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Toll-like receptor 2 heterodimers, TLR2/6 and TLR2/1 induce prostaglandin E production by osteoblasts, osteoclast formation and inflammatory periodontitis

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ABSTRACT

TLR2 forms heterodimers with TLR1 and TLR6, and regulates host defense mechanisms against pathogens. We examined the role of TLR2 heterodimer signaling in osteoclast formation and inflammatory periodontitis. In co-cultures of mouse bone marrow cells and osteoblasts, a TLR2/6 ligand (diacylated lipopeptide designed from Gram-positive bacteria) markedly induced osteoclast formation. A TLR2/1 ligand (triacylated lipopeptide designed from Gram-negative bacteria) also induced osteoclast formation. The osteoclast formation induced by TLR2/6 and TLR2/1 ligands was completely suppressed by indomethacin. Osteoblasts expressed TLR1, 2, 4, and 6 mRNAs, and both TLR2/6 and TLR2/1 ligands induced the expression of COX-2, mPGES-1, and RANKL mRNA, as well as PGE production in osteoblasts. Both TLR2/6 and TLR2/1 ligands induced the resorption of mandibular alveolar bone in organ cultures, and elicited inflammatory periodontitis *in vivo*. Therefore, TLR2 heterodimer signaling may play a key role in PGE-mediated inflammatory bone loss in periodontal disease.

1. Introduction

Bone remodeling is regulated by osteoclastic bone resorption and new bone formation. Osteoclasts are primary bone-resorbing cells, and are differentiated from the monocyte-macrophage lineage. Receptor activator of NF-κB ligand (RANKL) is expressed on the cell surface of osteoblasts in response to bone-resorbing factors, and plays a pivotal role in osteoclast differentiation [1]. Osteoclast precursors possess a receptor for RANKL (RANK), and can differentiate into mature osteoclasts via a mechanism involving RANK-RANKL recognition [2]. In the bone resorption associated with inflammation, prostaglandin E_2 (PGE_2) is produced by osteoblasts and acts as a potent stimulator of bone resorption [3]. Lipopolysaccharide (LPS) and inflammatory cytokines such as IL-1, IL-6 are known to induce PGE_2 production by osteoblasts, and this PGE_2 stimulates the expression of RANKL, leading to osteoclast differentiation [4,5].

PGE_2 synthesis is regulated by three metabolic steps; the release of arachidonic acid from the membranous phospholipids by phospholipase A_2 (PLA_2), the conversion of arachidonic acid to PGH_2 by cyclooxygenase (COX) and the synthesis of PGE_2 by PGE synthase (PGES) [6–8]. We have previously reported that the purpose of cytosolic PLA_2 ($cPLA_2$) expression in osteoblasts is to release arachidonic acid following PGE_2 production [9,10]. There are three forms of PGES, cytosolic PGES (cPGES), membrane-bound PGES (mPGES)-1 and mPGES-2 [11–13]. The expression of mPGES-1 is markedly induced by inflammatory stimuli, and is functionally coupled with COX-2. The coordinate induction of COX-2 and mPGES-1 is essential for the PGE_2 production associated with inflammation.

Toll-like receptors (TLRs; TLR1-TLR13) play critical roles in innate immunity, and various ligands for TLRs are thought to regulate the host defense mechanisms against pathogens. TLRs can be divided into two major subgroups, one consisting of TLR1, 2, 4, 5, 6 and 11, which are expressed on the cell surface, and the other is composed of TLR3, 7, 8 and 9, which are mainly expressed in intracellular vesicles [14]. TLR4 was identified as the receptor for LPS, which is an outer membrane component of Gram-negative bacteria, which are known to cause periodontitis. We have reported that LPS binds to the TLR4 expressed on osteoblasts, and that TLR4 signaling induces PGE_2 synthesis and RANKL expression in osteoblasts, leading to subsequent bone resorption in mouse alveolar bone [15]. Recently, TLR2 was shown to form heterodimers with TLR1 and TLR6 [16,17]. The TLR2-TLR1 heterodimer (TLR2/1) is known to recognize triacylated lipopeptides from Gram-negative bacteria [16]. On the other hand, the TLR2-TLR6

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heterodimer (TLR2/6) recognizes diacylated lipopeptides from Gram-positive bacteria [17]. Until now, the roles of TLR2 heterodimer signaling in bone tissue have been unknown.

Periodontal diseases are infectious diseases that develop as a result of bacterial plaque accumulation in periodontal pocket, which is located on the tooth surface in the junction between the gingiva and the tooth. Mixed Gram-negative anaerobic bacteria are considered to be pathogenic to the periodontal tissues, and are involved in the development and progression of periodontitis [18]. Periodontitis is a bone destructive disease, and LPS is a known pathogen-associated molecule involved in periodontitis. We have reported the development of an original mouse model of periodontitis that is associated with the resorption of alveolar bone that is induced by LPS injection into the lower gingiva, and we successfully detected alveolar bone loss induced by LPS [15]. In this model of periodontitis, LPS induced the loss of alveolar bone in wild-type mice, but not in mPGES-1-deficient mice, thus suggesting that mPGES-1-dependent PGE_2 production is essential for LPS-induced periodontal bone resorption [15].

In the present study, we demonstrate the effects of a TLR2/1 ligand and a TLR 2/6 ligand on osteoclast formation in co-cultures of mouse bone marrow cells and osteoblasts, and on COX-2- and mPGES-1-dependent PGE_2 production by osteoblasts. TLR2 heterodimer signaling induced the resorption of mandibular alveolar bone *in vitro*, and elicited inflammatory periodontitis *in vivo*, suggesting possible roles for TLR2 heterodimer signaling in the PGE-mediated inflammatory bone loss associated with periodontal disease.

2. Materials and methods

2.1. Animals and reagents

Newborn and 6-week-old mice of the *ddy* strain were obtained from Japan SLC Inc. (Shizuoka, Japan). All procedures were performed in accordance with the institutional guidelines for animal research. The TLR2/6 ligand, synthetic diacylated lipopeptide (Pam2CSK4: S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysineX3CF3COOH) is a diacylated lipopeptide designed from Gram-positive bacteria, and the TLR2/1 ligand, synthetic triacylated lipopeptide (Pam3CSK4: N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-systeinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine) is a triacylated lipopeptide designed from Gram-negative bacteria. Both ligands were obtained from InvivoGen Co. Ltd., CA, USA. LPS and PGE_2 were obtained from Sigma Aldrich Co. LLC., MO, USA.

2.2. Culture of primary mouse osteoblastic cells

Primary osteoblastic cells were isolated from newborn mouse calvariae after 5 routine sequential digestions with 0.1% collagenase (Roche Applied Science) and 0.2% dispase (Roche Diagnostics GmbH, Mannheim, Germany), as described previously [15]. Osteoblastic cells collected from fractions 2–4 were combined and cultured in α-modified MEM (αMEM), supplemented with 10% fetal calf serum (FCS) at 37 °C under 5% CO_2 in air.

2.3. Osteoclast formation in co-cultures of mouse bone marrow cells and osteoblasts

Bone marrow cells (3×10^5 cells) were isolated from 6-week-old mice and co-cultured with primary osteoblastic cells (5×10^3 cells) in αMEM containing 10% FCS [15]. After being cultured for 7 days, the cells adhering to the well surface were stained for tartrate-resistant acid phosphatase (TRAP). TRAP-positive multinucleated cells containing 3 or more nuclei per cell were counted as osteoclasts.

2.4. Measurement of PGE_2 production

The concentrations of PGE_2 in the culture medium were determined using an enzyme immunoassay (EIA) (GE Healthcare UK Ltd., Buckinghamshire, UK). The antibody had the following cross-reactivity calculated using the bound/free ratio: PGE_2, 100%; PGE_1, 7.0%; 6-keto-$PGF_{1\alpha}$, 5.4%; $PGF_{2\alpha}$, 4.3%; and PGD_2, 1.0%.

2.5. RT-PCR analysis

Total RNA was extracted from mouse osteoblasts using ISOGEN (Nippon Gene, Tokyo, Japan). cDNA was synthesized from 5 μg of total RNA by reverse transcriptase (Superscript II Preamplification System, Invitrogen, Carlsbad, CA) and amplified via PCR. The primers used for the PCR for the mouse COX-1, COX-2, mPGES-1, mPGES-2, cPGES, RANKL, and β-actin genes were reported previously [9,15]. The PCR product was run on a 1.5% agarose gel and stained with ethidium bromide.

2.6. Luciferase assay

Primary osteoblastic cells (5×10^4 cells) were cultured for 24 h before transfection with plasmids. Plasmid pNFκB-TA-Luc (0.2 μg) contained four tandem copies of the NFκB consensus sequence with the firefly luciferase reporter gene (Clontech Laboratories, Inc.) and the pGL4.74[hLuc/TK] plasmid (20 ng) contained the renilla luciferase reporter gene (Promega Corp.) as an internal control reporter vector. Both were transfected into mouse primary osteoblasts using FuGENE HD (Roche Corp.). The luciferase activity was measured with the Dual-luciferase Reporter Assay system (Promega Corp.) using an ARVO MX multilabel/luminescence counter (Perkin Elmer Corp.).

2.7. Bone-resorbing activity in organ cultures of mouse mandibular alveolar bone

To measure the bone-resorbing activity in organ cultures of the mandibular alveolar bone, mandibular alveolar bone specimens were collected from the mouse molar region under a microscope and cultured for 24 h in BGJb containing 1 mg/ml BSA. After 24 h, the alveolar bone was transferred to new media, with or without the TLR2/6 ligand or TLR2/1 ligand, and cultured for another 5 days. The bone-resorbing activity was determined by measuring the concentration of calcium in the conditioned medium [15].

2.8. Model of mouse experimental periodontitis

To develop the model of experimental periodontitis, LPS, the TLR2/6 ligand or the TLR2/1 ligand was dissolved in 50 μl of PBS and injected into the outside of the mouse lower gingiva on days 0, 2, and 4. As a control, PBS was injected into the lower gingiva at each time point in other mice. The mandibular alveolar bone with the tooth was collected from the mouse molar region 7 days after the first injection [15]. To examine the mass of the mandibular alveolar bone, three-dimensional (3D) reconstruction images of the alveolar bone were obtained by micro-computed tomography (μCT) (inspeXio SMX-90CT; Shimadzu, Kyoto, Japan). The bone mineral density (BMD) was measured by the 3D image in the bone of alveolar crest between 1st and 2nd molars of the mandibular bone.

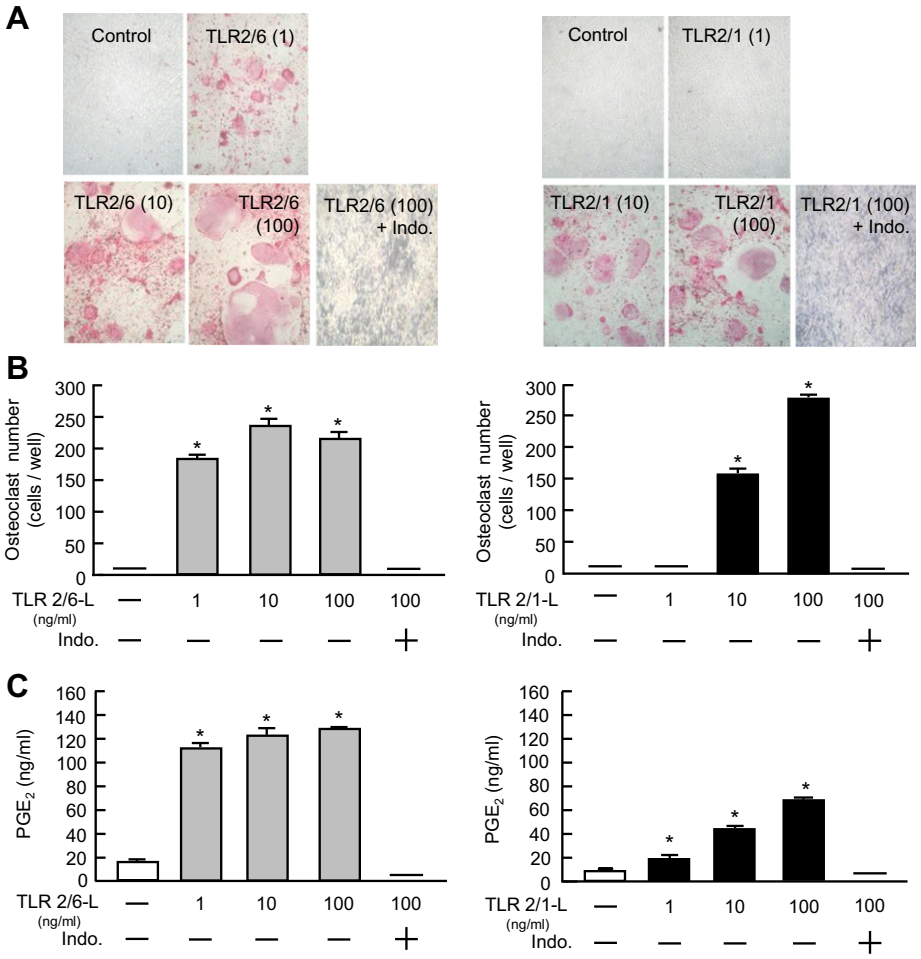

Fig. 1. Effects of TLR2/6 and TLR2/1 ligands on osteoclast formation and PGE_2 production in co-cultures of osteoblasts and bone marrow cells. Mouse bone marrow cells and osteoblastic cells were co-cultured for 7 days with 1, 10 and 100 ng/ml of TLR2/6 and TLR2/1 ligands. In some experiments, indomethacin (1 μM) was added to the cultures with 100 ng/ml of TLR2/6 or TLR2/1 ligand. (A) The cells were stained for tartrate-resistant acid phosphatase (TRAP), a specific marker for osteoclasts. (B) The number of TRAP-positive multinucleated cells containing 3 or more nuclei was counted. The data are expressed as the means ± SEM of 3 independent wells. (C) The level of PGE_2 was measured by EIA using the conditioned media from the co-cultures. The data are expressed as the means ± SEM of 4–5 independent wells. A significant difference between two groups is indicated, $^*p < 0.001$ vs control.

2.9. Statistical analysis

The data are expressed as the means + SEM. The significance of differences was analyzed using Student's *t*-test.

3. Results

3.1. Effects of the TLR2/6 and TLR2/1 ligands on osteoclast formation and PGE production in co-cultures of mouse bone marrow cells and osteoblasts

In the co-cultures of mouse bone marrow cells and osteoblasts, the TLR 2/6 ligand, at concentrations of 1–100 ng/ml, markedly induced osteoclast formation, and numerous TRAP-positive osteoclasts were formed (Fig. 1A,B). The TLR2/1 ligand, at concentrations of 10–100 ng/ml, also induced osteoclast formation (Fig. 1A,B). The addition of indomethacin, a nonsteroidal anti-inflammatory drug (NSAID), completely suppressed the osteoclast formation induced by the TLR 2/6 and TLR2/1 ligands (Fig. 1A,B). Because LPS, a TLR4 ligand, -dependent osteoclast formation is closely related to PGE_2 production [15], we measured the levels of PGE_2 using the conditioned media from the co-cultures of mouse bone marrow cells and osteoblasts. The level of PGE_2 was markedly elevated in the cultures treated with the TLR 2/6 ligand and TLR 2/1 ligand, and indomethacin completely suppressed the induction of PGE_2 by the respective TLR2 heterodimer ligands (Fig. 1C).

3.2. The TLR2/6 and TLR2/1 ligands induce the expression of COX-2, mPGES-1, and RANKL mRNAs, and the production of PGE_2 in mouse osteoblasts

We performed a RT-PCR analysis to examine the effects of the TLR 2/6 ligand and the TLR2/1 ligand on the expression of COX-1, COX-2, mPGES-1, mPGES-2, cPGES, and RANKL in mouse primary osteoblasts. A concentration of 100 ng/ml of both the TLR 2/6 ligand and TLR2/1 ligand markedly induced the expression of COX-2, mPGES-1, mPGES-2, and RANKL mRNAs in the mouse osteoblasts (Fig. 2A). The mouse primary osteoblasts expressed TLR1, TLR2, TLR4, and TLR6 mRNAs, as determined by RT-PCR (data not shown), indicating that the TLR2/6 and TLR2/1 ligands can bind to TLR1, TLR2, and TLR6 to be recognized by the respective heterodimers of TLR2/6 and TLR2/1.

To confirm the effects of the TLR 2/6 and TLR2/1 ligands on PGE_2 production by osteoblasts, the levels of PGE_2 in the conditioned medium of cultured osteoblasts were measured after 24 h in culture. We detected a marked elevation of PGE_2 in the conditioned

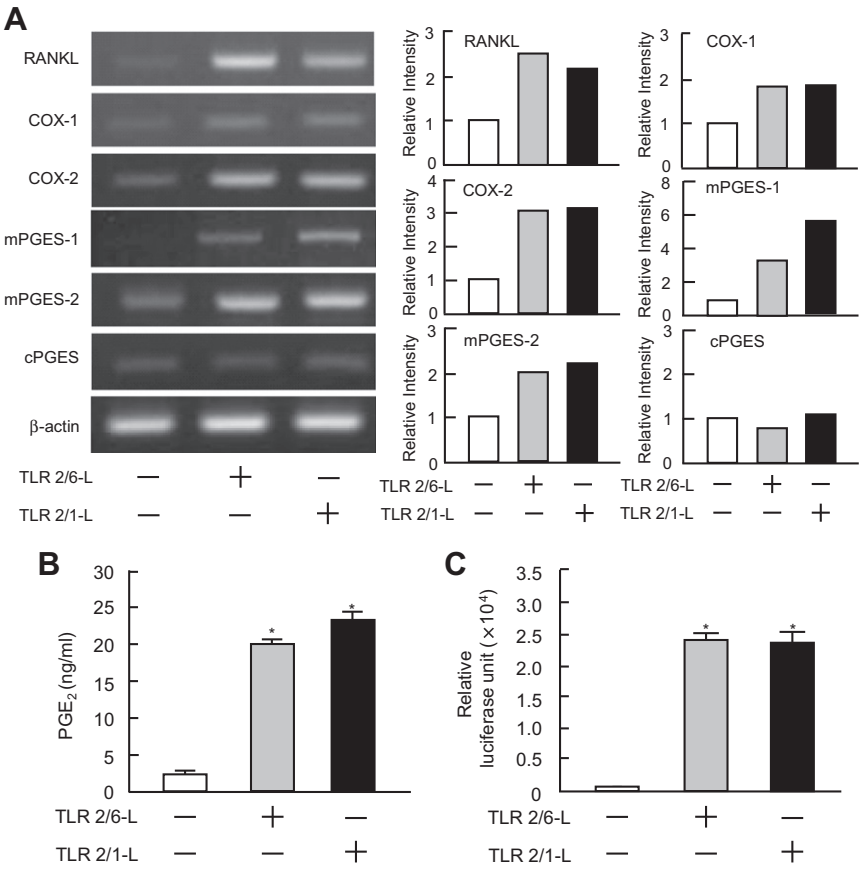

Fig. 2. Effects of TLR2/6 and TLR2/1 ligands on the expression of COXs and mPGESs mRNAs, the production of PGE_2, and the NFκB activity in mouse primary osteoblasts. (A) Mouse osteoblasts were treated with 100 ng/ml of TLR2/6 ligand or TLR2/1 ligand for 24 h, and the total RNA was extracted. The mRNA expression levels of RANKL, COX-1, COX-2, mPGES-1, mPGES-2, and cPGES were detected by RT-PCR. The relative intensity of the respective mRNA expression levels was calculated. (B) Mouse osteoblasts were treated with 100 ng/ml of TLR2/6 ligand or TLR2/1 ligand for 24 h, and the levels of PGE_2 were measured using the conditioned media. (C) Mouse osteoblasts were transfected with pNFκB-TA-Luc or pGL4.74 [hLuc/TK] vectors. Osteoblasts were cultured for 22 h with 100 ng/ml of TLR2/6 ligand or TLR2/1 ligand, and NFκB activation was measured by the luciferase assay. The data are expressed as the means ± SEM of 4–6 independent wells. A significant difference between the 2 groups is indicated, $^{*}p < 0.001$ vs control.

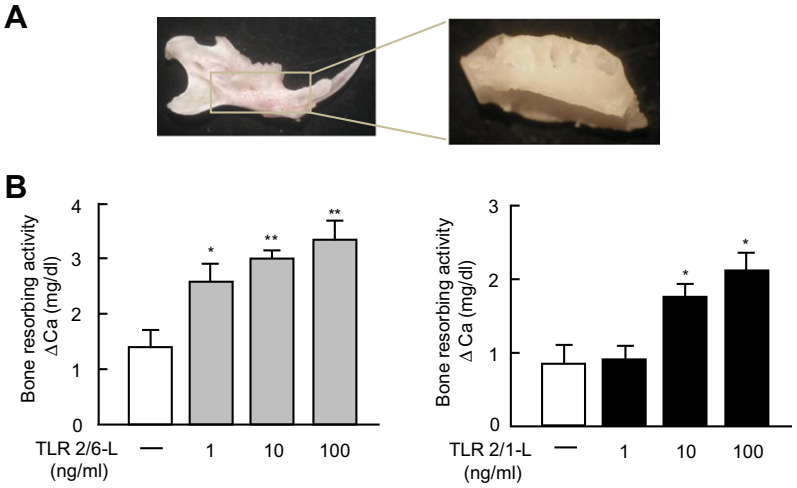

Fig. 3. Effects of TLR2/6 and TLR2/1 ligands on the bone-resorbing activity in organ cultures of mouse mandibular alveolar bone. (A) Mandibular alveolar bone specimens were collected from the molar region of the lower jaw under a microscope. (B) The mandibular alveolar bone collected was cultured for 24 h in BGJb containing 1 mg/ml of BSA. After 24 h, the alveolar bone was transferred to new media, and cultured for five days with 1, 10 and 100 ng/ml of the TLR2/6 ligand or TLR2/1 ligand. The concentration of calcium in the media was measured to calculate the bone-resorbing activity. The data are expressed as the means ± SEM of 3–4 independent cultures. A significant difference between the two groups is indicated, $^{*}p < 0.05$, $^{**}p < 0.01$ vs control.

medium of mouse osteoblasts treated with the respective TLR2 heterodimer ligands (Fig. 2B).

Activation of NFκB is critical for the expression of various inflammatory genes, including COX-2 and mPGES-1. In osteoblasts, inflammatory stimuli such as IL-1 and LPS induce NFκB activation. Therefore, we examined the effects of TLR2 heterodimer ligands on the NFκB-dependent gene expression in osteoblasts using a luciferase assay. When mouse osteoblasts were treated for 22 h with

the TLR2/6 ligand or the TLR2/1 ligand, marked activation of NFκB was detected (Fig. 2C).

3.3. Effects of the TLR2/6 and TLR2/1 ligands on the bone-resorbing activity in organ cultures of mouse mandibular alveolar bone

We have previously established an organ culture system for mouse mandibular alveolar bone, and reported that LPS induced bone-resorbing activity in the mandibular alveolar bone *in vitro* [15]. To examine the effects of the TLR2/6 and TLR2/1 ligands on the resorption of mandibular alveolar bone, the mandibular alveolar bone was collected from the mouse lower mandible (Fig. 3A), and cultured with or without the respective TLR2 heterodimer ligands. Bone resorption was detected by measuring the calcium level in the medium. We observed a marked increase in the bone resorbing activity in the TLR2/6 ligand (1–100 ng/ml)- and TLR2/1 ligand (10–100 ng/ml)-treated mandibular alveolar bone samples (Fig. 3B). The effects of the respective TLR2 heterodimer ligands were similar to those induced by LPS, a TLR4 ligand (data not shown).

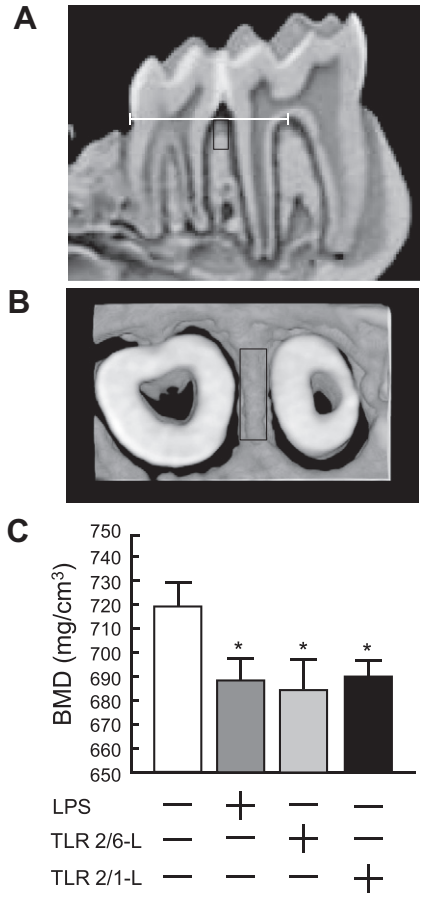

Fig. 4. Administration of the TLR2/6 ligand or TLR2/1 ligand induced the loss of mandibular alveolar bone in mice. In the model of experimental periodontitis, the TLR2/6 ligand (1 μg/mouse), TLR2/1 ligand (1 μg/mouse) and LPS (25 μg/mouse) were injected into the mouse lower gingiva on days 0, 2, and 4. As a control, PBS was injected into the lower gingiva at each time point. The mandibular alveolar bone with the tooth was collected 7 days after the first injection, and the mass of mandibular alveolar bone was measured. (A) A three-dimensional (3D) μCT device reconstructed 2D image of longitudinal section of the 1st and 2nd molar region of alveolar bone in the control mouse. (B) A 3D μCT reconstruction image of cross section of the molar region shown in the white bar scale of panel A. The rectangular area indicates the alveolar crest area between 1st and 2nd molars in the mandibular bone used for the 3D analysis. (C) To measure the mass of mandibular alveolar bone, the bone mineral density (BMD) was calculated by a 3D quantitative analysis of the respective μCT images in the identical black rectangular area shown in panels A and B. The data are expressed as the means + SEM of 4–5 mice. A significant difference between the 2 groups is indicated; $^{*}p < 0.05$, vs control.

3.4. The TLR2/6 ligand and TLR2/1 ligand induce experimental periodontitis in the mouse model

In prior *in vivo* experiments, we found that the injection of LPS into the gingiva of the mouse lower mandible induced the loss of alveolar bone [15]. To examine the effects of the TLR2/6 and TLR2/1 ligands on the experimental periodontitis, we injected these ligands or LPS into the gingiva of the mouse lower mandible, and the alveolar bone with the tooth was collected on day 7 after the injection. As shown in Fig. 4A,B, 3D reconstructed images of the alveolar bone were obtained by μCT, and the bone of alveolar crest between 1st and 2nd molars, shown as a square, was defined as the area for the measurement of the alveolar bone mass. Then, the BMD was measured in the selected region. In this analysis, both the TLR2/6 ligand and the TLR2/1 ligand significantly suppressed the BMD of the mandibular alveolar bone, similar to LPS (Fig. 4C).

4. Discussion

In this study, we showed that both a TLR2/6 ligand and a TLR2/1 ligand acted on osteoblasts to induce the production of PGE_2 and RANKL-dependent osteoclastogenesis (Figs. 1 and 2). We confirmed that the osteoblasts expressed TLR1, 2, 4, and 6 mRNAs, and that the TLR2/6 ligand and TLR2/1 ligand induced osteoclastic bone resorption in mouse calvarial organ cultures (data not shown). In osteoblasts, the TLR2 heterodimer signaling markedly induced NFκB-dependent gene transcription (Fig. 2C). The mouse COX-2 gene promoter possesses functional regulatory elements for NFκB [19]. Therefore, TLR1, 2, and 6 expressed in osteoblasts may transmit TLR2 heterodimer signaling to induce osteoclastic bone resorption by a PGE-dependent and RANKL-mediated mechanism in bone.

In healthy mouse periodontal tissues, the roots of the molar teeth embed into individual sockets in the mandibular alveolar bone. However, the teeth roots are exposed by the bone resorption of alveolar crest due to inflammation, and the loss of teeth is the end-point of periodontal bone resorption. It is known that most periodontal pathogens are Gram-negative anaerobic bacteria, such as *Actinobacillus actinomycetemcomitans*, *Parphyromonas gingivalis*, and *Tannerella forsythia*, and that the outer membranes of these bacteria possess LPS, a known pathogen-associated molecule involved in periodontitis [18].

In our study model of mouse periodontitis, the injection of LPS into the lower mandibular gingiva induces alveolar bone loss, during which TLR4 signaling elicits COX-2- and mPGES-1-mediated PGE_2 synthesis [15]. Regarding the presence of TLR2, Asai et al. [20] have shown that human gingival epithelial cells express TLR2, and that the cells produce IL-8 when stimulated with *P. gingivalis* fimbrial protein through TLR2, suggesting that TLR2 is involved in the inflammation in gingival tissues. Studies using TLR2-deficient mice have shown that the cytokine production and alveolar bone loss induced by Gram-negative bacteria such as *P. gingivalis* and T. *forsythia* was attenuated [21,22]. In the present study, both a TLR2/6 ligand (diacylated lipopeptide designed from Gram-positive bacteria) and a TLR2/1 ligand (triacylated lipopeptide designed from Gram-negative bacteria) induced alveolar bone loss in our model of periodontitis. We speculate that TLR2/6 and TLR2/1 ligands induce the inflammation of gingival tissues, and enhance the inflammatory bone resorption in alveolar bone. Therefore, it is possible that not only Gram-negative bacteria, but also Gram-positive bacteria, are involved in the pathogenesis of

periodontitis, and that both mechanisms involve TLR2 heterodimer signaling. Therefore, both TLR4 and TLR2 signaling may be involved in the pathogenesis of periodontitis. Further studies are needed to identify the ligand of the TLR2 heterodimer that acts as the inducible pathogen in human periodontitis.

Previous investigations of the roles of TLRs in osteoclastic bone resorption have mostly focused on TLR4, because LPS is known to be a bone resorbing factor associated with inflammation and infection. Kikuchi et al. [23] reported that LPS induced the expression of RANKL on mouse osteoblasts. We have reported that LPS binds to the TLR4 expressed on osteoblasts, and that TLR4 signaling induces PGE_2 synthesis and RANKL expression in osteoblasts, leading to subsequent bone resorption in mouse calvaria and alveolar bone [15]. Because mPGES-1-deficient mice are resistant to the osteoclast formation induced by LPS, the PGE_2 produced by osteoblasts is essential for TLR4-mediated osteoclast formation [15]. Sato et al. [24] have shown that MyD88 is essential for the osteoclastogenesis and osteoclast survival induced by LPS in mice. Park et al. [25] reported that TLR4 and myeloid differentiation factor 2 (MD-2) form a heterodimer that recognizes LPS molecules. On the other hand, the role of TLR2 signaling in bone metabolism was unknown. Signal transduction of the TLR2 heterodimer was reported to be similar to that of TLR4, and NFκB may be involved in the process [14]. In this study, we showed that TLR2 heterodimer ligands activate NFκB, leading to the expression of COX-2, mPGES-1, and RANKL in osteoblasts, and these signals could induce osteoclastic bone resorption.

We herein described the effects of TLR2 heterodimer signaling by both TLR2/6 and TLR2/1, on PGE_2-mediated and RANKL-dependent osteoclast differentiation. In the mouse model of periodontal bone resorption, TLR2 heterodimer signaling induced the bone resorption of mandibular alveolar bone *in vitro*, and elicited mouse experimental periodontitis *in vivo*. This knowledge can be exploited for the development of novel therapeutic approaches for clinical periodontitis.

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