarbio Science & Technology (Beijing, China). TRAP staining was done as previously described. The images were captured using a microscope (Olympus, Tokyo, Japan). All analyses were performed using OsteoMeasure (Osteometrics, Decatur, GA, USA).

Cell culture

Osteogenesis was assessed via osteogenic differentiation of mesenchymal stem cells from mandibular bone. The primary cells were from Cola1a-GFP mice, which expressed green fluorescent protein (GFP) under the control of the Collagen 1a1 promoter, as a marker for osteogenesis. Bone marrow cells flushed from mandibular bone were suspended in alpha-MEM (HyClone, Logan, UT, USA) containing 10% foetal calf serum (FCS, HyClone), 1% penicillin and streptomycin (HyClone). Cells were seeded in 12-well plates with osteogenic medium consisting of alpha-MEM supplemented with 10% foetal bovine serum (FBS, HyClone), 1% penicillin and streptomycin (HyClone). Cells were seeded in 12-well plates with osteogenic medium consisting of alpha-MEM supplemented with 10% foetal bovine serum (FBS, HyClone), 1% penicillin and streptomycin (HyClone).

Cell culture was performed as follows. Briefly, bone marrow cells flushed from mandibular bone were suspended in alpha-MEM containing 10% FCS and 1% penicillin and streptomycin. The cultures were fed fresh medium with cytokines every 3 days and harvested on days 9 to 11 of culture. Coculture was performed as follows. Briefly, bone marrow cells flushed from mandibular bone were suspended in alpha-MEM containing 10% FCS, 1% penicillin and streptomycin. TLR4−/− cells were obtained from a TLR4-deficient model (B6.B10ScN-Tlr4lps-del/JthJ, stock #007227). This strain was homozygous for a dele-
tion allele, Tlr4ips-del, that caused spontaneous deletion of TLR4, resulting in the absence of TLR4 mRNA and protein. The strain was purchased from the Jackson Laboratory and backcrossed with mice on the C57BL/6J genetic background.

Quantitative polymerase chain reaction (qPCR)

Gene expression was analysed using real-time quantitative polymerase chain reaction (qPCR) using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA, USA), as previously described. Relative gene expression was normalised to that of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene using the comparative cycle threshold (Ct) method (delta Ct).

Statistics

The data are presented as mean ± standard deviation (SD) based on at least three independent experiments. Statistical differences were calculated using a two-tailed Student t test in Microsoft Excel (Microsoft Redmond, WA, USA) and P < 0.05 was considered statistically significant.

Results

Total bone mass of alveolar bone is decreased in LPS-induced periodontitis

The samples of periodontitis were collected 8 weeks after periodontitis-inducing surgery. MicroCT showed that the total bone mass of alveolar bone was much lower in periodontitis than in the control group (Fig 1). The parameters of alveolar bone among the roots of the first molar were also analysed. The ratio of bone volume to total volume, trabecular number and trabecular thickness were all significantly decreased. Accordingly, trabecular spacing increased significantly.

Pathological changes in alveolar bone in periodontitis

Immunohistochemistry (IHC) staining showed that the Runx2 expression of alveolar bone in the periodontitis
group was decreased, which indicated that bone formation in the periodontitis group was suppressed (Fig 2). In contrast, the osteoclasts in alveolar bone increased, as detected by TRAP staining. Accordingly, more vesicular bone appeared in the periodontitis group, which suggested more active bone resorption. Statistical analysis confirmed that the ratio of osteoclast surface to bone surface was significantly increased, as was that of the number of osteoclasts to the bone perimeter.

**Effects of LPS on osteogenesis and osteoclastogenesis**

To further study the pathological changes to bone formation and bone resorption in periodontitis, an in vitro cell culture was performed to induce osteogenesis or osteoclastogenesis. Figure 3 shows that LPS suppressed osteogenesis of mesenchymal stem cells from mandibular bone. The expression of Col1a1, a marker of osteogenesis, was significantly decreased, according to the statistical analysis. Accordingly, TRAP+ osteoclasts, differentiated from bone marrow macrophages, gradually increased as LPS concentration increased. The statistical analysis further revealed that both TRAP+ preosteoclasts and mature osteoclasts were significantly enhanced.

**Effect of LPS on osteoclastogenesis via osteoblast paracrine signalling**

BMMs and osteoblasts were cocultured to induce osteoclastogenesis. Figure 4 shows that LPS significantly suppressed osteoclast formation. TRAP staining revealed that osteoclasts gradually decreased as LPS concentration increased. qPCR showed that the expression of cathepsin K (CTSK), as a marker of osteoclasts, was significantly decreased and the expression of Opg was significantly increased while RANKL was significantly decreased, which indicated that LPS weakened osteoclastogenesis via paracrine signalling in osteoblasts. Additionally, the expression of TLR4, a target of LPS, was significantly increased.

**TLR4−/− osteoblasts rescue osteoclast formation in coculture**

As shown in Fig 5, TRAP staining revealed that LPS stimulation of osteoblasts resulted in fewer osteoclasts differentiated from BMMs in coculture. However, knockout of TLR4 in LPS-induced osteoblasts enhanced osteoclast formation and resulted in more mature osteoclasts. Statistical analysis indicated that TLR4 knockout significantly enhanced osteoclast formation compared with that in wild-type (WT) LPS-induced osteoblasts. Accordingly, the expression of Opg was significantly decreased, while RANKL was significantly increased in the TLR4−/− LPS-induced osteoblast group compared with the WT LPS-induced osteoblast group.

**Discussion**

Periodontitis is the most common chronic bacterial infection. The transition from health to periodontitis is associated with a gradual change from a symbiotic to a pathogenic host–microbial relationship. LPS accumulates during the overgrowth of bacteria. In this study, 8 weeks after surgery to induce periodontitis, the phenotype was obvious. Thus, our experiment showcases a representative model for periodontitis. In this model, the morphological and pathological changes to alveolar bone in periodontitis were examined. Alveolar bone is
the basic support tissue for teeth and is related to many dental diseases\textsuperscript{15}. It is maintained by intramembranous bone regeneration, which is different to endochondral bone regeneration in long bone\textsuperscript{16}. Under physiologic conditions, alveolar bone closely surrounds teeth. In our in vivo periodontitis model, alveolar bone loss occurred, the gap between teeth and alveolar bone increased and bone mineral density decreased. TRAP staining suggested that bone resorption increased. Loss of bone mass may result not only from overactive osteoclastic bone resorption, but also from retarded bone formation\textsuperscript{17}. Thus, we examined the cellular processes of intramembranous bone regeneration of alveolar bone in LPS-induced periodontitis via cell culture. Our results showed that LPS suppressed the osteogenesis of MSCs from alveolar bone but accelerated the osteoclastogenesis of BMMs. Thus, our results suggest that LPS could disturb alveolar bone regeneration by directly suppressing new alveolar bone formation and promoting bone resorption. These effects could account for the bone loss encountered in periodontitis.

Osteoclast differentiation requires a variety of factors, including several key cytokines produced by osteoblasts\textsuperscript{18,19}. The process of osteoclast differentiation requires osteoblasts as an inducing support system\textsuperscript{18}. Thus, an osteoblast/osteoclast coculture could show the regulatory mechanisms underlying osteoclastogenesis more effectively than a RANKL-stimulated osteoclast culture\textsuperscript{20}. As such, we went a step further to characterize the regulation of osteoclastogenesis via paracrine signalling in LPS-induced osteoblasts. Our results revealed that LPS suppressed osteoclastogenesis by stimulating paracrine activity in osteoblasts. This inhibitory effect increased with greater concentrations of LPS. The mRNA expression of CTSK increased accordingly, as a marker of osteoclasts\textsuperscript{21}. Moreover, our qPCR results showed that LPS suppressed the mRNA expression of RANKL in osteoblasts while promoting the mRNA expression of Opg, which confirmed the anti-osteoclastogenic effect of LPS-stimulated osteoblast paracrine signalling. It is known that Opg and RANKL are secreted by osteoblasts, and Opg suppresses osteoclastogenesis whereas RANKL promotes it. The Opg-RANKL axis orchestrates osteoclastogenesis. Thus, we conducted qPCR and found that the mRNA expression of RANKL was suppressed while the mRNA expression of Opg was promoted in the LPS-treated group compared to the control group.
Opg ratio was consequently decreased and was responsible for suppressing osteoclastogenesis. Meanwhile, the mRNA expression of TLR4 was decreased. These results suggest that LPS suppresses the paracrine activity of osteoblasts via the TLR4 pathway.

Previous research has mainly focused on the auxoaction of LPS on osteoclastogenesis in view of the phenotype of bone loss in vivo. However, the present study also found that LPS suppressed osteoclastogenesis via crosstalk between osteoblasts and osteoclasts. To confirm this result, we used TLR4−/− osteoblasts to recover this effect of LPS in cell coculture. Our results showed that TLR4−/− osteoblasts stimulated with LPS promoted more osteoclast formation than WT osteoblasts stimulated with LPS. Moreover, our qPCR results showed that TLR4 knockout accordingly rescued the mRNA expression of RANKL and Opg in LPS-induced osteoblasts. Thus, our experiment suggested that TLR4 was the downstream target of LPS in osteoblasts, which in turn regulated osteoclastogenesis via crosstalk between osteoblasts and osteoclasts.

**Conclusion**

In conclusion, the present study established a representative periodontitis model via LPS induction and revealed the morphological and pathological changes to alveolar bone. It also found that LPS suppressed osteogenesis but played a bidirectional role in osteoclastogenesis. LPS promoted osteoclastogenesis via an osteoclast-autonomous mechanism but suppressed osteoclastogenesis by impacting the paracrine signalling of osteoblasts via TLR4. The combined effects of LPS on osteogenesis, osteoclastogenesis and their crosstalk account for alveolar bone loss in periodontitis. *P < 0.05.

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Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Dr Chen Chen ZHOU collected the data and drafted the manuscript; Drs Ruo Shi XU, Zu Ping WU and Zhao Wei ZHANG analysed and interpreted the data; Drs Quan YUAN, Shu Juan ZOU and Jing XIE critically revised the manuscript; Dr De Mao ZHANG created the concept and designed the study. All authors read and approved the final manuscript.

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