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Signaling pathways in osteoblast proinflammatory responses to infection by *Porphyromonas gingivalis*

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Introduction: We recently investigated global gene expression in ST2 mouse stromal cells infected by the periodontal pathogen *Porphyromonas gingivalis* using microarray technology, and found that the bacterium induces a wide range of proinflammatory gene expression. Here, we reported the signaling pathways involved in those proinflammatory responses.
Methods: ST2 cells and primary calvarial osteoblasts from C3H/HeN, C57BL/6, and MyD88-deficient (MyD88$^{-/-}$) mice were infected with *P. gingivalis* ATCC33277 and its gingipain-deficient mutant KDP136. Expression of the chemokines CCL5 and CXCL10, and matrix metalloproteinase-9 (MMP9) were quantified by real-time polymerase chain reaction, while phosphorylation of protein kinases and degradation of an inhibitor of nuclear factor-κB, IκB-α, were detected by Western blotting, and activation of transcriptional factors was determined by a luciferase reporter assay. The effects of inhibitors of transcriptional factors and protein kinases were also investigated.
Results: Infection by *P. gingivalis* elicited gene expression of CCL5, CXCL10, and MMP9 in both ST2 cells and osteoblasts. Western blot and reporter assay results revealed activation of nuclear factor-κB (NF-κB) and activator protein-1 transcription factors. The NF-κB inhibitor suppressed the expression of CCL5 and MMP9, but not that of CXCL10, whereas *P. gingivalis* infection induced significant CCL5 expression in MyD88$^{-/-}$ osteoblasts. In addition, activation of protease-activated receptors by trypsin elicited significant induction of CXCL10.
Conclusion: Our results suggest that various proinflammatory responses in *P. gingivalis*-infected stromal/osteoblast cells are NF-κB-dependent, but not always dependent on the Toll-like receptor/MyD88 pathway, while some responses are related to the activation of protease-activated receptors. Thus, *P. gingivalis* does not fully utilize well-established pathogen recognition molecules such as Toll-like receptors.

Key words: inflammatory response; nuclear factor-κB; *Porphyromonas gingivalis;* signaling pathway

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Marginal periodontitis, a chronic inflammatory disease of the supporting tissues of the teeth, is one of the most common types of human infection (14, 33). Chronic inflammation leads to destruction of periodontal tissue and alveolar bone resorption. A limited group of periodontal bacteria is considered to play an important role in the pathogenesis of the disease, with *Porphyromonas gingivalis* well-established as a pathogen related to severe forms of adult periodontitis (3, 16, 24).

P. gingivalis is a gram-negative, black-pigmented anaerobe that colonizes periodontal pockets and spreads into deeper tissues, including the connective and bone tissues (16, 24). This bacterium is also associated with periapical periodontitis and is isolated from root canals (20). Chronic infection by *P. gingivalis* and other periodontal pathogens has been

proposed to cause local inflammatory responses in gingival tissue. A number of *in vitro* studies have revealed that *P. gingivalis* infection triggers the production of a wide variety of cytokines and chemokines, including interleukin-1 (IL-1), IL-6, and CCL2 (MCP-1; monocyte chemoattractant protein-1), in gingival fibroblasts, epithelial cells, and endothelial cells, and suggested that *P. gingivalis* cell surface components, such as lipopolysaccharide (LPS) and fimbriae, are potent stimulators of production of these proinflammatory mediators (5, 9, 10, 15, 17, 22, 29, 30, 39, 42).

Recent advances in our understanding of innate immunity have shown that bacterial cell components such as LPS, peptidoglycans, and bacterial DNA are recognized by a family of innate pathogen recognition molecules named Toll-like receptors (TLRs) (2, 27). LPS and fimbriae of *P. gingivalis* have been reported to be recognized by TLRs, resulting in host cell activation and proinflammatory responses (10, 15, 30, 39). Myeloid differentiation factor 88 (MyD88) is an adaptor molecule critical for signaling from all TLRs except TLR3 (2, 27, 41), and MyD88-deficient ($MyD88^{-/-}$) mice do not respond to LPS or other microbial components such as peptidoglycan (21, 41).

Several recent studies revealed that osteoblasts and bone marrow stromal cells are responsible for the development of local inflammatory responses including bone resorption (13, 31, 32, 40, 45), and therefore, it is interesting to know the proinflammatory responses in osteoblasts and stromal cells induced by the local and chronic infection by periodontal pathogens. We previously profiled the global change of gene expression in a mouse ST2 osteogenic stromal cell line following infection with *P. gingivalis* using microarray technology (31). Those results showed that the expression of a variety of genes for chemokines, cytokines, and other proinflammatory factors were upregulated following *P. gingivalis* infection. Further, our findings suggested that the proinflammatory response to *P. gingivalis* cells might be more complex than that elicited by isolated bacterial components such as LPS and fimbriae (31). Although previous studies (29, 30, 44) have reported that cellular signaling factors such as nuclear factor-κB (NF-κB) were activated by stimulation with either *P. gingivalis* whole cells or isolated bacterial components, it is unknown whether a number of other signaling molecules are also activated by that infection.

In the present study, we investigated the signaling pathways involved in inflammatory gene expression in mouse stromal cells and primary osteoblasts in response to *P. gingivalis*. The major aim was to understand the activation profiles of the signaling pathways in cells infected by *P. gingivalis*. In our experiments, we focused on signal transduction that mediates the gene expression of CCL5, CXCL10 and matrix metalloproteinase 9 (MMP-9) because these proinflammatory factors were found to be clearly up-regulated by *P. gingivalis* infection in our previous study (31), and are known to be involved in the pathogenesis of human periodontitis and infectious bone disease (4, 12, 13, 26).

Materials and methods

Reagents and antibodies

SP600125, PD98059, SB203580, and GF109203X were purchased from Sigma-Aldrich (St Louis, MO). Carbobenzoxyl-L-leucinyl-L-leucinyl-L-leucinal (Z-LLL-al), an inhibitor of NF-κB (19), was purchased from the Peptide Institute (Osaka, Japan). Rabbit antibodies against total and phosphorylated kinases, including extracellular signal regulated protein kinase (ERK), p38 mitogen-activated protein kinase (MAPK), c-Jun, c-Jun N-terminal kinase (JNK), and protein kinase B (PKB/Akt kinase), were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated anti-rabbit antibody and the antibody against the inhibitor of NF-κB-α (IκB-α) were also purchased from Cell Signaling Technology. LPS from *Escherichia coli* O111:B4 was obtained from Sigma-Aldrich, it was also extracted from lyophilized cells of *P. gingivalis* ATCC33277 using a hot phenol–water procedure, and the crude extract was treated with nuclease and washed extensively with pyrogen-free water by ultracentrifugation, after which the chemical and immunobiological properties of the prepared LPS were confirmed, as described previously (22). Other chemicals were obtained from Sigma-Aldrich or Wako Pure Chemicals (Osaka, Japan).

Bacterial strains

P. gingivalis ATCC33277, KDP136 (a triple deficient mutant lacking three gingipains, Arg-gingipain-A, Arg-gingipain-B, and Lys-gingipain, of ATCC33277) (37) were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD) supplemented with yeast extract (0.1%; BBL), hemin (5 μg/ml; Sigma-Aldrich), and menadione (1 μg/ml; Sigma-Aldrich), as described previously (32). Bacterial cells were harvested by centrifugation, then washed with phosphate-buffered saline (PBS) and re-suspended in alpha minimum essential medium (αMEM; Gibco-BRL, Grand Island, NY) containing no antibiotics. The numbers of bacteria were determined using a spectrophotometer at an optical density of 600 nm, based on a standard curve established by colony formation on bacterial plates (28).

Cell culture and RNA preparation

Mouse ST2 cells (a bone-marrow-derived stromal cell line) and C3H10T1/2 cells (an embryo fibroblast cell line) were obtained from the Riken BioResource Center (Tsukuba, Japan). ST2 is an osteogenic clonal stromal cell line that supports osteoclast differentiation and has some pre-osteoblastic character. Primary calvarial osteoblasts were prepared from C3H/HeN and C57BL/6 mice (Japan SLC, Hamamatsu, Japan) as reported previously (32). Primary calvarial osteoblasts from $MyD88^{-/-}$ mice were kindly provided by Dr N. Udagawa (Matsumoto Dental College, Shiojiri, Japan) (36), after receiving permission from Drs S. Akira and K. Takeda (Osaka University, Suita-Osaka, Japan) (2). The cells were grown in six-well culture plates in an incubator at 37°C with 5% CO_2 in αMEM supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco-BRL), penicillin G(100 U/ml), and streptomycin (100 μg/ml). To avoid non-specific biological effects of growth factors or hormones contained in FCS, infection of cells with *P. gingivalis* was performed in serum-free medium containing no antibiotics. The cells were washed three times with serum-free αMEM containing no antibiotics, cultured for 24 h in the serum-free medium, subjected to infection with viable *P. gingivalis* at a multiplicity of infection (MOI) of 100, and cultured for a further 6 h in the presence of the bacterium in an incubator at 37°C in an atmosphere with 5% CO_2.

Total RNA was prepared from the cells using Trizol reagent (Invitrogen Co, Carlsbad, CA), then reverse-transcribed in the presence of oligo $(dT)_{15}$ using the Super-Script first-strand synthesis system (Invitrogen), according to the manufacturer's instructions. To quantify messenger RNA (mRNA), real-time polymerase chain reaction (PCR) was performed using a Light-Cycler (Roche Molecular Biochemicals,

Table 1. Sequence and expected fragment sizes of synthetic oligonucleotides used for real-time PCR and RT-PCR

Target mRNA	Primer sequence	Size (bp)
TLR2	5′-TTTGCTGGGCTGACTTCTCT-3′ 5′-AAATCTCCAGCAGGAAAGCA-3′	149
TLR4	5′-GGTTGAGAAGTCCCTGCTGA-3′ 5′-CGAGGCTTTTCCATCCAATA-3′	144
TLR6	5′-ACACAATCGGTTGCAAAACA-3′ 5′-GCAGCACTTAATCCCAGGAA-3′	143
PAR1	5′-CTCCTCAAGGAGCAGACCAC-3′ 5′-AGACCGTGGAAACGATCAAC-3′	172
PAR2	5′-CATTCCAGCCTTGAACATCA-3′ 5′-GCACGTAGGCAGATGCAGTA-3′	158
PAR3	5′-TGCAGTGCTCCTGAAATGAC-3′ 5′-AGCTGGACAGGCTAAAGCAA-3′	169
PAR4	5′-TGGTACTGTTCTCGGCAGTG-3′ 5′-AGCTGTTGAGGGTGCTGAGT-3′	159
β-actin	5′-TCCTGTGGCATCCATGAAACT-3′ 5′-AACGCAGCTCAGTAACAGTC-3′	340

Mannheim, Germany) with SYBR Green PCR reagent (QIAGEN, GmbH, Hilden, Germany). Samples were subjected to 40 cycles of amplification at 95°C for 15 s followed by 55°C for 30 s and 72°C for 30 s. Each assay was normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA. The normalized data are expressed as the fold increase against the mRNA level of uninfected cells. The primer sequences for CCL5, CXCL10, MMP9, and GAPDH were described in our previous study (31). Other sequences are listed in Table 1.

Western blot analysis

ST2 cells were cultured with or without stimulants, then washed with ice-cold PBS (pH 7.2) containing 10 mM *p*-tosyl-L-lysine-chloromethylketone (TLCK) (Sigma-Aldrich), and dissolved in 50 mM of Tris–HCl, pH 6.8, containing 2% Triton X-100, 10 μM TLCK, 6.25 mM NaF, 12.5 mM β-glycerophosphate, 12.5 mM *p*-nitrophenyl phosphate, 1.25 mM $NaVO_3$, and a 1% protease inhibitor cocktail (Roche Molecular Biochemicals). The soluble fraction was collected by centrifugation at 16,000 ***g*** for 5 min at 4°C. The cell extract was denatured in sodium dodecyl sulfate (SDS) sample buffer, resolved by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis, and electrotransferred to a polyvinylidene fluoride membrane. The membrane was blocked with SeaBlock blocking buffer (Pierce Chemical, Rockford, IL) for 1 h, then incubated with primary antibodies (1 : 500 dilution in PBS containing 0.05% Tween-20 and 20% SeaBlock) overnight at 4°C. After washing, horseradish-peroxidase-linked anti-rabbit immunoglobulin G (diluted 1 : 1000 in PBS containing 5% skimmed milk) was added to the membrane. Detection of proteins or phosphorylated proteins was performed using SuperSignal™ West Dura Extended Duration Substrate (Pierce Chemical).

Luciferase assay

Luciferase reporter plasmids containing NF-κB (pNF-kB-Luc), activating protein 1 (AP-1; pAP-1-Luc), cAMP response element (CRE; pCRE-Luc), glucocorticoid response element (GRE; pGRE-Luc), heat-shock response element (HSE; pHSE-Luc), and serum response element (SRE; pSRE-Luc) as a *cis*-acting enhancer element were purchased from Strategene (La Jolla, CA). C3H10T1/2 cells (2×10^4 cells/well) were seeded in 24-well culture plates (Asahi Techno Glass, Tokyo, Japan). After incubation for 24 h, the monolayers were washed three times with αMEM and then transfected with 0.1 μg of reporter plasmids using FuGene 6 transfection reagent (Roche). The efficacy of transfection was confirmed by co-transfection of the control plasmid pRL-TK (Promega, Madison, WI) containing the *Renilla reniformis* luciferase gene driven by the promoter of herpes simplex virus thymidine kinase. After an initial incubation for 18 h, the cells were incubated with *P. gingivalis* (MOI = 100) at 37°C for 6 h, and lysed in cell lysis reagent (Promega), after which luciferase activity was determined using a dual-luciferase reporter assay system (Promega). Luciferase activities were calculated as fold induction as compared with the unstimulated control vector.

Quantification of chemokine CCL5

C57BL/6 osteoblasts were grown in six-well culture plates to 70–80% confluence and then exposed for 3 h to viable *P. gingivalis* (MOI = 100) in serum-free αMEM containing no antibiotics. Following infection, the cells were washed with the medium and grown in αMEM containing 10% FCS and 10 μM leupeptin (Sigma) for 24 h. The culture supernatants were then centrifuged (10,000 ***g***, 5 min) and stored at –80°C. CCL5 was measured using enzyme-linked immunosorbent assay (ELISA) kits for RANTES (CCL5) (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Statistical analyses

All data are presented as the mean ± SD. Statistical analyses were performed using an unpaired Student's *t*-test.

Results

Signal transduction pathways activated by *P. gingivalis* infection

To determine the involvement of transcription factors and MAPK pathways in the proinflammatory response to *P. gingivalis* infection, mouse ST2 cells were stimulated with *P. gingivalis* ATCC33277, its gingipain-deficient mutant KDP136, or *P. gingivalis* LPS for 0–60 min, then analyzed for the degradation of IκB-α and phosphorylation of ERK, p38 MAPK, c-Jun, JNK, and PKB/Akt. Western blot analysis detected the degradation of IκB-α 30 min after infection, suggesting that *P. gingivalis* elicited activation of the NF-κB transcription factor (Fig. 1A). Further, phosphorylation of c-Jun (Fig. 1A) and JNK (data not shown) was also detected 30 min after infection. ERK and p38 MAPK were found to be spontaneously phosphorylated in unstimulated cells, thus the effect of *P. gingivalis* infection could not be evaluated (data not shown). No phosphorylation of PKB/Akt was detected (data not shown).

Next, to profile the effects of *P. gingivalis* infection on signal transduction pathways, several reporter vectors that contained a specific DNA sequences of NF-κB, AP-1, CRE, GRE, HSE, and SRE as *cis*-acting enhancer elements, as well as a sensitive luciferase reporter gene, were used. Mouse C3H10T1/2 cells were transiently transfected with luciferase reporter plasmids, then infected with *P. gingivalis* ATCC33277 or the gingipain-deficient mutant KDP136. Consistent with the results of the Western blot analysis, both the wild-type and the mutant *P. gingivalis* showed activation of both NF-κB and AP-1, whereas negligible induction of reporter

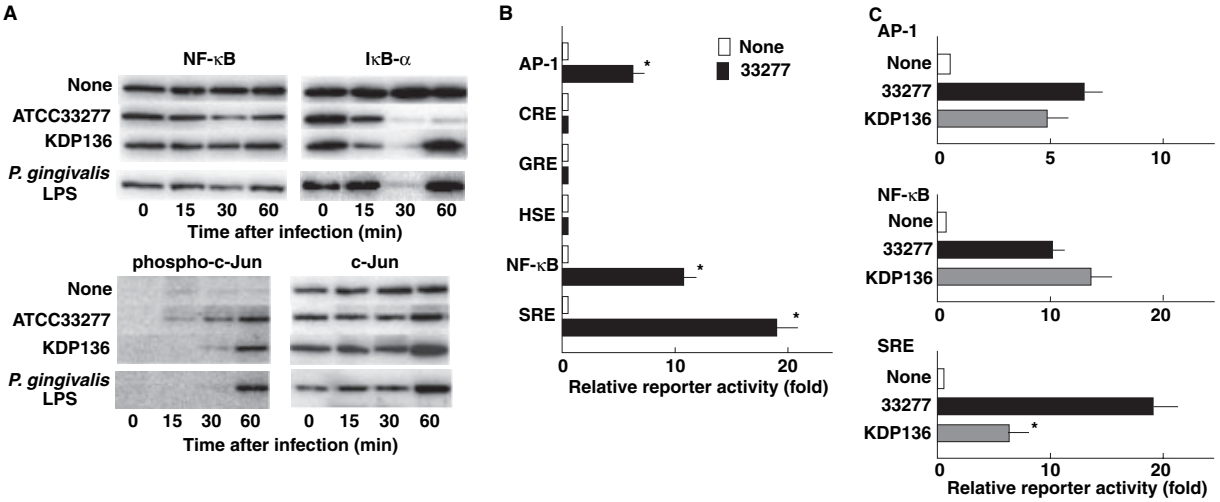

Fig. 1. Activation of signaling molecules in cells infected with *Porphyromonas gingivalis*. (A) Western blot analysis of degradation of IκB-α and phosphorylation of c-Jun. ST2 cells were infected with *P. gingivalis* ATCC33277 for 0, 15, 30, and 60 min at an MOI of 100. Degradation of IκB-α and phosphorylation of c-Jun were analyzed by Western blotting with antibodies against NF-κB (p65), IκB-α, total c-Jun, and phosphorylated c-Jun. (B) Reporter assay of *P. gingivalis*-infected cells. pAP-1-Luc, pCRE-Luc, pGRE-Luc, pHSE-Luc, pNF-κB-Luc, and pSRE-Luc reporter plasmids were introduced into C3H10T1/2 cells. The cells were then infected with *P. gingivalis* ATCC33277 for 6 h in serum-free αMEM containing no antibiotics. Relative reporter activity is expressed as the fold increase in relative luciferase units of non-infected control cells. Results are expressed as the means ± SD for triplicate assays. $*P < 0.05$ in comparison with uninfected control. (C) Reporter assay of AP-1, NF-κB, and SRE in *P. gingivalis*-infected cells. The transformed cells were infected with *P. gingivalis* ATCC33277 and KDP136 for 6 h. $*P < 0.05$ between 33277 and KDP136.

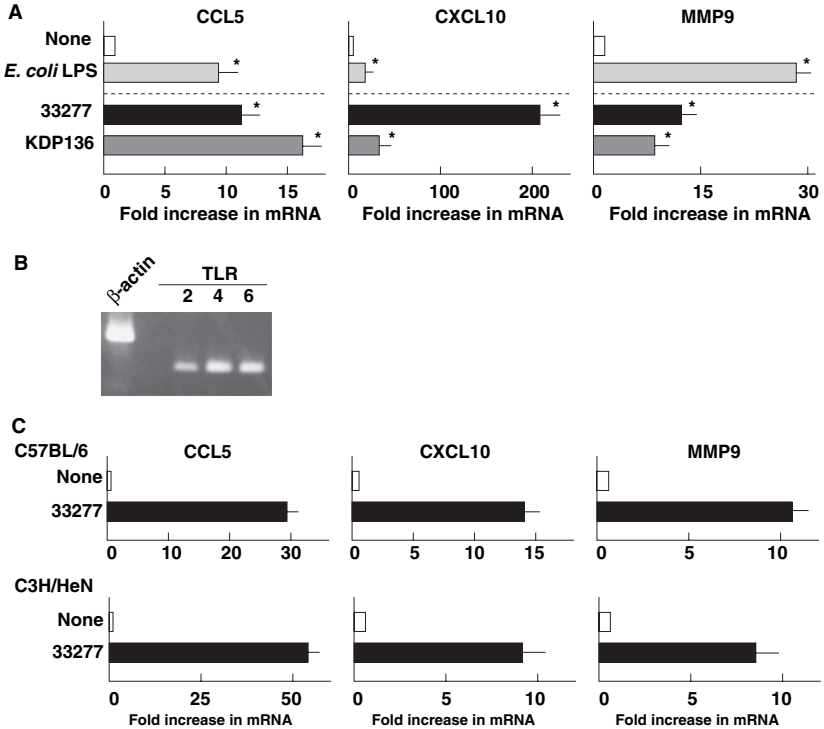

Fig. 2. Proinflammatory response in ST2 cells, and C57BL/6 and C3H/HeN osteoblasts. (A) ST2 cells were stimulated with *Escherichia coli* LPS, *Porphyromonas gingivalis* ATCC33277, and gingipain-deficient KDP136 in serum-free αMEM containing no antibiotics, then total RNA was extracted 6 h after stimulation. RNA samples were reverse-transcribed, and the expression of CCL5, CXCL10, and MMP9 was determined using real-time PCR. The expression of each gene in non-stimulated cells is expressed as the ratio of *GAPDH* gene expression, which was given the value of 1.0. Changes in mRNA levels are expressed as the fold-increases relative to expression values in non-infected cells. Results are expressed as the means ± SD for triplicate assays. $*P < 0.05$ in comparison with uninfected control. (B) RT-PCR analysis of TLR mRNA in ST2 cells. Total RNA from ST2 cells was reverse-transcribed and the expression of TLRs was detected using PCR. (C) Expression of proinflammatory genes in C57BL/6 and C3H/HeN primary osteoblasts infected with *P. gingivalis* ATCC33277. Cells were infected with *P. gingivalis* ATCC33277 at an MOI of 100 for 6 h. Changes in mRNA levels of the infected cells are expressed as the fold-increases relative to that of non-infected cells.

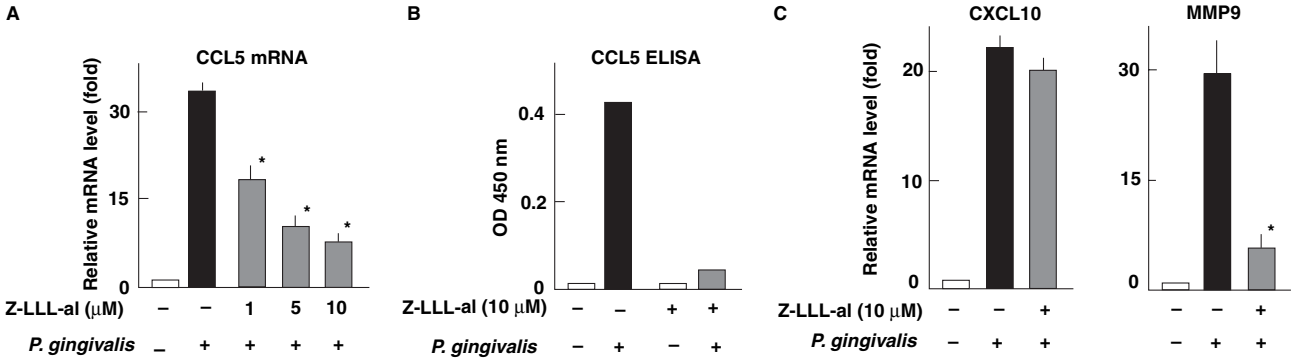

Fig. 3. Effects of NF-κB inhibitor (Z-LLL-al) on proinflammatory gene expression. C57BL/6 osteoblasts were pretreated with Z-LLL-al (1–10 μM) for 1 h in serum-free αMEM containing no antibiotics, then the cells were infected with *Porphyromonas gingivalis* ATCC33277 (MOI = 100) for 6 h. (A) Effect of Z-LLL-al on CCL5 gene expression. Changes in mRNA levels in the infected cells are expressed as the fold-increases relative to that of non-infected cells. *$P < 0.05$ in comparison with the absence of the inhibitor. (B) Effect of Z-LLL-al on CCL5 production. C57BL/6 osteoblasts were infected for 6 h with *P. gingivalis* (MOI = 100) in serum-free αMEM containing no antibiotics. Following infection, the cells were washed with the medium, and grown in αMEM containing 10% FCS and 10 μM leupeptin (Sigma) for 24 h. The concentrations of CCL5 in the culture supernatants were measured using ELISA kits for RANTES (CCL5). (C) Effects of Z-LLL-al on CXCL10 and MMP9 gene expression. C57BL/6 osteoblasts were pretreated with Z-LLL-al (10 μM) for 1 h in serum-free αMEM containing no antibiotics, then the cells were infected with *P. gingivalis* ATCC33277 (MOI = 100) and total RNA was extracted 6 h after infection. *$P < 0.05$ in comparison with the absence of the inhibitor.

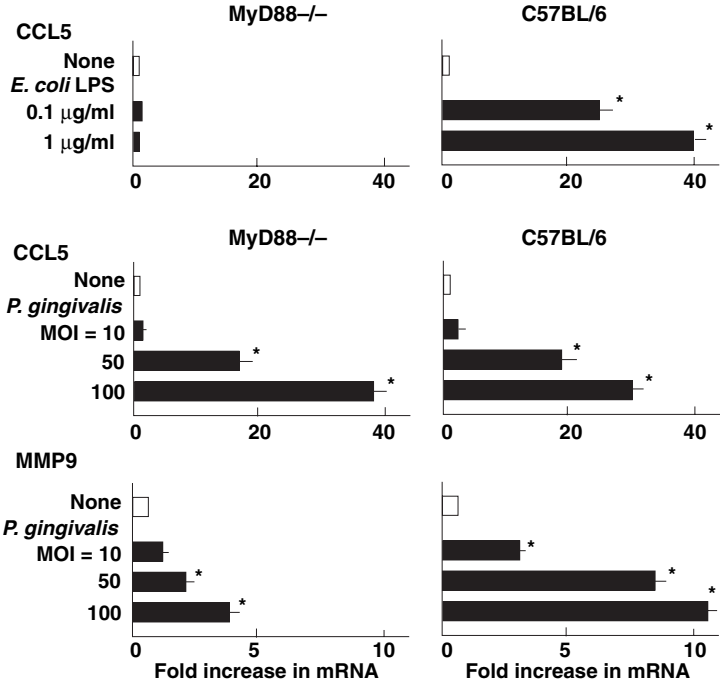

Fig. 4. Effects of *Porphyromonas gingivalis* infection on proinflammatory gene expression in MyD88$^{-/-}$ osteoblasts. Osteoblasts from MyD88$^{-/-}$ and C57BL/6 mice were infected with *P. gingivalis* ATCC33277 (MOI = 10, 50, and 100), and total RNA was extracted 6 h after infection. RNA samples were reverse-transcribed, then subjected to quantitative real-time PCR. To confirm the unresponsiveness of the MyD88$^{-/-}$ osteoblasts against TLR ligands, *Escherichia coli* LPS (1 and 10 μg/ml) was also examined. Changes in mRNA levels are expressed as the fold-increases relative to that of non-infected cells. Results are expressed as the means ± SD for triplicate assays. *$P < 0.05$ in comparison with the uninfected control.

activity was detected for CRE, GRE, and HSE (Fig. 1B). Furthermore, SRE promoter activity was enhanced by *P. gingivalis* ATCC33277; however, that activity was reduced in cells infected with KDP136 (Fig. 1C).

Expression of CCL5, CXCL10, and MMP9 in ST2 cells and primary osteoblasts infected with *P. gingivalis*

We previously described the strong induction of chemokines, cytokines, and MMPs in *P. gingivalis*-infected ST2 cells (29). In the present study, LPS from *E. coli* elicited a strong expression of CCL5 and MMP9; however, its induction of CXCL10 was marginal (Fig. 2A). On the other hand, the *P. gingivalis* wild-type strain apparently induced mRNA expression of those three, whereas the gingipain-deficient mutant showed a markedly reduced induction of CXCL10, when compared to the wild-type. As expected, the reverse transcription PCR assay results showed that the ST2 cells expressed mRNA for TLR2, TLR4, and TLR6 (Fig. 2B). Next, primary osteoblasts prepared from C57BL/6 and C3H/HeN were infected with *P. gingivalis*, and inflammatory gene expression was compared to that of ST2 stromal cells. As shown in Fig. 2C, infection with *P. gingivalis* ATCC33277 stimulated the expression of CCL5, CXCL10, and MMP9, suggesting that the responses of primary osteoblasts were similar to those of the ST2 stromal cells. However, the CXCL10 expression level in primary osteoblasts appeared to be lower than that in ST2 cells.

Effect of NF-κB inhibitor on inflammatory gene expression

NF-κB and AP-1 transcription factors were activated in cells infected with *P. gingivalis*, as shown in Fig. 1. To confirm the involvement of NF-κB transcription factor in these proinflammatory responses, C57BL/6 osteoblasts were treated with the NF-κB inhibitor Z-LLL-al before infection with *P. gingivalis*. Figure 3A shows that the expression of CCL5 was significantly suppressed by the

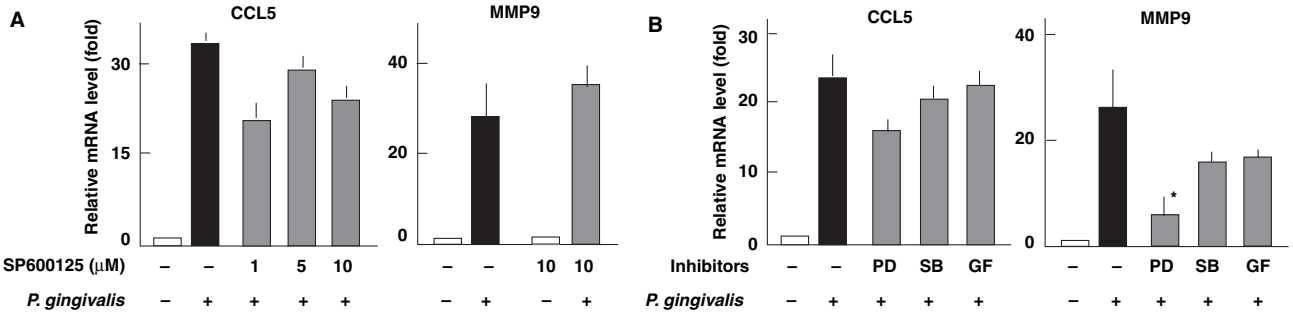

Fig. 5. Effects of various inhibitors on proinflammatory gene expression. (A) Effects of SP600125 (JNK inhibitor) on CCL5 and MMP9 gene expression. C57BL/6 osteoblasts were pretreated with SP600125 (1–10 μM) for 1 h in serum-free αMEM containing no antibiotics, then the cells were infected with *Porphyromonas gingivalis* ATCC33277 (MOI = 100), and total RNA was extracted 6 h after infection. RNA samples were reverse-transcribed and subjected to quantitative real-time PCR. Changes in mRNA levels are expressed as the fold-increases relative to that of non-infected cells. Results are expressed as the means ± SD for triplicate assays. (B) Effects of PD98059 (PD) (ERK inhibitor), SB203580 (SB) (p38 MAPK inhibitor), and GF109203X (GF) (protein kinase C inhibitor) on CCL5 and MMP9 gene expression. C57BL/6 osteoblasts were pretreated with the inhibitors (10 μM) for 1 h, then the cells were infected with *P. gingivalis* ATCC33277 (MOI = 100) and total RNA was extracted 6 h after infection. *$P < 0.05$ in comparison with the absence of the inhibitor.

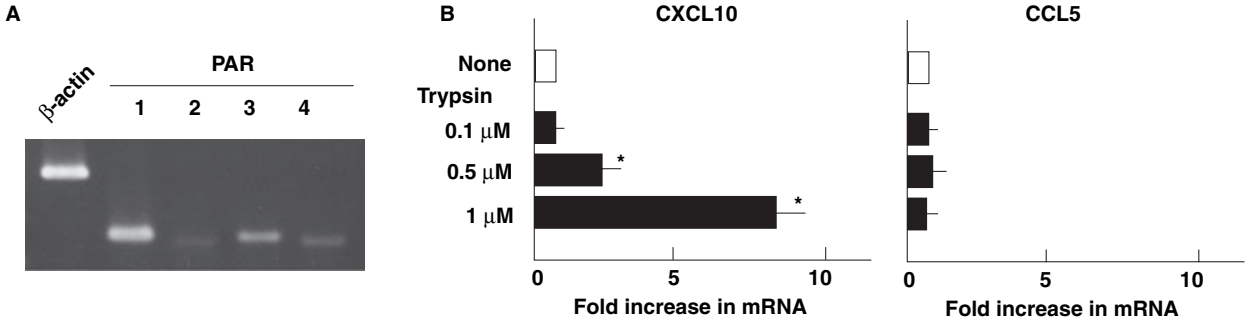

Fig. 6. Involvement of PARs in CXCL10 expression. (A) RT-PCR analysis of PAR mRNA in ST2 cells. Total RNA from ST2 cells was reverse-transcribed and the expression of PARs was analyzed by PCR. (B) Effects of trypsin on expression of CCL5 and CXCL10 in ST2 cells. ST2 cells were cultured for 6 h in the presence of trypsin (0.1–1 μM), then total RNA was extracted and reverse-transcribed, and the mRNA levels of CCL5 and CXCL10 were quantified by a real-time PCR. Relative mRNA levels are expressed as the fold increases compared to the mRNA level of the chemokine in unstimulated cells. The values are shown as the means ± SD for triplicate assays. *$P < 0.05$ in comparison with uninfected control.

addition of Z-LLL-al in a dose-dependent manner. Furthermore, the ELISA demonstrated that the production of CCL5 was inhibited by Z-LLL-al (Fig. 3B). The effect of the NF-κB inhibitor was also observed on the expression of MMP9, but not on the expression of CXCL10 (Fig. 3C), suggesting that the expression of CCL5 and MMP9 is mainly under the control of the NF-κB transcription factor.

Inflammatory response in MyD88-deficient osteoblasts

It has been reported that fimbriae and LPS of *P. gingivalis* elicit inflammatory responses through the activation of TLR2 and TLR4, respectively (15, 29, 30, 38). In addition, our results suggest that the NF-κB transcription factor regulates the expression of CCL5 and MMP9 (Fig. 3). Therefore, we investigated the possible involvement of TLRs and MyD88 adaptor protein in the inflammatory response against *P. gingivalis* infection. First, we examined the proinflammatory responses in osteoblasts of TLR4-deficient C3H/HeJ mice infected with *P. gingivalis* ATCC33277 and found that their response was similar to normal C3H/HeN osteoblasts (data not shown), suggesting that *P. gingivalis* induces proinflammatory responses in a TLR4-independent manner. Next, primary osteoblasts prepared from MyD88-deficient mice were infected with *P. gingivalis*, after which we determined the expression of proinflammatory factors using real-time PCR. As shown in Fig. 4, *E. coli* LPS did not elicit CCL5 expression in MyD88$^{-/-}$ osteoblasts, whereas *P. gingivalis* apparently induced CCL5 expression, with no significant difference observed between C57BL/6 and MyD88$^{-/-}$ osteoblasts. These results suggest that CCL5 production is mediated through a MyD88-independent pathway, though it is dependent on NF-κB activation. On the other hand, the expression of MMP9 was reduced in MyD88$^{-/-}$ osteoblasts and the response seemed to be dependent on the MyD88 pathway.

Effects of JNK inhibitor and other inhibitors on proinflammatory responses

AP-1 transcription factor was found to be activated in cells infected with *P. gingivalis* (Fig. 1). Therefore, the effects of the JNK inhibitor SP600125 and kinase inhibitors PD98059 (ERK inhibitor), SB203580 (p38 MAPK inhibitor), and GF109203X (protein kinase C inhibitor) were investigated (Fig. 5). SP600125 showed no significant inhibition of expression of CCL5 or MMP9, suggesting that AP-1 signaling does not regulate the expression of these proinflammatory factors. SB203580 and GF109203X also showed no inhibitory effects. However, PD98059 significantly inhibited MMP9 expression in *P. gingivalis*-infected osteoblasts (Fig. 5B).

Activation of cellular signaling pathways in cells infected with *P. gingivalis*

	Receptors, Adaptors, Recognition molecules	Signaling molecules	Transcription factors
Possible involvement suggested by this study	TLRs, MyD88, PARs, Other receptors?	ERK, c-Jun, JNK	NF-κB, AP-1, SRE
No detectable involvement	-	p38 MAPK, Akt	CRE, HRE, GRE

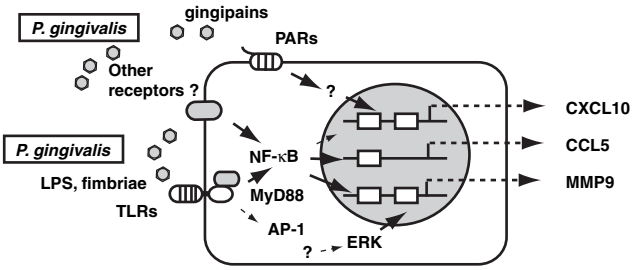

Fig. 7. Summary of the present results. Among the signaling molecules examined, *Porphyromonas gingivalis* infection was found to induce the activation of NF-κB and AP-1 transcription factors. Furthermore, the NF-κB pathway was associated with the expression of CCL5 and MMP9. Results with MyD88$^{-/-}$ osteoblasts suggest that some unknown innate immune systems beside TLR/MyD88 systems are involved in the proinflammatory responses. In addition, activation of PARs by gingipains may contribute to the expression of CXCL10. The involvement of AP-1 transcription factors in the proinflammatory responses was not revealed in the present study.

Involvement of protease-activated receptors in CXCL10 expression

Since the induction of the chemokine CXCL10 was limited in ST2 cells infected with the gingipain-deficient mutant KDP136 (Fig. 2A), gingipains were considered to be involved in its expression. ST2 cells were shown to express all types of protease-activated receptors (PARs) (Fig. 6A), with expression levels similar to those observed in primary osteoblasts (data not shown). Furthermore, we also found that trypsin, an enzyme activator of PAR1, PAR2, and PAR4 (8), elicited the expression of CXCL10 (Fig. 6B). Although these findings suggest the involvement of PARs in CXCL10 expression in *P. gingivalis*-infected cells, the inhibitors for JNK, ERK, p38 MAPK, and PKC demonstrated no significant inhibition of expression of that chemokine (data not shown).

Discussion

We investigated the involvement of various transcription factors and MAPKs with regard to the expression of proinflammatory factors of *P. gingivalis*-infected ST2 stromal cells and primary osteoblasts, and used CCL5, CXCL10, and MMP9 as indicators of the proinflammatory response, because they have been reported to be involved in marginal periodontitis and infectious bone diseases, and have shown strong responses against *P. gingivalis* infection (4, 12, 13, 26, 31). The present study results are summarized in Fig. 7. We found that the NF-κB transcription factor was an important regulator of the responses of those proinflammatory indicators, and also that *P. gingivalis* infection induced the activation of AP-1 and SRE transcription, whereas activation of other transcription factors, such as CRE, HSE, and GRE, was not detectable. To our surprise, even though the expression of CCL5 was dependent on NF-κB activation, there was no significant involvement of the MyD88 adaptor protein in the expression of that chemokine, suggesting that *P. gingivalis* is able to induce proinflammatory responses in a MyD88-independent manner. In addition, CXCL10 expression seemed to be related to the PAR signaling pathway more than the NF-κB pathway. As reported previously (31), the expression of CCL2 and MMP13 was also strongly upregulated by the infection of *P. gingivalis*, and our preliminary study suggested that the expression patterns of CCL2 and MMP13 were similar to those of CCL5 and CXCL10, respectively (data not shown). Therefore, the present results show that *P. gingivalis* utilizes multiple signaling pathways for different proinflammatory mediators.

The NF-κB transcription factor regulates a number of genes involved in a wide variety of biological processes (35), and is a predominant transcriptional factor that functions in immune and inflammatory responses by mediating signaling from TLRs in response to microbial ligands (2, 27). TLRs recognize bacterial cell components such as LPS, peptidoglycans, and lipopeptides, as well as flagella and bacterial DNA (2, 27, 41), while MyD88 is a critical adaptor molecule in TLR signaling (21, 41). It has been reported that both LPS and fimbriae of *P. gingivalis* are recognized by TLRs, resulting in host cell activation and proinflammatory responses (10, 15, 29, 30, 39). Our current results also indicate the involvement of NF-κB in proinflammatory responses by cells infected with *P. gingivalis*. In addition, it should also be noted that the present findings showed that *P. gingivalis* elicited some proinflammatory responses in a MyD88-independent manner. It is known that Toll/IL-1 receptor (TIR) domain-containing adaptor-inducing interferon-β (TRIF) is a critical adaptor molecule in the MyD88-independent signaling pathways of TLR3 and TLR4 (2), and it has also been reported that mouse osteoblasts do not express the TRIF-related adaptor molecule (TRAM), which has been suggested to be required for TRIF-mediated action (36). Therefore, TLR-mediated signals in osteoblasts are likely dependent on the MyD88 molecule. Our results indicate that *P. gingivalis* infection induces significant proinflammatory responses in MyD88$^{-/-}$ osteoblasts, suggesting that the bacterium is able to stimulate proinflammatory responses in a MyD88- and TLR-independent manner. The nucleotide-binding oligomerization domain (NOD) proteins NOD1 and NOD2 are also important molecules in innate immunity, in which they function as sensors of microbial components such as muramyl dipeptide, a structural unit of peptidoglycan. NOD1 has also been shown to participate in host defense against infection with *Helicobacter pylori* (38), whereas NOD2 is reported to be expressed in osteoblasts, and its signals are related to the muramyl dipeptide-mediated expression of the receptor activator of NF-κB ligand (RANKL), a cytokine that induces osteoclast differentiation (45). Therefore, it is important to investigate the possible involvement of NOD1 and NOD2 in *P. gingivalis*-induced proinflammatory responses.

It should also be noted that *P. gingivalis* has mechanisms to downregulate the host immune response, such as suppression of IL-8 expression induced by commensal bacteria (9, 17). In addition, *P. gingivalis*

produces numerous proteases, including arginine-specific and lysine-specific gingipain proteases. These proteolytic activities may confer on *P. gingivalis* its unique additional mechanisms of interaction with host cells, and some investigators have suggested that *P. gingivalis* uses proteases for the induction of some inflammatory mediators (18, 25, 43). Moreover, proteases such as trypsin and thrombin are reported to stimulate osteoblasts through the activation of PARs to produce cytokines such as IL-6 (1, 6, 8, 23). The present findings also suggest that *P. gingivalis* proteases are involved in the regulation of CXCL10 expression in osteoblasts. Therefore, *P. gingivalis* does not fully utilize well-established pathogen recognition molecules such as TLRs.

MAPKs, including the serine-threonine kinases ERK, JNK, and p38 MAPK, are central to many host cell signaling pathways (7, 11, 34). In the present study, we found that *P. gingivalis* induced activation of the JNK and AP-1 transcription factors. Previously, Watanabe et al. (44) reported that JNK was activated in human epithelial cells 5 min after infection with *P. gingivalis* and suggested that it is associated with the invasive process of the pathogen. Although our present findings showed that CCL5 and MMP9 expression was independent of the JNK and AP-1 signaling pathways, we previously found that AP-1 was involved in the expression of RANKL in *P. gingivalis*-infected osteoblasts (32). Therefore, the expression of some other proinflammatory factors may be regulated by the AP-1 transcription factor. In addition, because other pathogenic bacteria involved in the infectious bone diseases such as *Staphylococcus aureus* and *Salmonella* are reported to induce proinflammatory responses in osteoblasts (13), it should be investigated whether there are significant differences in the abilities of these pathogens to induce the proinflammatory responses in osteoblasts and stromal cells.

In summary, proinflammatory responses against *P. gingivalis* infection showed differential dependence on NF-κB transcription factor. Furthermore, TLR-independent innate immune systems such as PARs and NODs may differentially influence the proinflammatory responses induced by *P. gingivalis* infection. These findings drive us to investigate further the mechanism of local inflammation in periodontitis.

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References

1. Abraham LA, Chinni C, Jenkins AL et al. Expression of protease-activated receptor-2 by osteoblasts. Bone 2000: **26**: 7–14.
2. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006: **124**: 783–801.
3. Amano A. Molecular interaction of *Porphyromonas gingivalis* with host cells: implication for the microbial pathogenesis of periodontal disease. J Periodontol 2003: **74**: 90–96.
4. Birkedal-Hansen H. Role of matrix metalloproteinases in human periodontal diseases. J Periodonotol 1993: **64**: 474–484.
5. Choi E-K, Park S-A, Oh W-M et al. Mechanisms of *Porphyromonas gingivalis*-induced monocyte chemoattractant protein-1 expression in endothelial cells. FEMS Immunol Med Microbiol 2005: **44**: 51–58.
6. Chung WO, Hansen SR, Rao D, Dale BA. Protease-activated receptor signaling increases epithelial antimicrobial peptide expression. J Immunol 2004: **173**: 5165–5170.
7. Cobb MH, Goldsmith EJ. How MAP kinases are regulated. J Biol Chem 1995: **270**: 14843–14846.
8. Cocks TM, Moffatt JD. Protease-activated receptors sentries for inflammation? Trends Pharmacol Sci. 2000: **21**: 103–108.
9. Darveau RP, Belton CM, Reife RA, Lamont RJ. Local chemokine paralysis, a novel pathogenic mechanism for *Porphyromonas gingivalis*. Infect Immun 1998: **66**: 1660–1665.
10. Darveau RP, Pham T-TT, Lemley K et al. *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. Infect Immun 2004: **72**: 5041–5051.
11. Davis RJ. The mitogen-activated protein kinase signal transduction pathway. J Biol Chem 1993: **268**: 14553–14556.
12. Garlet GP, Martins W Jr, Ferreira BR, Milanezi CM, Silva JS. Patterns of chemokines and chemokine receptors expression in different forms of human periodontal disease. J Periodontal Res 2003: **38**: 210–217.
13. Gasper NA, Petty CC, Schrum LW, Marriott I, Bost KL. Bacterium-induced CXCL10 secretion by osteoblasts can be mediated in part through toll-like receptor 4. Infect Immun 2002: **70**: 4075–4082.
14. Genco RJ. Current view of risk factors for periodontal diseases. J Periodontol 1996: **67**: 1041–1049.
15. Hajishengallis G, Tapping RI, Harokopakis E et al. Differential interactions of fimbriae and lipopolysaccharide from *Porphyromonas gingivalis* with the toll-like receptor 2-centred pattern recognition apparatus. Cell Microbiol 2006: **8**: 1557–1570.
16. Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of *Porphyromonas gingivalis*. Periodontology 2000 1999: **20**: 168–238.
17. Huang GT, Kim D, Lee JK, Kuramitsu HK, Haake SK. Interleukin-8 and intercellular adhesion molecule 1 regulation in oral epithelial cells by selected periodontal bacteria: multiple effects of *Porphyromonas gingivalis* via antagonistic mechanisms. Infect Immun 2001: **69**: 1364–1372.
18. Imamura T. The role of gingipains in the pathogenesis of periodontal disease. J Periodontol 2003: **74**: 111–118.
19. Jimi E, Nakamura I, Ikebe T, Akiyama S, Takahashi N, Suda T. Activation of NF-κB is involved in the survival of osteoclast promoted by interleukin-1. J Biol Chem 1998: **273**: 8799–8905.
20. Jung IY, Choi BK, Kum KY et al. Molecular epidemiology and association of putative pathogens in root canal infection. J Endodontol 2000: **26**: 599–604.
21. Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 1999: **11**: 115–122.
22. Koga T, Nishihara T, Fujiwara T et al. Biochemical and immunobiological properties of lipopolysaccharide (LPS) from *Bacteroides gingivalis* and comparison with LPS from *Escherichia coli*. Infect Immun 1985: **47**: 638–647.
23. Kozawa O, Tokuda H, Kaida T, Matsuno H, Uematsu T. Thrombin regulates interleukin-6 synthesis through phosphatidylcholine hydrolysis by phospholipase D in osteoblasts. Arch Biochem Biophys 1997: **345**: 10–15.
24. Lamont RJ, Jenkinson HF. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. Microbiol Mol Biol Rev 1998: **62**: 1244–1263.
25. Lourbakos A, Potempa J, Travis J et al. Arginine-specific protease from *Porphyromonas gingivalis* activates protease-activated receptors on human oral epithelial cells and induces interleukin-6 secretion. Infect Immun 2001: **69**: 5121–5130.
26. Makela M, Salo T, Uitto V-J, Larjava H. Matrix metalloproteinases (MMP2 and MMP9) of the oral cavity: cellular origin and relationship to periodontal status. J Dent Res 1994: **73**: 1397–1406.
27. Miyake K. Innate recognition of lipopolysaccharide by toll-like receptor 4-MD-2. Trends Microbiol 2004: **12**: 186–192.
28. Nakagawa I, Amano A, Kuboniwa M, Nakamura T, Kawabata S, Hamada S. Functional differences among FimA variants of *Porphyromonas gingivalis* and their effects on adhesion to and invasion of human epithelial cells. Infect Immun 2002: **70**: 277–285.
29. Ogawa T, Asai Y, Hashimoto M, Uchida H. Bacterial fimbriae activate human peripheral blood monocytes utilizing TLR2, CD14 and CD11a/CD18 as cellular receptors. Eur J Immunol 2003: **32**: 2543–2550.
30. Ogawa T, Asai Y, Hashimoto M et al. Cell activation by *Porphyromonas gingivalis* lipid A molecule through toll-like receptor 4- and myeloid differentiation factor

88-dependent signaling pathway. Int Immunol 2002: **14**: 1325–1332.
31. Ohno T, Okahashi N, Kawai S et al. Proinflammatory gene expression in mouse ST2 cell line in response to infection by *Porphyromonas gingivalis*. Microbes Infect 2006: **8**: 1025–1034.
32. Okahashi N, Inaba H, Nakagawa I et al. *Porphyromonas gingivalis* induces receptor activator of NF-κB ligand expression in osteoblasts through the activator protein-1 pathway. Infect Immun 2004: **72**: 1706–1714.
33. Pihlstrom BL, Michalowizc BS, Hohnson NW. Periodontal diseases. Lancet 2005: **366**: 1809–1820.
34. Robinson MJ, Cobb MH. Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 1997: **9**: 180–186.
35. Ruland J, Mak TW. Transducing signals from antigen receptors to nuclear factor κB. Immunol Rev 2003: **193**: 93–100.
36. Sato N, Takahashi N, Suda K et al. MyD88 but not TRIF is essential for osteoclastogenesis induced by lipopolysaccharide, diacyl lipopeptide, and IL-1α. J Exp Med 2004: **200**: 601–611.
37. Shi Y, Ratnayake DB, Okamoto K, Abe N, Yamamoto K, Nakayama K. Genetic analyses of proteolysis, hemoglobin binding, and hemagglutination of *Porphyromonas gingivalis*. J Biol Chem 1999: **274**: 17 955–17 960.
38. Strober W, Murray PJ, Kitani A, Watanabe T. Signalling pathways and molecular interactions of NOD1 and NOD2. Nat Rev Immunol 2006: **6**: 9–20.
39. Tabeta K, Yamazaki K, Akashi S et al. Toll-like receptors confer responsiveness to lipopolysaccharide from *Porphyromonas gingivalis* in human gingival fibroblasts. Infect Immun 2000: **68**: 3731–3735.
40. Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat Rev Immunol 2007: **7**: 292–304.
41. Takeuchi O, Akira S. MyD88 as a bottle neck in Toll/IL-1 signaling. Curr Top Microbiol Immunol 2002: **270**: 155–167.
42. Taxman DJ, Zhang J, Champagne C et al. ASC mediates the induction of multiple cytokines by *Porphyromonas gingivalis* via caspase-1-dependent and -independent pathways. J Immunol 2006: **177**: 4252–4256.
43. Uehara A, Muramoto K, Imamura T et al. Arginine-specific gingipains from *Porphyromonas gingivalis* stimulate production of hepatocyte growth factor (scatter factor) through protease-activated receptors in human gingival fibroblasts in culture. J Immunol 2005: **175**: 6076–6084.
44. Watanabe K, Yilmaz O, Nakhajiri SF, Belton CM, Lamont RJ. Association of mitogen-activated protein kinase pathways with gingival epithelial cell responses to *Porphyromonas gingivalis* infection. Infect Immun 2001: **69**: 6731–6737.
45. Yang S, Takahashi N, Yamashita T et al. Muramyl dipeptide enhances osteoclast formation induced by lipopolysaccharide, IL-1α, and TNF-α through nucleotide-binding oligomerization domain 2-mediated signals in osteoblasts. J Immunol 2005: **175**: 1956–1964.