**Porphyromonas gingivalis** Stimulates Bone Resorption by Enhancing RANKL through Activation of Toll-like Receptor 2 in Osteoblasts*

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**Key words:** Toll-like receptors; Porphyromonas gingivalis; osteoclast; bone resorption; inflammation

**Background:** Inflammation causes bone loss through enhanced osteoclast formation. **Results:** Porphyromonas gingivalis stimulates osteoclast formation through Toll-like receptor 2. **Conclusion:** Activation of Toll-like receptors may represent a mechanism for inflammation-induced bone loss in diseases like rheumatoid arthritis and periodontitis. **Significance:** A thorough understanding of the mechanisms involved in inflammation-induced bone loss will lead to improved treatment.

Periodontitis has been associated with rheumatoid arthritis (RA). In experimental arthritis, concomitant periodontitis caused by oral infection with Porphyromonas gingivalis (P.g.) enhances articular bone loss. The aim of this study was to investigate how lipopolysaccharide (LPS) from P.g. stimulates bone resorption. The effects by LPS P.g. and four other TLR2 ligands on bone resorption, osteoclast formation and gene expression in *wild type* and *Thr2* deficient mice were assessed in *ex vivo* cultures of mouse parietal bones and in an *in vivo* model in which TLR2 agonists were injected *s.c.* over the skull bones. LPS P.g. stimulated mineral release and matrix degradation in the parietal bone organ cultures by increasing differentiation and formation of mature osteoclasts, a response dependent on increased RANKL. LPS P.g. stimulated RANKL in parietal osteoblasts dependent on the presence of TLR2 and through a MyD88 and NF-κB mediated mechanism. Similarly, the TLR2 agonists HKLM, FSL1, Pam2 and Pam3 stimulated RANKL in osteoblasts and parietal bone resorption. LPS P.g. and Pam2 robustly enhanced osteoclast formation in periosteal/endosteal cell cultures by increasing RANKL. LPS P.g. and Pam2 also upregulated RANKL and osteoclastic genes *in vivo*, resulting in increased number of periosteal osteoclasts and immense bone loss in *wild type* mice but not in *Thr2* deficient mice. These data demonstrate that LPS...
Porphyromonas gingivalis (P.g.) stimulates periosteal osteoclast formation and bone resorption by stimulating RANKL in osteoblasts via TLR2. This effect might be important for periodontal bone loss and for the enhanced bone loss seen in RA patients with concomitant periodontal disease.

Rheumatoid arthritis (RA)¹, psoriatic arthritis, septic arthritis, reactive arthritis, periodontitis, peri-implantitis, joint prosthetic loosening and osteomyelitis are bone-related inflammatory processes associated with infiltration of a wide variety of cells involved in the innate and acquired immune responses. Breakdown of supporting tissues such as cartilage, juxta-articular bone, jaw bone and bone retaining prosthesis and tooth implants is the reason for joint destruction and for the loosening of teeth and implants (1-4). Bone loss is mainly due to increased formation and activity of osteoclasts generated by fusion of hematopoietic myeloid mononuclear progenitor cells (5). M-CSF is required for proliferation and survival of the progenitors, and the receptor activator of NF-κB ligand (RANKL) is required for fusion and differentiation to osteoclasts (1-5). The decoy receptor osteoprotegerin (OPG) binds and neutralizes RANKL.

In inflammatory conditions, osteoclastogenesis is believed to be caused by increased expression of cytokines, which increases RANKL/OPG ratio in either osteoblasts or in other resident cells, such as synovial fibroblasts or periodontal ligament cells. To this group of cytokines belong IL-1β, IL-6, IL-11, IL-17, TNF-α, LIF, oncostatin M (OSM) and cardiotoxin-1 (CT-1) (5-7). During recent years, the potential role of the innate immune system for inflammation-induced bone resorption has attracted increasing interest. Resident cells and infiltrating leukocytes express pattern recognition receptors (PRRs), including TLRs that respond to pathogen-associated molecular patterns (PAMPs) expressed by bacteria, viruses, and fungi (8). These receptors also respond to host-derived molecules generated during cell death, inflammation and tissue damage (9, 10). It has been repeatedly shown that LPS from different bacteria can stimulate osteoclast formation and bone resorption in vitro and in vivo and that the effect is due to activation of TLR4 (11-13).

Porphyromonas gingivalis (P.g.) is a gram-negative bacteria present in the biofilm on teeth and associated with periodontitis (14, 15). LPS preparations from P.g. is different to LPS from other bacteria and can be either an agonist or antagonist of TLR4, or even without affinity to TLR4, depending on modifications of the lipid A moiety caused by environmental conditions. LPS preparations from P.g. often are potent agonists of TLR2 due to contamination with a lipoprotein with affinity to TLR2 (16). Oral infection with P.g. in mice causes inflammation-induced alveolar bone loss through activation of TLR2 (17-19). The mechanism by which P.g. induces bone loss is not fully understood since the role of TLR2 in osteoclastogenesis has been studied less as compared to TLR4. P.g., the synthetic TLR2 ligand Pam3 (palmitoyl-3-Cys-Ser-(Lys)₄) and lipoteichoic acid from S. aureus, similar to LPS from E. coli, inhibit RANKL-stimulated osteoclast formation in mouse bone marrow macrophage (BMM) cultures (20, 21). At variance, heat shock protein 60 potentiates RANKL-stimulated osteoclast formation in mouse BMM cultures, an effect not observed using cells from Tlr2 deficient mice (22). It was recently reported that the lipopolysaccharides Pam2 (palmitoyl-2-Cys-Ser-...
(Lys)₃ and Pam3 stimulate local and systemic bone loss, as evidenced by micro computed tomography (µCT) when administered s.c. or i.p., respectively (23). This effect was mainly attributed to a direct effect by Pams on osteoclast progenitors since Pam2 and Pam3, similar to LPS *E. coli*, stimulated osteoclast formation in RANKL-primed BMM cultures.

Clinical and epidemiological data indicate that periodontitis is associated to RA (24). Periodontal disease is more common and severe in RA patients than in healthy controls (25-27) and management of periodontitis seems to decrease the severity of RA (28, 29). Several lines of evidence indicate that the link between periodontitis and RA could be the periodontitis-associated bacteria *P. g.* DNA from *P. g.* has been detected in serum and synovial fluid from patients with RA (30, 31) and enhanced antibody titers against *P. g.* have been found in RA patients (32, 33). Moreover, periodontitis and RA have been suggested to involve citrullination of proteins by the peptidylarginine deiminase expressed by *P. g.*, which then could drive autoimmunity in RA (34). Experimentally, it has been shown that preexisting s.c. inflammation due to infection with heat-killed *P. g.* resulted in more severe adjuvant arthritis (35) and that preexisting periodontitis caused by oral infections with *P. g.* caused more advanced arthritis in a mouse model of collagen antibody-induced arthritis (36). Similar observations have been made in mice with concurrent periodontitis caused by oral *P. g.* infection and collagen type II induced arthritis (37), where mice with periodontitis exhibited more severe arthritic bone loss with no effect on cartilage destruction.

Data showing stimulatory or inhibitory effects on osteoclastogenesis by stimulation of TLR4 and TLR2 have been obtained using osteoclast progenitor cells from either bone marrow or peripheral blood. Functional osteoclasts are only formed on bone surfaces. We, therefore, focused our studies on the effect by LPS *P. g.* on periosteal osteoclast formation and bone resorption using ex vivo cultures of mouse parietal bones and an in vivo model using local injections with P.g. Our aim was also to evaluate if LPS *P. g.* could enhance osteoclastogenesis not only directly on primed osteoclast progenitors but also indirectly through increased RANKL production in resident cells. We report here that LPS *P. g.* stimulates periosteal osteoclast formation ex vivo and in vivo due to induction of RANKL in osteoblasts by activation of TLR2.

**MATERIALS AND METHODS**

*Materials*: Recombinant mouse cytokines and neutralizing antibodies, Quantikine® ELISA kits for RANKL and OPG (R&D Systems); BMS-345541 and Celastrol (Sigma-Aldrich); α-MEM, fetal calf serum (FCS), zolendronic acid, and indomethacin (Invitrogen); [³⁵Ca]CaCl₂ (Amersham Biosciences); oligonucleotide primers and probes (Invitrogen or Applied Biosystems); LPS *P. g.* (version 10G20-MT), other TLR2 and TLR4 agonists and primers (InvivoGen, and R&D systems); RatLaps™ CTX ELISA kit (Immonodiagnosticesystems); Prostaglandin E2 [³²P]-RIA® kit (PerkinElmer); RNAqueous–4 PCR® kit (Ambion); High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems); Kapa2G™ Robust HotStart PCR kit, Kapa™ Probe Fast qPCR kit (KapaBiosystems); TaqMan® Fast Advanced Master Mix (Life Technologies); RNAlater®, RNaseasy®, Cignal Lenti Reporter Assay® kits (Qiagen); Luciferase Assay System (Promega).

*Animals*: CsA mice from our own inbred colony were used for most experiments. CB57BL/6J and B6.129 Tlr²⁻⁻/⁻/⁻ mice were purchased from Jackson Laboratories. MyD88⁻⁻ mice (38) and their wild type C57BL/6 mice were bred at the Laboratory for Experimental Biomedicine, Sahlgrenska Academy at the University of Gothenburg. Animal care and experiments were approved and conducted in accordance with accepted
standards of humane animal care and used as deemed appropriate by the animal care and use committees of Umeå University, Umeå, Sweden, and the University of Gothenburg, Gothenburg, Sweden.

Osteoclast formation and bone resorption in cultured mouse bones - Parietal bones from 5-7 day-old mice were microdissected, cut into either parietal halves or quarters and then cultured as previously described (39, 40).

Mineral mobilization was assessed by analyzing the release of $^{45}$Ca from bones pre-labelled in vivo with 1.5 $\mu$Ci $^{45}$Ca. For the time-course experiments, mice were injected with 12.5 $\mu$Ci $^{45}$Ca, and radioactivity was analyzed at different time points by extraction of small amounts of culture medium.

Bone extracellular matrix degradation was assessed by analyzing the amount of type I collagen degradation fragments (CTX) in culture media released from parietal halves using the RatLaps™ kit.

Osteoclast formation was assessed by counting the number of cathepsin K positive osteoclasts and osteoclast differentiation by analyzing expression of osteoclastic and osteoclastogenic genes.

Osteoblast isolation and culture - Bone cells were isolated from 2-3 day-old mouse parietal bones by time sequential digestion with bacterial collagenase (41). Cells from digestions 6 to 10 were used and plated at a density of $10^6$ cells/cm$^2$. At the end of the cultures, RNA was isolated for gene expression analysis.

Osteoclast formation in periosteal cell cultures - Periosteal and endosteal cells were isolated from 2-3 day-old mice and cells from all 10 digestions were pooled (41). These isolations contain both osteoblast and osteoclast progenitors and stimulation by RANKL results in robust formation of bone-resorbing osteoclasts. The periosteal cells were seeded at a density of $10^5$ cells/cm$^2$ and incubated for 9 days. At the end of the cultures, cells were stained for tartrate-resistant acid phosphatase (TRAP) and TRAP$^+$ cells with more than three nuclei were counted (TRAP$^+$MuOCL). RNA was also isolated for gene expression analysis.

Osteoclast formation in bone marrow macrophage cultures - Mouse bone marrow cells were incubated with 30 ng/ml M-CSF for two days (42). The adhering macrophages were incubated for 3-4 days with 200 µl of medium containing either M-CSF (30 ng/ml) or M-CSF+ RANKL (4 ng/ml), with or without TLR-2 agonists. At the end of the cultures, the cells were stained for TRAP and TRAP$^+$MuOCL counted.

Osteoclast differentiation and bone loss in vivo - Five week-old male mice were injected with 100 µl of LPS $P.g.$ (500 µg), Pam2 (50 µg) or NaCl s.c. over the skull bones and sacrificed after 6 days. The skull bones were dissected and analyzed for the number of TRAP$^+$ osteoclasts, for bone loss and for gene expression.

High-resolution µCT analysis - Skull bones were scanned by high-resolution µCT (Skyscan 1172) at 50 kV, 201µA and with 13.5 µm voxel size. Image reconstructions were made by NRecon software.

Immunostaining of osteoclasts - Parietal bones from newborn mice were immunostained for cathepsin K as previously described (43). The number of cathepsin K positive multinucleated cells per section was determined. Control stainings without primary antibody did not show any positive reaction.

Enzyme histochemistry - Skull bones from 5 week-old mice were fixed, decalcified in 10% EDTA and TRAP$^+$MuOCL detected using the Naphtol AS-BI phosphate method. Staining was performed by Histocenter AB, Gothenburg, Sweden according to its accredited protocol.

Gene expression analyses - RNA was isolated from parietal bones and cell cultures using either the RNAqueous®–4 PCR kit or
RNeasy kit. RNA from 5 week-old mice skull bones was prepared in Trizol, after homogenization, and purified using the RNeasy® kit. RNA from unstimulated and stimulated groups was isolated at each time point. Single-stranded cDNA was synthesized from 0.1-0.5 µg of total RNA using a High-Capacity cDNA Reverse Transcription Kit.

Semi-quantitative RT-PCR analyses of the mRNA expression were performed using the Kapa2G™ Robust HotStart PCR kit. Sequences of the primers are given upon request.

Quantitative real-time PCR analyses were performed using either the Kapa™ Probe Fast qPCR kit or the TaqMan® Fast Advanced Master Mix with primers and probe as described previously (44, 45). Amplifications were performed with the ABI PRISM 7900 HT Sequence Detection System and Software or with the StepOnePlus Real-Time PCR system. β-actin was used as housekeeping gene in all analyses.

**RANKL and OPG protein analyses** - Half parietal bones were cultured for 96 h. Bones cells were lysed with 0.2% Triton X-100 and the amount of RANKL and OPG protein was assessed by measuring the levels of RANKL and OPG in the bone lysates using Quantikine® ELISA kits.

**Analysis of prostaglandin E₂** - The formation of prostaglandin E₂ (PGE₂) was assessed by analyzing the release of PGE₂ from cultured parietal bones to culture media, using the RIA-kit.

**Neutralizing antibody experiments** - Initial control experiments ensured that the antibodies used specifically abolished mRNA expression of Tnfsf11 (encoding RANKL) in parietal bones stimulated by either IL-1β, IL-6+sIL-6R, IL-11, LIF, OSM or TNF-α, respectively. The antibodies were then added solely, or in different combinations, with LPS P.g. or Pam2 to parietal bones or isolated osteoblasts. The effects on mineral release and Tnfsf11 mRNA expression were then assessed.

**Reporter gene experiments** - Cells were transduced by lentiviral vectors expressing the luciferase reporter gene under the control of either the NF-κB response elements or positive or negative control at a multiplicity of infection of 10 for 24 h. Cells were then incubated in vector-free media with the TLR2 agonist. Luciferase was measured after harvesting at different time points by using the Luciferase Assay System and Mithras LB940 luminometer.

**Statistics** - All statistical analysis was performed using One-Way ANOVA with Shapiro-Wilk’s normality test and post hoc Holm-Sidak’s test or a Paired t-test (SigmaPlot, Systat Software Inc.). All experiments were performed at least 3-5 times with comparable results, and all data are presented as means+SEM.

**RESULTS**

**Stimulation of bone resorption in parietal bones by LPS Porphyromonas gingivalis** - LPS P. g. increased the release of ⁴⁵Ca from parietal bones in a time- and concentration-dependent manner (Fig. 1A and B). LPS P.g. also enhanced the release of CTX (Fig. 1C).

Stimulation of ⁴⁵Ca release caused by LPS P.g. was inhibited by the bisphosphonate zoledronic acid (Fig. 1D).

LPS P.g. significantly enhanced the number of cathepsin K positive multinucleated osteoclasts on bone surfaces (Fig. 1E and F).

We next analyzed the effects by LPS P.g. on gene expression by isolating RNA from the parietal bones. The mRNA expression of Ctsk (encoding cathepsin K) was time- and concentration- dependently increased by LPS P.g. (Fig. 1G and H). LPS P.g. also increased the mRNA expression of Ap5 (encoding TRAP; Fig. 1). The mRNA expression of the early response gene c-Fos
was increased by LPS P.g. at 1 h, and still at 48 h (Fig. 1f), a response dependent on the concentration of LPS P.g. (Fig. 1k).

**Bone resorption induced by LPS P. gingivalis is due to increased RANKL.** Gene expression analyses using RNA from the parietal bones showed that LPS P.g. enhanced the mRNA expression of Tnfrsf11a (encoding RANK), Tnfsf11 (encoding RANKL), Csf1 (encoding M-CSF), Csf1r (encoding the M-CSF receptor c-Fms) and Oscar, whereas Tnfrsf11b (encoding OPG) mRNA was unaffected (Fig. 2A). q-PCR analyses showed that LPS P.g. caused a time- and concentration-dependent, robust increase of Tnfsf11 mRNA expression (Fig. 2B and C). In contrast, Tnfrsf11b mRNA was unaffected (Fig. 2B and C). q-PCR also confirmed that LPS P.g. enhanced Tnfrsf11a, Csf1r, Oscar and Csf1 mRNA (data not shown).

LPS P.g. significantly enhanced RANKL protein in the parietal bones (Fig. 2D), but OPG protein was not significantly changed (Fig. 2E).

The increased release of $^{45}$Ca induced by LPS P.g. was abolished by the addition of OPG (Fig. 2F). The inhibition of $^{45}$Ca release by OPG was associated with decreased mRNA expression of Ctsk (Fig. 2G), but not of Tnfsf11 (Fig. 2H), showing that OPG acted downstream RANKL formation to inhibit osteoclast formation.

**The importance of TLR2 for the stimulatory effect of LPS P. gingivalis in parietal bones.** TLR2 forms heterodimers with either TLR1 or TLR6 (46). Mouse parietal bones express Tlr1, Tlr2, Tlr6 and Tlr4 mRNA (Fig. 3A). TLR2-TLR1 and TLR2-TLR6 heterodimers recognize triacylated and diacylated lipopeptides, respectively. Pam2 time-dependently stimulated $^{45}$Ca release (Fig. 3B) and increased bone matrix degradation (Fig. 3C) in the organ cultured parietal bones. RNA was isolated from the parietal bones and Pam2 was found to increase the mRNA expression of Ctsk (Fig. 3D). In agreement with this finding, Pam2 increased the number of cathepsin K positive osteoclasts in the parietal bones (Fig. 3E).

Pam2, similar to LPS P.g., increased the mRNA expression in the parietal bones of Tnfrsf11a, Tnfsf11, Csf1, Csf1r and Oscar, without affecting that of Tnfrsf11b (Fig. 3F). Pam2 caused a robust, time-dependent enhanced Tnfsf11 mRNA expression, but did not affect Tnfrsf11b mRNA (Fig. 3G). q-PCR also confirmed that Pam2 increased the mRNA expression of Tnfrsf11a, Csf1r, Oscar and Csf1 mRNA (data not shown). Pam2 significantly enhanced RANKL protein without affecting OPG in the parietal bones (Fig. 3H and I).

Three additional TLR2 agonists, Pam3, HKLM (heat-killed preparation of *Listeria monocytogenes*) and FSL-1 (a synthetic lipoprotein from *Mycoplasma salivarium*), stimulated $^{45}$Ca release from mouse parietal bones (Fig. 3J) and robustly increased Tnfrsf11 mRNA in the parietal bones (Fig. 3K), but did not impact Tnfrsf11b mRNA (Fig. 3L).

**The stimulatory effect by LPS P. gingivalis is not mediated by osteotropic cytokines or prostaglandins.** LPS P.g. and Pam2 rapidly (1 h) and concentration-dependently increased the mRNA expression in the parietal bones of Il1b, Il6, Il11, Lf, Osm and Tnfsf2 (encoding TNF-α) (data now shown), all known to stimulate bone resorption (4, 5). LPS P.g. and Pam2 also enhanced the expression of Ptg2 (encoding cyclooxygenase-2) and the release of PGE$_2$ from the parietal bones (data not shown). Neutralizing IL-1β, IL-6, IL-11, LIF, OSM and TNF-α by specific antibodies, either one-by-one (data not shown) or by adding all together (Fig. 3M) showed that the effects of LPS P.g. and Pam2 on mineral release (Fig. 3M) and on Tnfrsf11 mRNA expression (data now shown) were independent of these proinflammatory mediators. Blocking prostaglandin synthesis with indomethacin did not affect LPS P.g. and Pam2 stimulated $^{45}$Ca release (Fig. 3N) but it did partially reduce Tnfrsf11 mRNA (data not shown).
LPS P. gingivalis stimulates RANKL in parietal osteoblasts through TLR2 - Since osteoblasts have been shown to produce RANKL in response to a variety of bone resorbing-hormones and cytokines (4, 5, 47, 48), we investigated if these cells responded to the different TLR2 agonists with increased RANKL. Mouse parietal osteoblast cultures expressed Tlr1, Tlr2, Tlr4 and Tlr6 mRNA and Tlr2 mRNA was upregulated by LPS P.g. and Pam2 (Fig. 4A). LPS P.g. also caused a time- and concentration-dependent increase of Tnfsf11 mRNA expression in the isolated osteoblasts, without affecting Tnfsf11b mRNA (Fig. 4B and C). Similarly, Pam2 increased Tnfsf11mRNA but not Tnfsf11b mRNA in these cells (Fig. 4D). Increased Tnfsf11 mRNA in osteoblasts was also observed when cells were stimulated by Pam3, HKLM and FSL-1 (Fig. 4F).

LPS P.g. and Pam2, increased the mRNA expression of Il1b, Il6, Il11, Lif, Osm and Tnfsf2 in the parietal osteoblasts (data not shown). Also Pam3, HKLM and FSL-1 increased the mRNA expression of Il1b, Il6 and Tnfsf2 in the parietal osteoblasts (data not shown). Neutralization of IL-1β, TNF-α, IL-11, Lif, OSM and IL-6 did not affect LPS P.g. or Pam2 induced Tnfsf11 mRNA in the osteoblasts (Fig. 4F-H).

Using osteoblasts isolated from Tlr2 deficient mice we found that Tnfsf11 mRNA induced by LPS P.g., Pam2, Pam3, HKLM and FSL-1, but not by LPS E.coli, was dependent on Tlr2 expression (Fig. 4I).

LPS P. gingivalis stimulates RANKL in parietal periosteal osteoblasts through MyD88 and NF-κB - We next sought to determine by which mechanism stimulation of TLR2 in osteoblasts results in increased Tnfsf11 expression. First, we found that stimulation of Tnfsf11 mRNA in osteoblasts by LPS P.g., Pam2, Pam3, HKLM and FSL-1 was critically dependent on the presence of MyD88 (Fig. 4J). Next, we showed that LPS P.g. and Pam2 activated NF-κB as assessed both by increased mRNA expression of p50, p52, p65 and RelB (Fig. 4K) and by activation of a luciferase reporter gene driven by NF-κB (Fig. 4L). Stimulation of Tnfsf11 mRNA, as well as of the well-known NF-κB target Il6, by LPS P.g. and Pam2 was abolished by BMS and Celastrol, two inhibitors of NF-κB activation acting either on IKKα/IKKβ (BMS) or on TAK1 upstream of IKKβ involved in canonical activation of NF-κB (Celastrol) (Fig. 4M and N).

P. gingivalis and Pam2 increase osteoclast formation in vivo by a TLR2 dependent mechanism - To investigate the in vivo relevance of our in vitro findings we injected LPS P.g. and Pam2 s.c. over skull bones in 5 week-old mice. Six days after the injections, the number of TRAP+ osteoclasts on the periosteal surface of the skull bones was enhanced by LPS P.g. and Pam2 (Fig. 5A and B). LPS P.g. and Pam2 increased the mRNA expression of c-Fos, Nfatc1, Atp5, Ctsk and Tnfsf11 in the skull bones, effects absent in Tlr2 knockout mice (Fig. 5C-G). The enhanced number of osteoclasts resulted in extensive loss of bone in wild type compared to Tlr2 deficient mice as assessed by μCT analyses in LPS P.g. and Pam2 treated mice (Fig. 5H).

P. gingivalis stimulates osteoclast formation in periosteal/endosteal cell cultures through RANKL - It has been reported that P.g. bacteria and Pam3 inhibit RANKL-induced osteoclast differentiation in BMM cultures (20) in contrast both to the RANKL-dependent stimulation of periosteal osteoclast formation observed in ex vivo cultures of mouse parietal bones and to the induction of osteoclastic genes observed in vivo in the present study. We, therefore, compared effects by LPS P.g. and Pam2 on osteoclast formation using cells from either periosteum/endosteum or bone marrow.

Co-stimulation with RANKL and either LPS P.g. or Pam2 did not affect RANKL-induced 45Ca release from parietal bones (Fig. 6A). Nor was RANKL-induced osteoclast formation (Fig. 6B and C) or the expression of osteoclastic genes Ctsk and Atp5 (Fig. 6D and E) in isolated periosteal/endosteal cell cultures affected by
co-treatment with LPS P. g. or Pam2. In agreement with previous findings (20), LPS P. g. and Pam2 abolished osteoclast formation and mRNA expression of Ctsk and Acp5 in BMM cultures stimulated by M-CSF/RANKL (Fig. 6F-H).

In the absence of exogenous RANKL, LPS P. g. and Pam2 stimulated formation of TRAP+ MuOCLs (Fig. 6I and J) and the expression of Acp5 and Ctsk (Fig. 6K and L) in periosteal/endosteal cell cultures, an effect associated with increased mRNA expression of Tnfsf11 (Fig. 6M). Osteoclast formation in these cultures by LPS P.g. and Pam2 was abolished by adding OPG (Fig. 6N).

DISCUSSION

Previous studies have shown that oral infection with P. g. not only causes local alveolar bone loss (17-19) but also enhances articular bone loss in arthritic mice (35-37). These studies do not reveal by which mechanisms P. g. infection causes decreased bone mass. In the present study, we show that locally injected LPS P. g. s.c. above mouse skull bones induces bone loss and excessive osteoclast formation, due to enhanced osteoclastogenesis as assessed by increased expression of osteoclastogenic transcription factors and osteoclastic genes. Increased osteoclastogenesis might be due either to a direct effect by P. g. on osteoclast progenitors or by an indirect effect due to increased RANKL/OPG ratio. In favor of the latter view, we here show for the first time that LPS P.g. robustly enhances the mRNA expression of Tnfsf11 in vivo. To investigate if P.g. can affect RANKL/OPG in osteoblasts, we studied the effect by LPS P.g. in ex vivo cultures of mouse parietal bones and in isolated mouse parietal osteoblasts.

In the parietal bones, LPS P.g. stimulated the release of mineral and the degradation of bone matrix. Similar to the observations in vivo, LPS P.g. enhanced the number of mature osteoclasts and the expression of osteoclastic genes, in addition to the osteoclastogenic transcription factor c-Fos. In the organ-cultured bones, LPS P.g. enhanced the RANKL/OPG ratio by a mechanism due exclusively to increased RANKL.

Since osteoblasts/osteocytes are important for RANKL production in physiological bone remodeling (47, 48) we assessed if osteoblasts also could produce RANKL in pathological bone resorption induced by P.g. Challenge of the osteoblasts with LPS P.g. increased Tnfsf11 mRNA expression with no effect on Tnfrsf11b mRNA, which demonstrates that osteoblasts are target cells for P.g. induced RANKL production. Moreover, exogenous OPG abolished both LPS P.g. induced mineral release and the upregulation of Ctsk mRNA expression without affecting the enhanced Tnfsf11 mRNA, showing that the bone-resorptive response by LPS. P.g. was totally dependent on increased RANKL.

We further demonstrated the important role of RANKL for P.g. induced osteoclast formation by using a cell culture system based upon isolation of periosteal/endosteal cells from mouse parietal bones containing both osteoblasts and osteoclast progenitor cells. LPS P.g. robustly increased the formation of osteoclasts, the expression of osteoclastic genes and Tnfsf11 mRNA, similar to the observations in the intact bones in vivo and ex vivo. Also in this system, the LPS P.g. induced osteoclast formation was totally dependent on RANKL since osteoclast formation was abolished by adding OPG. Although we here demonstrate the potent stimulatory effect by LPS P.g. on RANKL formation in osteoblasts we cannot exclude, however, that other cells present in vivo in the inflammatory reaction also contribute to the RANKL response.

Since previous studies have shown that co-stimulation of osteoclast progenitors from bone marrow with RANKL and P.g. inhibits osteoclast differentiation (20), we wondered why differentiation of osteoclast progenitors present on the surfaces of parietal bones was not inhibited, but on the contrary, stimulated. The fact that RANKL-stimulated mineral release in the parietal
bones and that RANKL-stimulated osteoclast formation in the periosteal/endosteal cell cultures was unaffected by co-stimulation with LPS P.g., whereas co-stimulation in the BMM cultures abolished osteoclast formation, show that the osteoclastic P.g. response in osteoclast progenitors on the bone surfaces are different from that in bone marrow progenitors. We do not know if the difference is because periosteal/endosteal osteoclast progenitors lack TLR2 or if the surrounding cells make them insensitive to P.g. induced inhibition. The fact that cocultures of mouse BMM and mouse parietal osteoblasts respond to the TLR2 agonists Pam2 and Pam3 with enhanced RANKL production and increased osteoclast formation argues for the latter explanation (23). It seems that observations made in BMM cultures might not be fully relevant to osteoclastogenesis at the bone surface. Since mature osteoclasts are formed only at bone surfaces our findings suggest that studies on osteoclastogenesis also should include studies with osteoclast progenitors present at bone surfaces.

Similar to LPS P.g., four other TLR2 agonists (HKLM, FSL-1, Pam2 and Pam3) stimulated mineral release and Tnfsf11 mRNA in the parietal bones and in parietal osteoblasts, an effect lost in osteoblasts from Tlr2 deficient mice. These data show that TLR2 activation in osteoblasts is linked to RANKL formation, osteoclast formation and bone resorption. In agreement with these findings, bone loss, enhanced mRNA expression of Asep5 and Cisk, as well as increased Tnfsf11 mRNA, were not observed in skull bones in Tlr2 deficient mice when LPS P.g. or Pam2 were injected s.c. Similarly, decreased alveolar bone volume observed in mice with oral infection of P.g. was not seen in Tlr2 deficient mice (17, 19). All together, these findings show that P.g. can stimulate osteoclast formation, bone loss and RANKL production by activating TLR2, although we can not exclude that P.g. bacteria can affect bone cells also through other PRRs than TLR2.

We next evaluated by which mechanism LPS P.g. and Pam2 stimulate Tnfsf11 mRNA in osteoblasts and found the presence of the adapter protein MyD88 to be crucial, similar to the inhibitory effect in RANKL-stimulated BMM and the stimulatory effect in RANKL-primed BMM (20, 23). We then evaluated the importance of NF-κB and found that LPS P.g. and Pam2 activated NF-κB as demonstrated by increased mRNA expression of the four NF-κB subunits p50, p65, p52 and RelB and by activation of a NF-κB reporter gene transfected in the osteoblasts. The crucial role of NF-κB was shown by the finding that two NF-κB inhibitors, BMS and Celastrol, abolished LPS P.g. and Pam2 induced Tnfsf11 mRNA. BMS inhibits both canonical and non-canonical NF-κB pathways by inhibiting IKKα and IKKB, whereas Celastrol inhibits TAK1 which is upstream IKKB activation in the canonical pathway (49).

Recently it was shown in an elegant study that Tlr2−/− mice become sensitive to P.g. induced alveolar bone loss after adoptive transfer of wild type bone marrow-derived macrophages (18). This finding suggests an important role of macrophages in P.g. induced bone loss in mice with global deletion of Tlr2, including in osteoblasts. One possibility might be that P.g. stimulates macrophages to differentiate to mature osteoclasts. Another reason might be that P.g. stimulates macrophages to release cytokines, thus enhancing RANKL production in osteoblasts. The knowledge about the relative role of osteoblasts and macrophages as primary targets in P.g. induced bone loss has to await studies using mice with cell-specific deletion of Tlr2.

Here, we report that activation of TLR2 in osteoblasts by P.g. increases RANKL production, osteoclast formation and bone loss both ex vivo and in vivo. Our findings provide explanation why P.g. can stimulate alveolar bone loss, but might also contribute to our understanding of why oral infection with P.g. seems to cause a more severe loss of juxta-articular bone in RA. TLR2, which
is highly expressed in RA synovium (50-52), not only activated by PAMPs such as P.g. but also by endogenous ligands present in RA synovium such as gp96 (53) and Snapin (54). Our data may also help to explain the role of endogenous ligands in the pathogenesis of RA bone erosions.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

AK performed most of the experiments and analyses; PH performed the gene reporter and MyD88 osteoblast experiments; PL and PS contributed to study conception and design; CL performed immunohistochemical analysis and contributed to study conception and design; UHL designed and supervised the project. All authors were involved in drafting the article or revising it critically for important intellectual content.

REFERENCES


TLR2 stimulates bone resorption

**FIGURE 1.** LPS from *Porphyromonas gingivalis* (P.g.) stimulates bone resorption, osteoclast formation and expression of osteoclastic and osteoclastogenic genes in organ cultures of neonatal mouse parietal bones. (A-C) LPS P.g. time- and concentration-dependently increased 45Ca and CTX release from the parietal bones. (D) The stimulatory effect by LPS P.g. on 45Ca release was inhibited by zoledronic acid (0.2 µmol/l). (E) The number of cathepsin K positive (CTSK+) osteoclasts was enhanced by LPS P.g. (G and H) LPS P.g. time- and concentration-dependently enhanced the mRNA expression of *Ctsk*. (I and J) The mRNA expression of *Aap5* (48 hr) and *c-Fos* (1 and 48 hr) was increased by LPS P.g. (K) LPS P.g. concentration-dependently increased *c-Fos* mRNA. *P<0.05; **P<0.01 compared to unstimulated controls (C-E, I, and J) or to LPS P.g. stimulated bones (D). LPS P.g. was used at a concentration of 10 µg/ml in Fig. 1A, C-G, I and J. Data are means of 4-5 observations and SEM is given as vertical bars when larger than the radius of the symbol.

**FIGURE 2.** The stimulatory effect on bone resorption in neonatal mouse parietal bones by LPS from *Porphyromonas gingivalis* (P.g.) is dependent on increased RANKL. (A) LPS P.g. enhanced the mRNA expression of *Tnfrsf11a*, *Tnfsf11*, *Csf1*, *Csf1r* and *Oscar*, without affecting *Tnfrsf11b*. (B and C) LPS P.g. time- and concentration-dependently enhanced *Tnfsf11* mRNA with no effect on *Tnfrsf11b* mRNA. (D and E) LPS P.g. enhanced the cellular level of RANKL protein without affecting OPG protein. (F-H) The stimulatory effect by LPS P.g. on 45Ca release and *Ctsk* mRNA was inhibited by adding exogenous OPG (300 ng/ml) to the culture medium whereas *Tnfrsf11* mRNA was unaffected. **P<0.01; ***P<0.001 compared to unstimulated controls (D and F-H) or to LPS P.g. stimulated bones (F and G). LPS P.g. was used at a concentration of 10 µg/ml in Fig. 2A, B, and D-H. Data are means of 4-5 observations and SEM is given as vertical bars when larger than the radius of the symbol.

**FIGURE 3.** The lipopeptide palmitoyl-2-Cys-Ser-(Lys)₄ (Pam2) and three additional TLR2 agonists stimulate bone resorption, osteoclast formation and expression of osteoclastic and osteoclastogenic genes in organ cultures of neonatal mouse parietal bones by an effect dependent on RANKL but independent on cytokine and prostaglandin formation. (A) LPS P.g. and Pam2 did not affect the mRNA expression of *Tlr1*, *Tlr2*, *Tlr4* and *Tlr6*. (B-E) Pam2 increased the release of 45Ca (B) and CTX (C), upregulated *Ctsk* mRNA (D) and enhanced the number of cathepsin K positive (CTSK+) osteoclasts (E). (F) Pam2 increased the mRNA expression of *Tnfrsf11a*, *Tnfrsf11*, *Csf1*, *Csf1r* and *Oscar* without affecting *Tnfrsf11b*. (G-I) Pam2 time-dependently increased *Tnfsf11* mRNA (G) resulting in increased RANKL protein after 48 hr (H), without affecting *Tnfrsf11b* mRNA (G) or OPG protein (I). (J-L) Palmitoyl-3-Cys-Ser-(Lys)₄ (Pam3; 10 ng/ml), heat-killed *Listeria monocytogenes* (HKLM; 10⁷ UFC) and a synthetic lipoprotein from *Mycoplasma salivarium* (FSL-1; 0.1 µg/ml) stimulated 45Ca release (J) and the mRNA expression of *Tnfrsf11* (K) without affecting *Tnfrsf11b* (L). (M and N) The stimulatory effect by LPS P.g. and Pam2 on 45Ca release was unaffected by adding a cocktail of antibodies neutralizing IL-1β, IL-6, IL-11, LIF, OSM and TNF-α or by adding indomethacin (1 µmol/l). *P<0.05; **P<0.01; ***P<0.001 compared to unstimulated controls (C, E, H, J, K, M, and N). LPS P.g. was used at a concentration of 10 µg/ml in Fig. 3A, M and N. Pam2 was used at a concentration of 10 ng/ml in Fig. 2A-I, M, and N. Data are means of 4-5 observations and SEM is given as vertical bars when larger than the radius of the symbol.
FIGURE 4. Five different TLR2 agonists enhanced Tnfsf11 mRNA expression in mouse parietal osteoblasts by a mechanism dependent on TLR2, MyD88 and NF-κB but independent on cytokine formation. (A) LPS P.g. and Palmitoyl-2-Cys-Ser-(Lys)₄ (Pam2) upregulated Tlr2 without affecting Tlr1, Tlr4 or Tlr6. (B and C) LPS P.g. time- and concentration-dependently enhanced Tnfsf11 mRNA without affecting Tnfrsf11b. (D) Pam2 time-dependently increased Tnfsf11 mRNA without affecting Tnfrsf11b. (E) Palmitoyl-3-Cys-Ser-(Lys)₄ (Pam3; 10 ng/ml), heat-killed Listeria monocytogenes (HKLM; 10⁷ UFC) and a synthetic lipoprotein from Mycoplasma salivarium (FSL-1; 0.1 µg/ml) stimulated Tnfsf11 mRNA. (F-H) The stimulatory effect by LPS P.g. and Pam2 on Tnfsf11 mRNA was unaffected by adding antibodies neutralizing IL-1β, IL-6, IL-11, LIF, OSM and TNF-α. (I) LPS P.g., Pam2, Pam3 (10 ng/ml), HKLM (10⁷ UFC) and FSL-1 (0.1 µg/ml), but not LPS from E. coli (10 µg/ml), increased Tnfsf11 mRNA in osteoblasts from wild type (Wt) but not from Tlr2 deficient mice. (J) LPS P.g., Pam2, Pam3 (10 ng/ml), HKLM (10⁷ UFC) and FSL-1 (0.1 µg/ml) enhanced Tnfsf11 mRNA in osteoblasts from Wt mice but not from MyD88 deficient mice. (K) LPS P.g. and Pam2 increased the mRNA expression of the four NF-κB subunits p50, p52, p65 and RelB. (L) LPS P.g. and Pam2 increased NF-κB driven luciferase in transfected osteoblasts. (M and N) The stimulatory effect by LPS P.g. and Pam2 on Tnfsf11 (M) and Il6 mRNA (N) was inhibited by the two NF-κB inhibitors BMS (10 µmol/l) and Celastrol (0.2 µmol/l). **P<0.01; ***P<0.001 compared to unstimulated controls (E-K) or to LPS P.g. stimulated osteoblasts (M and N). LPS P.g. was used at a concentration of 10 µg/ml in Fig. 4A, B, and F-K. Pam2 was used at a concentration of 10 ng/ml in Fig. 4A, D, and F-N. Data are means of 4-5 observations and SEM is given as vertical bars when larger than the radius of the symbol.

FIGURE 5. Injection of LPS from Porphyromonas gingivalis (P.g.) and the TLR2 agonist palmitoyl-2-Cys-Ser-(Lys)₄ (Pam2) above skull bones stimulate osteoclast formation, expression of osteoclastic and osteoclastogenic genes, and bone loss in skull bones from 5 week-old mice. (A and B) LPS P.g. and Pam2 enhanced the number of tartrate resistant acid phosphatase positive, multinucleated osteoclasts (TRAP⁺MuOCL); in left panel of (B) is seen parietal bone six days after injection of vehicle and in right panel of (B) is shown osteoclasts in parietal bones six days after injection of LPS P.g. (C-G) Injection of LPS P.g. or Pam2 increased the mRNA expression of c-Fos, Nfatc1, Acp5, Cisk and Tnfsf11 after three days in skull bones from wild type (Wt) but not from Tlr2 deficient mice. (H) Injection of LPS P.g. or Pam2 resulted in bone loss after six days in skull bones from Wt but not in Tlr2 deficient mice. Images shown are representative of seven images per group. **P<0.01; ***P<0.001 compared to unstimulated controls (A and C-G). Data are means of 6-7 observations and SEM is given as vertical bars.
FIGURE 6. LPS from *Porphyromonas gingivalis* (*P. g.*) and the TLR2 agonist palmitoyl-2-Cys-Ser-(Lys)$_4$ (Pam2) regulate bone resorption and osteoclast formation differently in parietal bones, periosteal/endosteal bone cell and bone marrow cell cultures primed by RANKL (RL). (A) RL (10 ng/ml) stimulated $^{45}$Ca release from neonatal mouse parietal bones in organ culture was not affected by LPS *P. g.* (10 µg/ml) or Pam2 (10 ng/ml). (B-E) RL (10 ng/ml) stimulation of tartrate resistant acid phosphatase positive, multinucleated osteoclasts (TRAP$^+$MuOCL) and mRNA expression of *Ctsk* and *Acp5* in periosteal/endosteal cell cultures from mouse parietal bone were not affected by co-treatment with LPS *P. g.* (10 µg/ml) or Pam2 (10 ng/ml). (F-H) Increased formation of TRAP$^+$ multinucleated osteoclasts and mRNA expression of *Ctsk* and *Acp5* in M-CSF (30 ng/ml) and RL (4 ng/ml) stimulated mouse bone marrow cell cultures were abolished by co-treatment with LPS *P. g.* and Pam2. (I-M) LPS *P. g.* (10 µg/ml) and Pam2 (10 ng/ml) increased formation of TRAP$^+$ multinucleated osteoclasts and mRNA expression of *Acp5*, *Ctsk* and *Tnfsf11* in periosteal/endosteal cell cultures from mouse parietal bone. (N) The stimulatory effect by LPS *P. g.* and Pam2 on osteoclast formation in periosteal/endosteal cell cultures was abolished by adding OPG (300 ng/ml) to the culture medium. ***P<0.001 compared to unstimulated controls. Data are means of 6-7 observations and SEM is given as vertical bars.
Figure 3.

A

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B

No. of CTSK+ cells (per section)

C

CTX-release (% of control)

D

mRNA Ctsk/actin (% of control)

E

Gene expression (% of control)

G

Gene expression (% of control)

H

RANKL protein (pg/bone)

I

OPG protein (pg/bone)

J

45Ca-release (% of initial)

K

mRNA Tnfrsf11a -actin

L

mRNA Tnfrsf11b -actin

M

45Ca-release (% of control)

N

45Ca-release (% of control)

[Time], (h)

1 4 8 12 24 48

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Figure 4.
Figure 5.

A

No. of TRAP+MnOCL (per section)

Control LPS P.g. Pam2

***

B

Control LPS P.g. Pam2

C

mRNA c-Fos/actin (% of control)

wt Tlr2−/−

**

D

mRNA Nfatc1/actin (% of control)

wt Tlr2−/−

***

E

mRNA Tnfsf11/actin (% of control)

wt Tlr2−/−

***

F

mRNA Acp5/actin (% of control)

wt Tlr2−/−

***

G

mRNA Ctsk/actin (% of control)

wt Tlr2−/−

***

H

Wt

Control LPS P.g. Pam2

Tlr2−/−

Control LPS P.g. Pam2
Figure 6.
Porphyromonas Gingivalis Stimulates Bone Resorption by Enhancing RANKL through Activation of Toll-like Receptor 2 in Osteoblasts

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