Osteolytic diseases, including rheumatoid arthritis, osteomyelitis, and periodontitis, are usually associated with bacterial infections. However, the precise mechanisms by which bacteria induce bone loss still remain unclear. Evidence exists that Toll-like receptor (TLR) signaling regulates both inflammation and bone metabolism and that the receptor activator of NF-κB ligand (RANKL) and its receptor RANK are the key regulators for bone remodeling and for the activation of osteoclasts. Here, we investigate the direct effects of the periodontal pathogen Porphyromonas gingivalis on osteoclast differentiation and show that P. gingivalis differentially modulates RANKL-induced osteoclast formation contingent on the state of differentiation of osteoclast precursors. In addition, although an optimal induction of cytokines by P. gingivalis is dependent on TLR2 and TLR4, as well as myeloid differentiation factor 88 and Toll/IL-1R domain-containing adaptor-inducing IFN-β, P. gingivalis utilizes TLR2/myeloid differentiation factor 88 in modulating osteoclast differentiation. P. gingivalis modulates RANKL-induced osteoclast formation by differential induction of NFATc1 and c-Fos. More importantly, RANKL-mediated lineage commitment also has an impact on P. gingivalis-induced cytokine production. RANKL inhibits P. gingivalis-induced cytokine production by down-regulation of TLR/NF-κB and up-regulation of NFATc1. Our findings reveal novel aspects of the interactions between TLR and RANK signaling and provide a new model for understanding the mechanism underlying the pathogenesis of bacteria-mediated bone loss.

Bone is a dynamic organ that constantly undergoes remodeling throughout life. Under normal conditions, bone is periodically resorbed while new bone is formed. Bone maintains its shape and mineralization through a subtle balance of bone formation and bone resorption. In pathogenic bone loss conditions, such as periodontal disease and rheumatoid arthritis, the balance is broken, and bone resorption outweighs bone formation, leading to bone erosion. One of the characteristic findings in these conditions is increased accumulation of osteoclasts at the site of focal erosion (1).

Osteoclasts are the sole bone resorbing cells of the body. They are large, multinucleated cells derived from hematopoietic precursor cells of the monocyte-macrophage lineage, the same precursor cells that also give rise to macrophages and dendritic cells (1–4). Osteoclast differentiation is regulated by two essential molecules: the macrophage colony-stimulating factor (M-CSF) and the receptor activator of NF-κB ligand (RANKL), a tumor necrosis factor (TNF) family cytokine also known as TNF-related activation-induced cytokine (3, 5). Although M-CSF promotes proliferation and survival of osteoclast progenitors, RANKL prompts the cells to differentiate along the osteoclast lineage (2). RANKL also acts as an activating and survival factor for mature osteoclasts. RANKL exerts its effect on osteoclasts by binding to the receptor RANK expressed on osteoclast precursors and mature osteoclasts. The interaction of RANKL and RANK results in recruitment of the TNF receptor-associated factor family proteins, which in turn activates multiple intracellular signaling pathways and transcription factors, leading to the differentiation and activation of osteoclasts (2, 3, 6).

Periodontitis, a chronic inflammatory disorder characterized by the destruction of periodontal connective tissues and the subsequent loss of alveolar bone, is one of the most common human infectious diseases and the primary cause of tooth loss in adults (7, 8). It is also significantly associated with several systemic disorders, e.g. cardiovascular disease, stroke, diabetes, and pregnancy complications (9–16). It is initiated by specific bacteria within the plaque biofilm and progresses because of abnormal immune responses to these bacteria (17). Although a number of Gram-negative, anaerobic bacteria have been implicated in the disease process, Porphyromonas gingivalis is con-
**P. gingivalis on Osteoclast Differentiation**

sidered a major etiologic agent of periodontitis (18, 19). This bacterium possesses multiple virulence factors, such as LPS, fimbriae, gingipains, and hemagglutinins, that are believed to contribute to the initiation and progression of periodontal diseases (8, 20, 21).

The ability of the immune system of the host to sense, recognize, and respond to periodontal-associated pathogens is an important determinant in the pathogenesis of periodontitis (22). This ability is largely mediated by the innate immune system via the expression of Toll-like receptors (TLRs) on antigen-presenting cells, including macrophages and dendritic cells (23, 24). Although Gram-negative bacteria are generally recognized by TLR4, there is some controversy regarding recognition of the Gram-negative bacterium *P. gingivalis* because studies (25–28) have shown that it can signal via TLR2, TLR4, or both. Recognition of conserved microbial products by TLRs tailors the response of the innate immune system through the use of different signaling components and adaptor molecules, such as the myeloid differentiation factor 88 (MyD88) and the Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF) (29–32). The MyD88-dependent signaling pathway is utilized by all known TLRs except TLR3, and the TRIF-mediated pathway, which is commonly known as the MyD88-independent pathway, plays an essential role in TLR3- and TLR4-mediated downstream signaling. Activation of TLRs triggers a variety of downstream signal transduction pathways, including the MAPKs, PI3K-Akt, and most notably the nuclear translocation of transcription factors, including NF-κB. Binding of transcription factors to specific DNA binding sites culminates in transcriptional activation of genes encoding inflammatory mediators and numerous other effectors of the immune response (33). The induction of inflammatory mediators by TLRs plays a major role in the initiation of protective immune responses. However, the extensive release of TLR-triggered inflammatory mediators may harm the host by accelerating inflammation and activating bone and tissue destruction, as seen in periodontal disease. The underlying mechanisms that regulate inflammation and bone resorption via TLR following pathogenic bacteria infection have not been delineated.

In this study, we investigated the direct effect of the periodontal pathogen *P. gingivalis* on RANKL-mediated osteoclast differentiation and the interaction between RANK and TLR signaling pathways in regulating osteoclastogenesis and *P. gingivalis*-induced cytokine responses.

**EXPERIMENTAL PROCEDURES**

**Bacteria—** *P. gingivalis* ATCC33277 was cultured and maintained on enriched trypticase soy agar plates containing trypticase soy agar, 1% yeast extract, 5% defibrinated sheep blood, 5 μg/ml hemin, and 1 μg/ml menadione, at 37 °C in an anaerobic atmosphere of 10% H₂, 5% CO₂, and 85% N₂. For the preparation of *P. gingivalis* for cell stimulation, the bacteria were harvested, centrifuged, and washed in PBS. The number of bacteria (colony-forming units/ml) was determined by measuring the optical density (A) at 580 nm and extrapolating using a standard curve.

**Mice—** C57BL/6 WT, TLR2 knockout (TLR2−/−), TLR4−/−, TLR2/4−/−, MyD88−/−, and TRIFΔp52 (TRIF−/−) mice were bred and maintained within an environmentally controlled, pathogen-free animal facility at the University of Alabama at Birmingham (UAB). The original TLR−/− and MyD88−/− breeding pairs were obtained under a material transfer agreement from Dr. Shikuo Akira (Osaka University, Osaka, Japan). The original TRIFΔp52 breeding pairs were purchased from the Jackson Laboratories (Bar Harbor, ME). Female mice were 8–10 weeks of age when used in the studies. All studies were performed according to the National Institutes of Health guidelines, and protocols were approved by the UAB Institutional Animal Care and Use Committee.

**Generation of BMMs—** Murine bone marrow cells were collected as described previously (34, 35). Briefly, mouse femurs and tibiae were removed and dissected free from adhering soft tissue. After soaking the bones in 70% ethanol for 2 min and rinsing in PBS, both ends were cut, and the marrow was flushed out with PBS using a 25-gauge needle. Single-cell suspensions were prepared by mechanically dispersing the bone marrow through a 100-μm cell strainer. Erythrocytes were lysed using M-lysis buffer (R&D Systems, Minneapolis, MN). To prepare osteoclast precursors, bone marrow cells were cultured in α-10 medium (α-minimum Eagle’s medium, 10% FCS, 1 X PenStrep) in a humidified 7.5% CO₂ incubator at 37 °C overnight. The next day, nonadherent cells were harvested and cultured with α-10 medium supplemented with 10% culture supernatant from CMG14–12 cells as the source of M-CSF. After 3 days of culturing, floating cells were removed, and attached cells were used as osteoclast precursor cells (BMMs).

**In Vitro Osteoclastogenesis Assays—** BMMs were cultured in 24-well plates (5 × 10⁵ cells/well) in α-10 medium in the presence of M-CSF (50 ng/ml). The cells were stimulated with *P. gingivalis* (m.o.i. = 50), RANKL (100 ng/ml), *P. gingivalis* plus RANKL, or pretreated with RANKL for 24 h and then stimulated with *P. gingivalis* for 24 h. Pam3CSK₄ (100 ng/ml) and *Escherichia coli* K12 LPS (100 ng/ml) (InvivoGen, San Diego, CA) were used as positive control agonists for TLR2 and TLR4, respectively. The cells were cultured for 3–10 days and stained for tartrate-resistant acid phosphatase (TRAP) activity using a leukocyte acid phosphatase kit (Sigma-Aldrich, St. Louis, MO).

**Cytokine Analysis—** Culture supernatants were assessed for the levels of TNF-α, IL-6 (eBiosciences, San Diego, CA), IL-10, and IL-12p40 (R&D Systems) by an ELISA according to the manufacturer’s instructions.

**Real-time Quantitative PCR—** Total RNA was extracted from 10⁶ cells at the indicated time points (see “Results”) using RNeasy Mini kits (Qiagen, Valencia, CA) according to the recommended procedure. cDNA was synthesized from 500 ng of total RNA by reverse transcription using QuantiTect RT kits (Qiagen). Real-time PCR was performed using a LightCycler (Roche) with a FastStart DNA Master SYBR Green I reagent (Roche). Relative quantities of the tested gene were normalized to β-actin mRNA. The normalized data were expressed using the comparative 2−ΔΔCT method.

**FACS Analysis—** BMMs were cultured with *P. gingivalis* (m.o.i. = 50), RANKL (100 ng/ml), *P. gingivalis* plus RANKL, or were pretreated with RANKL for 24 h and then stimulated with *P. gingivalis*. After 24 h of stimulation, cells were harvested,
washed, and suspended in FACS buffer and stained with phycoerythrin-labeled antibodies against TLR2, TLR4, or RANK (eBiosciences) for 30 min on ice. Cells were washed, suspended in FACS buffer, and immediately analyzed using a FACSCalibur (BD Biosciences). Data were analyzed with CellQuest software (BD Biosciences).

Cell Extract Preparation—BMMs were stimulated with P. gingivalis and/or RANKL for different periods of time, and whole cell lysates from 10⁶ cells were prepared as described previously (36, 37). Briefly, cells were washed with PBS and then lysed on ice for 10 min in radioimmune precipitation assay lysis buffer (Upstate, Lake Placid, NY) freshly supplemented with 1 mM PMSF, 1 mM Na₃VO₄, 1 mM NaF, and 1 μg/ml protease inhibitor cocktail (Roche). The whole cell lysates were transferred to tubes and incubated on ice for an additional 20 min, and following centrifugation, the supernatants were collected.

To prepare cytoplasmic and nuclear extracts, a nuclear extract kit (Active Motif, Carlsbad, CA) was used according to the manufacturer’s protocol. Specifically, cells were washed with PBS/phosphatase inhibitors and harvested by centrifugation. The cell pellet was then resuspended in hypotonic buffer and rotated at 150 rpm on ice for 15 min. Detergent was then added, and cells were mixed vigorously before centrifugation. The cytoplasmic extract was collected, and the nuclear pellet was resuspended in complete lysis buffer and rotated at 150 rpm on ice for 30 min. The nuclear fraction was collected after centrifugation at 14,000 rpm for 10 min at 4 °C.

Western Blot Analysis—Equivalent amounts of protein from cell lysates were separated by SDS-PAGE on a 10% Tris-HCl gel (Bio-Rad). Protein was electrotransferred to Immobilon-P membrane (Millipore, Bedford, MA) and probed with specific antibodies against total p38, β-actin, c-Fos, NFATc1, or the phosphorylated form of p38, ERK1/2, JNK, Akt (Ser-473), and NF-κB p65 (Ser-536). Detection was carried out using HRP-linked rabbit IgG antibody, followed by ECL Western blotting detection reagents (Amersham Biosciences). All antibodies were purchased from Cell Signaling Technology (Beverly, MA) except NFATc1 (Santa Cruz Biotechnology, Santa Cruz, CA). Densitometer scans of the blots were performed using the AlphaImager 2000 documentation and analysis system (Alpha Innotech, San Leandro, CA).

Retrovirus Production and Infection—The retroviral expression vectors pMSCV-GFP and pMSCV-caNFATc1 used in this study were a generous gift from Dr. Neil Clipstone, Loyola University, Chicago, IL (38, 39). Plat-E cells were cultured in Dulbecco’s modified Eagle’s medium with 10% FCS supplemented with puromycin and blasticidin, as described previously (40). Retrovirus vectors were transiently transfected into Plat-E cells using Lipofectamine Plus reagent (Invitrogen). Virus supernatant was harvested 48 and 72 h after transfection. For infection, BMMs were cultured with retroviral supernatant for 6 h in the presence of M-CSF and polybrene. Virus was then replaced, and the cells were cultured in regular growth medium. Infec tion efficiency of retrovirus was determined by confocal microscopy.

Statistical Analysis—Statistical significance was evaluated by analysis of variance and the Tukey multiple-comparisons test using the InStat program (GraphPad Software, San Diego, CA). Differences between groups were considered significant at a p value < 0.05.

RESULTS

P. gingivalis Differentially Regulates RANKL-induced Osteoclastogenesis—The involvement of osteoclasts in the pathogenesis of pathological bone loss conditions such as rheumatoid arthritis and periodontitis has been well recognized (41, 42). However, the precise role of bacterial infections on osteoclast differentiation remains unclear. To address this, BMMs from C57BL/6 mice were stimulated with recombinant RANKL and/or the periodontal pathogen P. gingivalis in the presence of M-CSF, and the formation of multinucleated cells (greater than three nuclei) positive for the osteoclast marker TRAP was analyzed. After 4 days of cultivating, RANKL efficiently induced the formation of TRAP-positive multinucleated cells, whereas P. gingivalis failed to induce osteoclast differentiation (Fig. 1A). However, when the precursor cells were treated with both P. gingivalis and RANKL at the same time, P. gingivalis completely inhibited RANKL-induced osteoclast formation (Fig.
1B). Conversely, when cells were pretreated with RANKL over-
night and then stimulated with P. gingivalis, significantly more
osteoclasts were induced compared with RANKL stimulation
alone (p < 0.05). These results indicate that P. gingivalis differen-
tially modulates RANKL-induced osteoclast formation either
by inhibiting the formation of osteoclasts from non-com-
mitted precursor cells or by promoting the differentiation
of osteoclasts from RANKL-committed cells.

RANKL Pretreatment Severely Impairs P. gingivalis-induced
Inflammatory Cytokine Response—Activation of host defense
and induction of cytokines have a great impact on pathological
bone resorption (1, 43, 44). Because osteoclasts are derived
from the monocyte/macrophage lineage with a prominent role
in the innate immune response, we next determined the induc-
tion of cytokines by P. gingivalis in osteoclast precursor cells
and the effect of RANKL treatment on P. gingivalis-induced
cytokine production. P. gingivalis induced a robust production
of proinflammatory cytokines IL-6, TNF-α, and IL-12p40 and a
limited amount of the anti-inflammatory cytokine IL-10 in
BMMs (Fig. 2A). Conversely, stimulation by RANKL did not
induce detectable levels of cytokine production (Fig. 2A). When
the cells were cocultured with P. gingivalis and RANKL at the
same time, the levels of the cytokines induced were similar to
those induced by P. gingivalis alone. However, pretreatment of
the cells with RANKL for 24 h resulted in a diminished produc-
tion of cytokines following P. gingivalis stimulation. These
results indicate that the ability of BMMs to produce inflamma-
tory cytokines in response to P. gingivalis infection is abrogated
by RANKL-induced commitment of the cells into the osteoclast
lineage.

We also assessed cytokine mRNA levels following P. gingen-
alis and/or RANKL stimulation at various times during a 24-h
period. Consistent with cytokine protein levels, stimulation
with P. gingivalis induced a robust expression of IL-6, IL-12p40,
TNF-α, and IL-10 mRNA, whereas no induction of mRNA for
these cytokines was seen with RANKL alone (Fig. 2B). Simulta-
neous stimulation with P. gingivalis and RANKL induced sim-
ilar or higher levels of cytokine mRNA as with P. gingivalis
alone. However, the level of cytokine mRNA was dramatically
decreased when the cells were primed with RANKL for 24 h
followed by P. gingivalis stimulation (Fig. 2B). These results
indicate that RANKL pretreatment regulates P. gingivalis-in-
duced cytokines at both the transcriptional and translational
levels.

P. gingivalis Inhibits RANKL-induced Osteoclast Differen-
tiation via TLR2/MyD88 but Not TLR4/TRIF—TLR signaling is a
major route by which the host innate immune system recog-
nizes microbial pathogens (24). Both osteoclast precursor cells
and mature osteoclasts express TLR2 and TLR4 (45). To
address the molecular mechanism governing the interaction
between bacterial infection and osteoclast differentiation, we
determined the involvement of TLR2 and TLR4 in
osteoclast precursor cells is mainly dependent
between bacterial infection and osteoclast differentiation, we
determined the involvement of TLR2 and TLR4 in
P. gingivalis, while TLR4 is expressed in osteoclasts but not in
cell lines. The level of TNF-α, IL-6, and IL-12p40 were signifi-
cantly lower in TLR2−/− cell cultures following P. gingivalis
stimulation compared with WT cells (p < 0.01) but higher
than those seen in TLR2−/− cultures (Fig. 3A). As expected,
Pam3CSK4 induced similar levels of cytokines in WT and
TLR4−/− but not TLR2−/− cells, whereas LPS induced cyto-
kine production in WT and TLR2−/− but not TLR4−/− cells.
No cytokine production was detected in TLR2−/−/− cell cultures
following P. gingivalis, Pam3CSK4, or LPS stimulation.
These results demonstrate that the cytokine response induced
by P. gingivalis in osteoclast precursor cells is mainly dependent
on TLR2 signaling and, to a lesser extent, on TLR4 signaling.

Because MyD88- and TRIF-dependent pathways are involved in
TLR4-mediated induction of inflammatory cyto-
kines whereas only MyD88 is involved in TLR2 signaling (24),
we next determined the involvement of these adaptor mole-

FIGURE 2. RANKL pretreatment of BMMs severely impairs P. gingivalis-induced cytokine response. A, RANKL pretreatment suppresses P. gingivalis-induced cytokine production. BMMs were treated with P. gingivalis (m.o.i. = 50), RANKL, P. gingivalis plus RANKL, or pretreated with RANKL for 24 h and then stimulated with P. gingivalis for 24 h. The levels of TNF-α, IL-6, IL-12p40, and IL-10 in the culture supernatants were determined by ELISA. Data are expressed as the mean ± S.D. of three independent experiments. **, p < 0.01 and ***, p < 0.001 compared with the cytokines in the cells treated with P. gingivalis only.

B

FIGURE 2. RANKL pretreatment of BMMs down-regulates P. gingivalis-induced cytokine mRNA expression. BMMs were treated with P. gingivalis, RANKL, P. gingivalis plus RANKL, or pretreated with RANKL for 24 h and then stimulated with P. gingivalis for 0, 2, 6, 12, and 24 h. Total RNA was isolated, and the presence of mRNA for TNF-α, IL-6, IL-12p40 and IL-10 was assessed by real-time PCR using SYBR Green. β-Actin was used as the endogenous control. The results are representative of three independent experiments.
cules in the induction of cytokines upon *P. gingivalis* stimulation. Cytokine production was essentially abolished in MyD88−/− cells, whereas in TRIF−/− cultures, the induction of cytokine production was differentially regulated (Fig. 3B). Significantly lower levels of TNF-α and IL-12p40 were seen after *P. gingivalis* stimulation in TRIF−/− cultures compared with WT cultures (*p* < 0.01). However, no significant difference in the levels of IL-6 and IL-10 was found between WT and TRIF−/− cell cultures following *P. gingivalis* stimulation. These results demonstrate that although *P. gingivalis*-induced cytokine production in osteoclast precursors signals predominantly through the MyD88-dependent pathway, there is nevertheless some involvement of the TRIF-dependent pathway. Collectively, our finding reveal that *P. gingivalis*-induced cytokine production is differentially regulated by TLR signaling. The involvement of TLR2 and TLR4, as well as the MyD88-dependent and MyD88-independent signaling pathways, seems necessary for the optimal induction of cytokines in osteoclast precursor cells following *P. gingivalis* stimulation.

Next, we determined the involvement of TLR2 and TLR4 in modulating osteoclast differentiation by *P. gingivalis*. RANKL was able to induce the formation of multinucleated cells in WT, TLR2−/−, TLR4−/−, and TLR2/4−/− cell cultures (Fig. 4A). Similarly, osteoclast formation was observed in cells primed with RANKL overnight under all conditions tested. However, simultaneous stimulation with *P. gingivalis* and RANKL induced osteoclast differentiation only in TLR2−/− and TLR2/4−/− cell cultures but not in WT and TLR4−/− cell cultures (Fig. 4A). The control Pam3CSK4 was able to induce RANKL-induced osteoclast formation of TLR2−/− and TLR2/4−/− cells, and LPS induced osteoclast differentiation in TLR4−/− and TLR2/4−/− cultures. Interestingly, we noticed that some cultures had a majority of multinucleated cells of medium (10–50 nuclei) to large (>50 nuclei) size, whereas others had osteoclasts of small (3–10 nuclei) to medium size (Fig. 4B). The reason for these differences is not known. Taken together, our results demonstrate for the first time that *P. gingivalis* abrogates osteoclast differentiation via TLR2 but not TLR4 signaling.

In terms of the involvement of MyD88 and TRIF in *P. gingivalis*-mediated modulation of osteoclast formation, *P. gingivalis* was able to block RANKL-induced osteoclast differentiation of WT and TRIF−/− BMMs in non-committed conditions. However, it induced osteoclast differentiation of MyD88−/− cells (Fig. 5). These results indicated that the MyD88, but not the TRIF pathway, is required for the inhibitory effect exerted by *P. gingivalis* on osteoclast differentiation. Taken together, these findings demonstrate that the TLR2/MyD88 signaling pathway is critical for *P. gingivalis*-mediated inhibition of osteoclast differentiation.

**RANKL Pretreatment Inhibits *P. gingivalis*-induced Up-regulation of TLR2 and TLR4**—We next determined the effect of *P. gingivalis* and/or RANKL on the cell surface expression of TLR2 and TLR4. BMMs from WT mice were incubated with *P. gingivalis*, RANKL, *P. gingivalis* plus RANKL, or pretreated with RANKL for 24 h and then stimulated with *P. gingivalis*. Although RANKL by itself did not affect the expression of TLR2 and TLR4 on BMMs, *P. gingivalis* induced an up-regulation of TLR4 and especially TLR2 (Fig. 6A). Similar results were obtained when the cells were simultaneously treated with *P. gingivalis* and RANKL. However, when the cells were pretreated with RANKL overnight and then stimulated with *P. gingivalis*, no up-regulation of TLR2 and TLR4 was detected (Fig. 6A). These results suggest that RANKL pretreatment suppresses *P. gingivalis*-induced cytokine responses, possibly by making osteoclast precursor cells refractory to TLR signaling.

The above results prompted us to determine the effect of *P. gingivalis* on RANK expression by osteoclast precursor cells. RANKL did not induce an up-regulation of its receptor RANK on BMMs following 24 h (Fig. 6A) or 48 h (data not shown) stimulation. Conversely, *P. gingivalis* up-regulated the expression of RANK. A similar result was seen when the cells were simultaneously stimulated with RANKL and *P. gingivalis*, ruling out the possibility that *P. gingivalis* inhibits osteoclastogenesis by down-regulating RANK expression. Additionally, no up-regulation of RANK was observed when the cells were pretreated with RANKL and then stimulated with *P. gingivalis*.
Interestingly, except for TLR2 mRNA, there was little change in the expression of TLR4 and RANK at the mRNA level following P. gingivalis and/or RANKL stimulation (Fig. 6B), indicating that the level of TLR2, TLR4, and RANK protein may be regulated posttranslationally. Taken together, these data indicate that the inhibitory effect of P. gingivalis on osteoclastogenesis is not due to the regulation of RANK expression.

**P. gingivalis Modulates Osteoclastogenesis by Regulating NFATc1 and c-Fos**—RANKL has been shown to activate the MAPK and PI3K/Akt pathways and the transcription factor NF-κB to induce osteoclast differentiation (3, 46, 47). Furthermore, RANKL activates calcium (Ca^{2+}) signals that lead to the induction of the nuclear factor of activated T cell (NFAT) c1, an essential transcription factor for osteoclastogenesis (48, 49). Therefore, to explore the mechanism by which P. gingivalis affects RANKL-induced osteoclast differentiation, we examined the activation of these signaling molecules by RANKL and/or P. gingivalis. Although different activation kinetics were noted, RANKL and P. gingivalis each induced phosphorylation of p38, ERK1/2, JNK, Akt, and NF-κB p65 (Fig. 7A), indicating that the inability of BMMs to differentiate into osteoclasts in the presence of P. gingivalis is not due to a lack of activation of these molecules. Interestingly, the presence of P. gingivalis led to sustained phosphorylation of ERK, p38, and Akt compared with RANKL stimulation alone (Fig. 7A). In addition, although RANKL alone induced sustained NF-κB phosphorylation, P. gingivalis enhanced RANKL-induced NF-κB phosphorylation in unprimed cells after 30 min of stimulation (Fig. 7A). These data indicate that P. gingivalis does not inhibit osteoclastogenesis by suppressing RANKL-mediated activation of p38, ERK, Akt, or NF-κB. Moreover, although simultaneous stimulation of BMMs by P. gingivalis and RANKL induced a

**FIGURE 4. Role of TLR2 and TLR4 in P. gingivalis-mediated inhibition of osteoclastogenesis.** A, BMMs from WT, TLR2<sup>−/−</sup>, TLR4<sup>−/−</sup>, and TLR2/4<sup>−/−</sup> were treated with RANKL, P. gingivalis plus RANKL, or pretreated with RANKL for 24 h and then stimulated with P. gingivalis. Pam3CSK4 and LPS were used as control ligands for TLR2 and TLR4, respectively. The cultures were stained for TRAP activity on day 4, and the number of TRAP<sup>−</sup> MNC was counted. A representative area of the cultures from each condition at 40× magnification is shown. Data are expressed as mean ± S.D. of three independent experiments. *, p < 0.05 and ***, p < 0.001 compared with their RANKL control. B, a representative area at 40× and 100× magnification showing different sizes of osteoclasts.
transient JNK phosphorylation at 60 min, similar JNK activity was induced by stimulation with either RANKL or \textit{P. gingivalis} (Fig. 7A), ruling out the possibility that \textit{P. gingivalis} blocks osteoclastogenesis by inhibiting RANKL-mediated JNK activation. In RANKL-primed condition, \textit{P. gingivalis}-induced ERK activation was enhanced, whereas a trend of decreased activation of JNK, p38, and Akt was observed (Fig. 7B). Significantly, RANKL pretreatment severely suppressed \textit{P. gingivalis}-induced NF-κB activation (Fig. 7B). To confirm this finding, we next assessed the degradation and phosphorylation of IκBα, the documented upstream regulator of NF-κB. RANKL pretreatment decreased \textit{P. gingivalis}-induced IκBα degradation and phosphorylation (Fig. 7C), suggesting that RANKL pretreatment overrides \textit{P. gingivalis}-induced activation of NF-κB signaling.
NFATc1 has been described as the master switch for regulating terminal differentiation of osteoclasts downstream of RANKL, and its expression is regulated by c-Fos (48, 49). Therefore, we next examined the effect of \textit{P. gingivalis} on RANKL-induced expression of c-Fos and NFATc1. Stimulation of BMMs with RANKL alone enhanced c-Fos and NFATc1 expression (Fig. 7D). The expression peaked at 48 h and decreased to almost baseline level by 72 h. Conversely, \textit{P. gingivalis} did not induce an up-regulation of c-Fos and NFATc1 (Fig. 7D). Moreover, simultaneous treatment of cells with \textit{P. gingivalis} and RANKL suppressed RANKL-induced up-regulation of c-Fos and NFATc1 (Fig. 7D). However, when cells were primed with RANKL, \textit{P. gingivalis} sustained RANKL-induced c-Fos and NFATc1 expression (Fig. 7D). Because NFATc1 translocates into the nucleus following activation (49), we next assessed the subcellular localization of NFATc1 under various culture conditions. \textit{P. gingivalis} stimulation of unprimed BMMs blocked RANKL-induced nuclear translocation of NFATc1 (Fig. 7F, lane 5 versus lane 4). However, nuclear translocation of NFATc1 was observed in RANKL-primed cells stimulated with \textit{P. gingivalis} (Fig. 7F, Nuclear, lane 6). Taken together, these results provide evidence that \textit{P. gingivalis} modulates osteoclastogenesis by regulating RANKL-induced c-Fos and NFATc1 activity.

\textbf{NFATc1 Suppresses \textit{P. gingivalis}-induced NF-κB Activity—}

The MAPK and Akt pathways, as well as NF-κB, are critical in the regulation of TLR-mediated inflammatory cytokine responses (36, 50, 51). However, we questioned why RANKL activated these intracellular signaling pathways but induced minimal cytokine production by BMMs. The NFAT family of transcription factors has been shown to overlap functionally with the NF-κB family in coordinating the induction of many cytokines and immunoregulatory genes in activated T cells (52). However, in this study, a clear difference was noted in NFATc1 and NF-κB activities following \textit{P. gingivalis} stimulation in unprimed or RANKL-primed cells. Specifically, we observed an increased NF-κB but suppressed NFATc1 activity when unprimed BMMs were treated with \textit{P. gingivalis} (Fig. 7, A and D, respectively). Conversely, when the cells were primed with RANKL, NFATc1 activity was induced and \textit{P. gingivalis}-induced NF-κB activation was repressed (Fig. 7, E and B, respectively). These results led us to consider the existence of a negative feedback loop between NFATc1 and \textit{P. gingivalis}-induced NF-κB activity. To explore this possibility, we overexpressed constitutively active NFATc1 in BMMs using a retroviral system. Cells were then stimulated with \textit{P. gingivalis} for 30 min, and the activation of NF-κB was analyzed by Western blot. BMMs overexpressing constitutively active NFATc1 revealed a
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FIGURE 8. Overexpression of NFATc1 down-regulates P. gingivalis-induced NF-κB activity and cytokine production. A, BMMs were infected with GFP control virus or NFATc1 virus for 48 h, and the levels of NFATc1 and β-actin as well as phosphorylated NF-κB p65 following 30 min of P. gingivalis stimulation were assessed by Western blot analysis. B, BMMs infected with GFP virus or NFATc1 virus were stimulated with P. gingivalis for 12 h, and the levels of TNF-α, IL-6, IL-12 p40, and IL-10 in the culture supernatants were analyzed by cytokine ELISA. The results are representative of three independent experiments.

decrease in NF-κB activity compared with cells infected with GFP control virus (Fig. 8A), suggesting that NFATc1 negatively regulates P. gingivalis-induced NF-κB activity.

Considering that NF-κB signaling is critical for the regulation of TLR-mediated cytokine responses (36, 50, 51), we next determined whether NFATc1 affects P. gingivalis-induced cytokine production. BMMs overexpressing constitutively active NFATc1 showed decreased levels of TNF-α, IL-6, IL-12p40 and IL-10 production following 12 h of P. gingivalis stimulation, compared with cells infected with control virus (Fig. 8B), demonstrating that NFATc1 has an antagonistic effect on P. gingivalis-induced cytokine production. These data suggest that by inducing NFATc1, RANKL compromises the TLR-mediated cytokine response of BMMs. Furthermore, the cross-talk between P. gingivalis and RANKL occurs at least partially because of the differential induction of NF-κB and NFATc1.

DISCUSSION

Periodontopathic bacterial infection is closely associated with the inflammatory destruction of alveolar bone in periodontitis by both direct and indirect effects on osteoclast differentiation (53). Therefore, the identification and characterization of the direct interactions between bacterial stimulation and osteoclast differentiation, as well as the molecular mechanism underlying these interactions, is critical for an understanding of disease pathogenesis. Previous studies (45, 54–58) have shown a positive or negative role of bacterial components, especially LPS, on osteoclast differentiation. Although most studies considered LPS a stimulatory factor for the induction of osteoclastogenesis, we (59) and others (60) have recently demonstrated that E. coli LPS plays a bifunctional/dual role in this process. Furthermore, we have proposed that the commitment status of osteoclast precursors determines the outcome of LPS action in vivo in relation to osteoclastogenesis. However, it is not clear how whole bacteria can affect osteoclast differentiation.

In this study, we show that, similar to LPS, the periodontal pathogen P. gingivalis differentially modulates RANKL-induced osteoclastogenesis by inhibiting osteoclast differentiation of non-committed precursor cells and by potentiating osteoclast formation of RANKL-committed cells. Moreover, we have identified a new mechanism of homeostatic regulation of inflammation and osteoclastogenesis during bacterial infection. We demonstrate that RANKL-primed osteoclast precursors lose their ability to produce cytokines in response to P. gingivalis infection. Furthermore, RANKL-pretreatment of BMMs suppresses P. gingivalis-induced up-regulation of TLR2 and TLR4, which provides insight into the specific signaling molecules involved in the negative regulation of cytokine production.

This study also demonstrates that both TLR2 and TLR4 are essential for an optimal cytokine response of BMMs stimulated with P. gingivalis. Furthermore, although MyD88 is indispensable for P. gingivalis-induced cytokine production by BMMs, TRIF plays a minimal role in the regulation of certain cytokines. Moreover, TLR2/MyD88 but not TLR4/TRIF is the central pathway involved in P. gingivalis-mediated inhibition of osteoclast differentiation. Our results are in agreement with those of Sato et al. (54), indicating an involvement of MyD88 but not TRIF in LPS-mediated regulation of osteoclastogenesis, although a stimulatory effect of LPS on osteoclastogenesis was reported. These authors suggested that TRIF might be important for the function of immune cells but not nonimmune functions.

TLR ligands have been shown to inhibit osteoclastogenesis by down-regulating the expression of RANK (60, 61). However, we have shown that P. gingivalis up-regulates RANK expression. Although osteoclastogenesis is mediated via ligation of RANKL to its transmembrane receptor RANK, RANK expression is mainly controlled by M-CSF-mediated signaling (62). Therefore, it is likely that P. gingivalis inhibits RANKL activity through RANK-mediated downstream signaling. We have recently shown that E. coli LPS modulates osteoclast differentiation by interfering with RANKL-induced JNK activation and NFATc1 expression (59). However, distinct intracellular pathways may be elicited by different TLRs, as supported by the present findings. In this regard, we have shown that JNK is not involved in P. gingivalis-mediated inhibition of osteoclast differentiation. Furthermore, P. gingivalis suppresses RANKL-induced up-regulation of NFATc1 and c-Fos in unprimed BMMs while enhancing their expression in RANKL-primed cells, thus providing a molecular basis for understanding how P. gingivalis differentially modulates RANKL-induced osteoclastogenesis.

Importantly, in this study, we observed an interplay between NFATc1 and NF-κB activities following P. gingivalis stimulation in unprimed or RANKL-primed cells. This observation was somewhat surprising because inhibition of NF-κB has been shown to suppress NFATc1 expression in RANKL-induced osteoclastogenesis (63, 64). However, it has been shown that although NF-κB is recruited to the NFATc1 promoter 1 h after RANKL stimulation, this recruitment is not observed after 24 h. Instead, NFATc1 is recruited to its own promoter by that time, and this occupancy persists during the terminal differentiation of osteoclasts (65). Furthermore, RANKL has been shown to induce osteoclast formation of NF-κB knockout precursor cells as long as NFATc1 is expressed (66). Therefore, although NF-κB is absolutely required for the initial activation of NFATc1 in the early stages of osteoclast differentiation, the autoamplification mechanism of NFATc1 at the later stages
determines its essential role in osteoclastogenesis and is independent of NF-κB activity. Moreover, the robust induction of NFATc1 by RANKL negatively regulates NF-κB activity, which seems crucial for compromising TLR-mediated cytokine production and promoting RANKL-induced osteoclast differentiation. To our knowledge, this is the first study demonstrating that NFATc1 negatively regulates TLR signaling. Interestingly, a similar antagonistic correlation between NFATc1 and NF-κB activity was reported recently regarding Wnt5a-mediated regulation of chondrocyte differentiation (67), indicating that differential induction of NF-κB and NFATc1 may be critical in multiple cell fate determinations.

In summary, we have identified and characterized a novel cross-talk between the TLR and RANKL signaling pathways during osteoclastogenesis in the context of P. gingivalis infection. This cross-talk is accomplished through differential regulation of NFATc1 and NF-κB. Our findings provide a molecular basis to help understand the pathogenesis of bacteria-mediated bone loss diseases.

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