IL-1R/TLR2 through MyD88 Divergently Modulates Osteoclastogenesis through Regulation of Nuclear Factor of Activated T Cells c1 (NFATc1) and B Lymphocyte-induced Maturation Protein-1 (Blimp1)*

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Background: Interleukin-1 and *Porphyromonas gingivalis* both signal through the IL-1R/TLR superfamily but have distinct effects on osteoclastogenesis.

Results: IL-1 and *P. gingivalis* lipopolysaccharide (LPS-PG) differentially regulate osteoclast genes and osteoclastogenic transcription factor NFATc1, as well as transcription repressor Blimp1 and anti-osteoclastogenic genes.

Conclusion: Multiple signaling molecules are involved in distinct IL-1R/TLR2-mediated effects on osteoclastogenesis.

Significance: This is a novel mechanism for understanding infection/inflammation-mediated osteoclastogenesis.

Toll-like receptors (TLR) and the receptor for interleukin-1 (IL-1R) signaling play an important role in bacteria-mediated bone loss diseases including periodontitis, rheumatoid arthritis, and osteomyelitis. Recent studies have shown that TLR ligands inhibit the receptor activator of NF-κB ligand (RANKL)-induced osteoclast differentiation from un-committed osteoclast precursors, whereas IL-1 potentiates RANKL-induced osteoclast formation. However, IL-1R and TLR belong to the same IL-1R/TLR superfamily, and activate similar intracellular signaling pathways. Here, we investigate the molecular mechanisms underlying the distinct effects of IL-1 and *Porphyromonas gingivalis* lipopolysaccharide (LPS-PG) on RANKL-induced osteoclast formation. Our results show that LPS-PG and IL-1 differentially regulate RANKL-induced activation of osteoclast genes encoding Car2, Ctsk, MMP9, and TRAP, as well as expression of NFATc1, a master transcription factor of osteoclastogenesis. Regulation of osteoclast genes and NFATc1 by LPS-PG and IL-1 is dependent on MyD88, an important signaling adaptor for both TLR and IL-1 family members. Furthermore, LPS-PG and IL-1 differentially regulate RANKL-costimulatory receptor OSCAR (osteoclast-associated receptor) expression and Ca2+ oscillations induced by RANKL. Moreover, LPS-PG completely abrogates RANKL-induced gene expression of B lymphocyte-induced maturation protein-1 (Blimp1), a global transcriptional repressor of anti-osteoclastogenic genes encoding Bcl6, IRF8, and MafB. However, IL-1 enhances RANKL-induced blimp1 gene expression but suppresses the gene expression of bcl6, irf8, and mafb. Our study reveals the involvement of multiple signaling molecules in the differential regulation of RANKL-induced osteoclastogenesis by TLR2 and IL-1 signaling. Understanding the signaling cross-talk among TLR, IL-1R, and RANK is critical for identifying therapeutic strategies to control bacteria-mediated bone loss.

Chronic periodontitis, a bacteria-mediated inflammatory disease characterized by the destruction of periodontal connective tissues and the subsequent loss of alveolar bone, is a common human infectious disease and the primary cause of tooth loss in adults (1). Although infection and inflammation are prerequisites for periodontal bone loss, the precise molecular mechanisms underlying how bacterial infection and inflammation mediate osteoclast differentiation have not been fully elucidated. Excessive formation and activity of osteoclasts are major characteristics of pathogenic bone loss condition such as periodontitis and rheumatoid arthritis (2, 3). Osteoclast differentiation requires the presence of two essential molecules: the macrophage colony-stimulating factor (M-CSF) (2) and the receptor activator of NF-κB ligand (RANKL) (4, 5). Although M-CSF promotes proliferation and survival of osteoclast pre-

2 The abbreviations used are: M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of NF-κB ligand; NFATc1, nuclear factor of activated T cells c1; TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; TRIF, Toll/IL-1R domain-containing adaptor-inducing IFN-β; LPS-PG, *P. gingivalis* LPS; BMMs, bone marrow-derived macrophages; TRAP, tartrate-resistant acid phosphatase; Ctsk, cathepsin K; MIP9, matrix metalloproteinase 9; Car2, carboxic anhydrase II; ITAM, immunoreceptor tyrosine-based activation motif; TREM2, triggering receptor expressed in myeloid cells; OSCAR, osteoclast-associated receptor; Blimp1, B lymphocyte-induced maturation protein-1; IRF8, IFN regulatory factor 8; MafB, v-maf musculoaponeurotic fibrosarcoma oncogene family protein B; Bcl6, B cell lymphoma 6; HPRT, hypoxanthine guanine phosphoribosyltransferase.
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cursors, RANKL plays a key role in driving osteoclast precursors to differentiate along the osteoclast lineage and is also a potent regulator of the activation and survival of mature osteoclasts. Binding of RANKL to its receptor RANK activates multiple intracellular signaling cascades that result in the induction of the nuclear factor of activated T cells c1 (NFATc1), which serves as a “master regulator” of osteoclastogenesis (5, 6). NFATc1 in turn, induces the transcription of osteoclast-specific genes, leading to the differentiation and activation of osteoclasts (8).

*Porphyromonas gingivalis* is a Gram-negative, anaerobic bacterium associated with periodontitis (9, 10). We have shown that *P. gingivalis* differentially modulates RANKL-induced osteoclast formation contingent on the state of differentiation of osteoclast precursors (11). Thus, it inhibits the formation of osteoclasts from non-committed precursors cells but promotes the differentiation of osteoclasts from RANKL-committed cells. Furthermore, Toll-like receptor 2 (TLR2) and the myeloid differentiation factor 88 (MyD88) signaling pathway are critical for *P. gingivalis*-mediated inhibition of osteoclast differentiation. TLRs are a family of pattern recognition receptors by which the host innate immune system recognizes microbial pathogens (12, 13). TLRs tailor the innate immune response through the use of different signaling components and adaptor molecules, like MyD88 and the Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF). The MyD88-dependent signaling pathway is utilized by all known TLR 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. Interestingly, animal studies have shown that *P. gingivalis*-mediated periodontal bone loss is dependent on TLR2 signaling (14).

Interleukin-1 (IL-1) is a prototypic pro-inflammatory cytokine that regulates various immune and inflammatory responses (15–17). It is believed to be one of the most potent bone resorbing factors involved in bone loss associated with inflammation. Clinical and animal studies have shown that IL-1 levels are abnormally elevated at sites of inflammatory bone erosion (18, 19). In addition, inhibition of IL-1 decreases alveolar bone loss in several models of periodontal disease (20). IL-1 is believed to participate in multiple steps of osteoclast development and function. It stimulates the survival and function of osteoclasts by directly targeting RANKL-committed cells, but also promotes osteoclastogenesis from non-committed precursors in the presence of permissive levels of RANKL (21). IL-1 exerts its function by activating type I IL-1 receptor (IL-1R), its main signaling receptor (17). Notably, IL-1R and TLR belong to the same receptor superfamily called the IL-1R/TLR superfamily, based on the significant homology of their cytosolic domain, termed Toll/IL-1R (TIR) domain (17, 22, 23). In addition, IL-1R signaling also requires MyD88 to trigger the activation of downstream kinases and transcription factors such as NF-κB. However, it is not clear why TLR signaling inhibits RANKL-induced osteoclast differentiation from non-committed precursors, whereas IL-1R signaling potentiates this process.

*P. gingivalis* possesses multiple virulence factors, including lipopolysaccharide (LPS), fimbriae, gingipains, and hemagglutinins that are believed to be crucial for the initiation and progression of periodontitis (24). Among these, *P. gingivalis* LPS (LPS-PG) has been shown to be a potent stimulator of the innate host defense system and is critical in inducing multiple cells to secret pro-inflammatory cytokines that are implicated in potentiating bone loss (25–27). LPS-PG is very unique and has been shown to differ from *Escherichia coli* and other Gram-negative enterobacterial LPS in structure and functional activities (28–30). Although most enterobacterial LPS signal through TLR4, LPS-PG has been shown to signal via TLR2. In the present study, we investigated the effects of LPS-PG on RANKL-induced osteoclast differentiation and the molecular basis underlying the distinct regulation of osteoclastogenesis by LPS-PG and IL-1.

**Experimental Procedures**

*Mice—*C57BL/6 wild type (WT) and MyD88 knock-out (MyD88−/−) mice were bred and maintained within an environmentally controlled, pathogen-free animal facility at the University of Alabama at Birmingham (UAB). The original MyD88−/− breeding pairs were obtained under a material transfer agreement from Dr. Shikuo Akira (Osaka University, Osaka, Japan). 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 Bone Marrow-derived Macrophages (BMMs)—** Murine bone marrow cells were collected as previously described (11). 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). To prepare osteoclast precursors, bone marrow cells were cultured in α-10 medium (α-MEM, 10% FCS, 1× PenStrep) in a humidified 5% CO2 incubator at 37 °C overnight. Then, non-adherent 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 4 days of culturing, non-adherent cells were removed and adherent cells were used as osteoclast precursor cells.

**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. Cells were stimulated with recombinant IL-1α (R&D Systems), LPS-PG (InvivoGen), and/or recombinant RANKL as indicated in individual assays. The cells were cultured for 4 days and stained for tartrate-resistant acid phosphatase (TRAP) activity using a Leukocyte Acid Phosphatase kit (Sigma).

**Real-time Quantitative PCR—** Total RNA was extracted from 106 cells at the indicated time points (see “Results”) using RNeasy Mini kits (Qiagen) 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 done using a Lightcycler (Roche Molecular Biochemicals) with a FastStart DNA Master SYBR Green I reagent.
(Roche Applied Science). Relative quantities of the tested gene were normalized to hypoxanthine guanine phosphoribosyltransferase (HPRT) mRNA. The normalized data were expressed using the comparative $2^{-ΔΔCT}$ method.

**Cell Extract Preparation and Western Blot Analysis**—BMMs were stimulated with IL-1, LPS-PG, and/or RANKL for different periods of time, and whole cell lysates from $10^6$ cells were prepared as previously described (11, 31). Briefly, cells were washed with PBS and then lysed on ice for 10 min in RIPA lysis buffer (Upstate) freshly supplemented with 1 mM PMSF, 1 mM Na3VO4, 1 mM NaF, and 1 μg/ml of protease inhibitor mixture (Roche Applied Science). The whole cell lysates were transferred to tubes and incubated on ice for an additional 20 min, and then following centrifugation the supernatants were collected. Equivalent amounts of protein from cell lysates were separated by SDS-PAGE on a 10% Tris-HCl gel (Bio-Rad Laboratories). The whole cell lysates were transferred to Immobilon-P transfer membranes (Millipore), and probed with specific antibodies against β-Actin, NFATc1, or the phosphorylated form of p38, ERK1/2, JNK, Akt (Ser-473), NF-κB p65 (Ser-536). Detection was carried out using HRP-linked rabbit IgG antibody, followed by ECL Western blotting detection reagents (Amersham Biosciences UK Ltd.). All antibodies were purchased from Cell Signaling Technology, except NFATc1 (Santa Cruz Biotechnology).

**FACS Analysis**—BMMs were cultured with IL-1, LPS-PG, and/or RANKL. After 24 h of stimulation, cells were harvested, washed, and suspended in fluorescence-activated cell sorting (FACS) buffer and stained with phycoerythrin-labeled antibodies against c-fms or RANK (eBioscience) for 30 min on ice. Cells were washed, suspended in FACS buffer, and immediately analyzed using a FACS Calibur (BD Bioscience). Data were analyzed using CellQuest software (BD Bioscience).

**Ca2+ Mobilization Assay**—To measure cytosolic Ca2+ fluxes following IL-1, LPS-PG, and/or RANKL stimulation, BMMs were grown in glass bottom culture dishes (MatTek Corp.) and loaded with 2 μM Fura-2/AM (Invitrogen) for 30 min at 37 °C. Fluorescence imaging was recorded and analyzed using a digital fluorescence imaging system (InCyt Im2, Intracellular Imaging Inc.). The InCyt Im2 system has a pre-loaded software that converts the ratiometric signal into an actual calcium read out after the system is calibrated against a set of solutions that have known Ca2+ concentrations. RANKL, IL-1, LPS-PG, RANKL + IL-1, RANKL + LPS, or 10 μM ionomycin (positive control) was applied to the cells and Fura-2 fluorescence at an emission wavelength of 510 nm was induced by exciting Fura-2 alternately at 340 and 380 nm. The 340/380 nm ratio images were obtained on a pixel by pixel basis and converted to absolute Ca2+ concentrations by in vitro calibration with a Fura-2 Calcium Imaging Calibration kit (ThermoFisher Scientific) (32–34). The relative intracellular calcium levels in single cells were monitored for 300 s. Oscillating cells were defined as having oscillation amplitudes greater than 150 nm. The percentages of oscillating/non-oscillating cells in each condition were calculated. 10–20 single cells in each condition were captured and analyzed, and each condition was repeated at least 3 times.

**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**

**LPS-PG and IL-1 Differentially Regulate RANKL-induced Osteoclast Differentiation**—IL-1R and TLRs play an important role in innate immunity as well as in bone metabolism (16, 23, 35, 36). We and others have shown that P. gingivalis and TLR ligands such as E. coli LPS inhibit RANKL-induced osteoclast differentiation of un-committed osteoclast precursors (11, 37, 38). Conversely, IL-1 potentiates RANKL-induced osteoclast formation of un-committed osteoclast precursors (21). Noteworthy, IL-1R and TLRs belong to the same IL-1R/TLR superfamily, and activate similar intracellular signaling pathways (22, 35); however, it is unclear why they have different effects on RANKL-induced osteoclast formation. To understand the molecular mechanism underlying the distinct role of TLR and IL-1R signaling in RANKL-induced osteoclastogenesis, we examined the effect of LPS-PG, a TLR2 ligand on osteoclastogenesis and compared it with that of IL-1. BMMs from C57BL/6 mice were stimulated with LPS-PG or IL-1 in the presence/absence of optimal (100 ng/ml) or permissive (10 ng/ml) levels of RANKL, and the formation of multinucleated cells positive for TRAP staining was analyzed. Although BMMs treated with 100 ng/ml of RANKL formed numerous osteoclasts, 10 ng/ml of RANKL failed to induce multinucleated cell formation (Fig. 1, A–F). Similar to E. coli LPS or to P. gingivalis, LPS-PG alone was not able to induce osteoclast differentiation of murine BMM precursor cells (data not shown). In addition, LPS-PG suppressed RANKL-mediated osteoclast differentiation in a dose-dependent manner, with 1 μg/ml of LPS-PG completely abolishing RANKL-induced osteoclast formation (Fig. 1, A and C). However, unlike LPS-PG, IL-1 induced osteoclast differentiation in the presence of RANKL (Fig. 1, B and C). Substantial osteoclast formation was seen when BMMs were treated with IL-1 in the presence of optimal levels of RANKL. Furthermore, IL-1 was able to potentiate osteoclast formation in the presence of permissive levels of RANKL. These results demonstrate that TLR2 and IL-1R signaling have distinct effects on RANKL-induced osteoclast differentiation from un-committed osteoclast precursors.

**LPS-PG and IL-1 on RANKL-induced Osteoclast Genes**—Previous studies on gene expression profiling have established that RANKL stimulates osteoclast differentiation by altering the expression of numerous genes (8). In particular, RANKL upregulates the expression of genes encoding tartrate-resistant acid phosphatase (TRAP/acp5), cathepsin K (Ctsk), matrix metalloproteinase 9 (MMP9), and carbonic anhydrase II (Car2), which play important roles in osteoclast differentiation and/or function and are thus widely used as markers for osteoclasts. To understand the molecular basis of the differential role of LPS-PG and IL-1 in regulating osteoclastogenesis, we examined the effect of LPS-PG and IL-1 on the expression of these osteoclast genes on days 0, 2, and 4 of differentiation in the presence/absence of optimal or permissive level of RANKL. Our result showed that 100 ng/ml of RANKL stimulation strongly induced the expression of osteoclast genes by day 2, and the
expression of these genes was further enhanced by day 4 (Fig. 2A). Relatively lower levels of osteoclast gene expression were induced by 10 ng/ml of RANKL on days 2 and 4, as compared with stimulation by 100 ng/ml of RANKL (Fig. 2B). LPS-PG or IL-1 failed to induce the expression of these osteoclast genes. Furthermore, LPS-PG blocked RANKL-induced up-regulation of these genes. However, unlike LPS-PG, IL-1 was able to potentiate RANKL-induced osteoclast gene expression. In this regard, in the presence of 100 ng/ml of RANKL, we saw dramatically enhanced levels of osteoclast gene expression on day 2 by cells treated with IL-1 plus RANKL (R + IL-1), as compared with those cells treated with RANKL only (Fig. 2A). In the presence of permissive levels of RANKL, elevated levels of osteoclast genes were observed on both days 2 and 4 when IL-1 was added in the cultures (Fig. 2B). These results indicate that RANKL is able to render osteoclast genes responsive to IL-1, whereas LPS-PG inhibits RANKL-induced osteoclast gene expression.

**LPS-PG and IL-1 Differentially Modulate RANKL-induced NFATc1 Expression**—RANKL has been shown to induce osteoclastogenesis by activating multiple intracellular signaling pathways including MAPKs (p38, ERK, and JNK), Akt, NF-κB, and NFATc1 (7). To understand the molecular mechanism underlying the distinct effect of IL-1 and LPS-PG on RANKL-induced osteoclastogenesis, we next investigated the activation of these intracellular signaling pathways. As determined by Western blotting, stimulation of BMMs with IL-1 showed weaker phosphorylation of p38, ERK, JNK, Akt, and IκBα compared with that by RANKL stimulation. Yet, LPS-PG induced similar or stronger activation of these signaling pathways (Fig. 3A), even though it does not induce osteoclast differentiation of BMMs. These results indicate that the inability of LPS-PG or IL-1 to induce osteoclast differentiation is unlikely due to the inactivation or weaker/stronger activation of these signaling molecules. Stimulation of BMMs with LPS-PG and RANKL together (R + LPS-PG) led to enhanced and sustained phosphorylation of p38, ERK, JNK, Akt, and IκBα compared with RANKL stimulation alone (Fig. 3, A and B). Similarly, IL-1 enhanced RANKL-induced phosphorylation of MAPKs and Akt, as well as IκBα degradation (Fig. 3, A and B), ruling out the possibility that LPS-PG and IL-1 differentially regulate RANKL-induced osteoclastogenesis through regulating RANKL-mediated activation of MAPK, Akt, and NF-κB pathways. Taken together, although MAPK, Akt, and NF-κB pathways are prerequisites for RANKL-induced osteoclast differen-
Inactivation, activation of these signaling pathways does not necessarily lead to osteoclast differentiation. Next, we examined the effect of LPS-PG and IL-1 on RANKL-induced expression of NFATc1. RANKL stimulation alone was able to induce an up-regulation of NFATc1 expression at 24 and 48 h (Fig. 3C). However, LPS-PG induced minimal up-regulation of NFATc1, and no up-regulation of NFATc1 expression was observed in IL-1-treated cells. Moreover, simultaneous treatment of BMMs with RANKL and LPS-PG abrogated RANKL-induced up-regulation of NFATc1, whereas IL-1 potentiated RANKL-induced NFATc1 expression (Fig. 3C). A similar effect of LPS-PG and/or IL-1 on RANKL-induced mRNA expression of NFATc1 was observed (Fig. 4C). These results strongly suggest that LPS-PG and IL-1 differentially regulate RANKL-induced osteoclastogenesis through the modulation of NFATc1.

MyD88 in LPS-PG- and IL-1-mediated Regulation of Osteoclastogenesis—MyD88 is a key adaptor component for both TLR and IL-1R signaling pathways (39, 40). We have recently shown that MyD88 plays a crucial role in IL-1-mediated osteoclastogenesis of uncommitted BMMs (21). However, it is not clear whether MyD88 signaling is also involved in regulating IL-1-mediated osteoclastogenesis of uncommitted BMMs, and furthermore, the role of MyD88 signaling in LPS-PG-mediated regulation of osteoclastogenesis is unknown. Therefore, we next investigated the involvement of MyD88 in LPS-PG- and IL-1-mediated regulation of osteoclastogenesis of un-committed BMMs. Our findings showed that RANKL alone induced similar levels of TRAP-positive multinuclear cell formation in WT or MyD88−/− BMMs (Fig. 4A). However, LPS-PG inhibited RANKL-induced osteoclast formation in WT BMMs but not in MyD88−/− cells. Moreover, IL-1 enhanced RANKL-induced osteoclast formation in WT BMMs, but failed to do so in MyD88−/− cells, thus indicating that MyD88 signaling is critical for LPS-PG and IL-1-mediated regulation of osteoclast formation from non-committed osteoclast precursors.

Next, we investigated the involvement of MyD88 in the regulation of RANKL-induced osteoclast genes and activation of intracellular signaling pathways by LPS-PG and IL-1. Our results showed that although LPS-PG almost completely abolished RANKL-induced osteoclast gene expression in WT BMMs, no inhibition (car2 gene) or reduced levels of inhibition (trap, mmp9, and ctsk genes) were observed in MyD88−/− BMMs (Fig. 4B). Yet, IL-1 was able to potentiate RANKL-induced osteoclast gene expression in WT BMMs, but it failed to do so in MyD88−/− cells (Fig. 4B). Therefore, the inhibition or potentiation of RANKL-induced osteoclast gene expression by LPS-PG or IL-1, respectively, is dependent on MyD88. Interestingly, although a trend of decreased activation of JNK, p38, and IκBα was observed following R + LPS-PG or R + IL-1 stimulation in MyD88−/− BMMs com-
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LPS-PG and IL-1 on the Cell Surface Expression of c-fms and RANK—To understand the mechanism by which LPS-PG and IL-1 regulate RANKL-induced osteoclast genes and NFATc1, we next assessed the effect of LPS-PG and IL-1 on the cell surface expression of M-CSF receptor c-fms and RANKL receptor RANK. Stimulation of BMMs with RANKL, LPS-PG, IL-1, R + LPS-PG, or R + IL-1 had no effect on the cell surface expression of c-fms (Fig. 5A). When cells were stimulated with RANKL, IL-1, or R + IL-1, a minimal effect on RANK expression was seen (Fig. 5B). However, in the presence of LPS-PG, an up-regulation of RANK expression was seen (Fig. 5B). These results suggest that the differential regulation of osteoclastogenesis by LPS-PG and IL-1 is not due to the regulation of c-fms expression, and furthermore, RANK-mediated osteoclast differentiation of murine BMMs is not associated with the up-regulation of RANK expression.

Regulation of RANKL Co-stimulatory Receptors by LPS-PG and IL-1—Recently, the immunoreceptor tyrosine-based activation motif (ITAM)-dependent pathway has been identified as a costimulatory signaling for RANKL in inducing RANKL-induced osteoclast genes and NFATc1 expression (41, 42). Two ITAM-harboring transmembrane adaptors DNAX-activating protein 12 (DAP12) and Fc receptor common γ subunit (FcγR) have been found to be indispensable for RANKL-induced osteoclastogenesis (5, 41). By pairing with their co-receptors TREM2 (triggering receptor expressed in myeloid cells) and OSCAR (osteoclast-associated receptor), respectively, DAP12 and FcγR recruit the tyrosine kinase Syk and activate the PLCγ pathway. This results in the activation of calcium pathways and activates calcineurin to induce NFATc1 (41–43). To further understand the mechanisms by which LPS-PG and IL-1 regulate NFATc1, we next assessed the effect of LPS-PG and IL-1 on the gene expression of TREM2 and OSCAR. As shown in Fig. 6A, RANKL markedly up-regulated the expression of oscar, whereas LPS-PG or IL-1 alone failed to induce oscar gene expression. However, LPS-PG completely abolished RANKL-induced up-regulation of the oscar gene, yet, IL-1 was able to enhance RANKL-induced expression of the oscar gene (Fig. 6A). Interestingly, down-regulation of trem2 gene expression was observed with RANKL stimulation alone or in the presence of LPS-PG or IL-1 (Fig. 6B). Furthermore, LPS-PG or IL-1 alone also down-regulated trem2 gene expression. These results suggest that the induction of RANKL costimulatory receptor OSCAR expression is an important component involved in the differential regulation of RANKL-induced osteoclastogenesis by LPS-PG and IL-1.

Regulation of Calcium Oscillations by LPS-PG and IL-1—It has been reported that RANKL-induced Ca2+ oscillations are important for calcineurin-mediated activation of NFATc1, which in turn induce expression of osteoclast-specific genes (44–46). Therefore, we analyzed Ca2+ oscillations induced by RANKL in the presence or absence of LPS-PG or IL-1. Consistent with previous studies (44, 45), sustained Ca2+ oscillations were observed at 48 h of RANKL stimulation (Fig. 7, A and B). However, when cells were treated with LPS-PG or IL-1 alone, no significant induction of oscillations was observed. Noteworthy, whereas R + LPS-PG failed to induce significant Ca2+ oscillations, R + IL-1 were able to increase sustained Ca2+
oscillations were induced by RANKL, RANKL plus IL-1, or RANKL plus IL-1 plus RANKL. Furthermore, similar levels of increased Ca\(^{2+}\) expression. Figure 6 shows the effect of LPS-PG, IL-1, and/or RANKL on the surface expression of c-fms and RANK. BMMs from C57BL/6 WT mice were stimulated with RANKL (100 \(\mu\)g/ml), LPS-PG (1 \(\mu\)g/ml), IL-1 (10 ng/ml), RANKL plus LPS-PG (R+LPS-PG), or RANKL plus IL-1 (R+IL-1) for 24 h. The expression of c-fms and RANK on the cell surface was analyzed by FACS. The values in the histograms are the mean fluorescence intensity (MFI). The results are representative of three independent experiments.

Discussion

TLRs and IL-1R belong to the same IL-1R/TLR superfamily and are believed to play important roles in bacteria-mediated bone loss in diseases including periodontitis, rheumatoid arthritis, and osteomyelitis (14, 18, 42, 51–54). Studies have shown that TLR ligands inhibit RANKL-induced osteoclast differentiation from un-committed osteoclast precursors (11, 37, 38, 55), whereas IL-1 potentiates RANKL-induced osteoclast formation (21, 56, 57). However, the underlying mechanism accounting for their different outcomes in RANKL-mediated osteoclastogenesis is not fully understood.

In this study, we demonstrate that LPS-PG and IL-1 differentially regulate RANKL-induced osteoclastogenesis by regulating NFATc1, the master transcription factor of osteoclast differentiation. MAPKs, Akt, and NF-κB are critical intracellular signaling molecules regulating RANKL-induced NFATc1 expression (5, 58). Although studies have suggested a possible
involvement of ERK, JNK, p38, or NF-κB in TLR or cytokine-mediated regulation of RANKL-induced osteoclast differentiation (37, 59–62), it is unlikely that LPS-PG inhibits RANKL-induced osteoclastogenesis in murine bone marrow osteoclast precursor cells through MAPKs, Akt, or NF-κB.

TLRs have been shown to inhibit osteoclastogenesis by inhibiting the expression of RANK through down-regulation of c-fms and inhibition of M-CSF signaling (55, 63). However, in our studies, no inhibition of c-fms expression is observed with LPS-PG stimulation. In addition, consistent with our previous results with P. gingivalis (11), we show that LPS-PG along or RANKL plus LPS-PG up-regulate RANK expression. Furthermore, we did not see an up-regulation of c-fms and RANK expression by IL-1. Interestingly, although M-CSF is able to up-regulate RANK (data not shown), no further up-regulation of c-fms or RANK is induced by RANKL in the presence of M-CSF, indicating that RANKL induces osteoclastogenesis not by up-regulating c-fms and RANK. Previous studies have indicated that although osteoclastogenesis is mediated via ligation of RANKL to its transmembrane receptor RANK, RANK expression is mainly controlled by M-CSF-mediated signaling (64). Thus, RANK expression is necessary but not sufficient for osteoclast differentiation and that the timing of RANK binding to RANK is critical for the commitment of osteoclast precursors to the osteoclast lineage. Therefore, it is unlikely that TLR or IL-1R signaling regulates RANKL-induced osteoclastogenesis through modulation of RANK expression.

There is emerging evidence that induction of effective osteoclastogenesis by RANK requires costimulation by ITAM-coupled receptors, such as TREM2 and OSCAR (43, 65). TREM2, a DAP12-associated costimulatory receptor, has been reported to play an important role in human osteoclastogenesis, as patients with TREM2 loss-of-function mutations manifest Nasu-Hakola disease characterized by abnormalities in bone remodeling and defective osteoclastogenesis (66, 67). However, TREM2 appears to have a minimal role in mouse osteoclastogenesis. In this regard, studies have shown that TLR stimulation strongly inhibits TREM2 expression in human primary osteoclast precursors, but inhibition is less prominent in murine BMMs and RAW264.7 cells, a murine cell line with osteoclastogenic potential (55, 68, 69). In the present study, we observed a down-regulation of trem2 expression in response to LPS-PG alone. RANKL itself also suppresses trem2 expression, as shown in the present study and by others (70). Therefore, although RANKL plus LPS-PG down-regulate trem2 expression, it is unlikely that LPS-PG inhibits RANKL-induced osteoclastogenesis by down-regulation of trem2. In addition, our results rule out the possibility that IL-1 potentiates RANKL-induced osteoclastogenesis by regulation of TREM2. OSCAR is a potent FcRγ-associated receptor believed to be important in osteoclast
Importantly, in this study, we observed a differential role of LPS-PG and IL-1 on RANKL-induced Ca\(^{2+}\) oscillations. ITAM-mediated costimulatory signaling is believed to be necessary for inducing sustained Ca\(^{2+}\) oscillations, which are critical for sustained NFATc1 activation during osteoclastogenesis (45, 74). Therefore, it is likely that TLR2 and IL-1R signaling differentially regulate RANKL-induced osteoclast gene expression through the OSCAR-Ca\(^{2+}\) oscillations-NFATc1 pathway.

Accumulating evidence reveals that transcriptional repressors expressed constitutively in osteoclast precursors function to oppose the action of RANK and to restrain osteoclastogenesis (48). Thus, in addition to activating positive signaling pathways, RANK needs to overcome the “brakes” imposed on osteoclast differentiation by transcriptional repressors. IRF8, a transcription factor specially expressed in immune cells, is a key negative regulator for osteoclastogenesis (75, 76). TLR4 activation has been reported to induce IRF8 expression and abrogate RANKL-induced down-regulation of IRF8 (76). Recent studies have identified Blimp1 as a global transcriptional repressor of osteoclastogenesis through targeting multiple negative regulators including Bcl6, IRF8, and MafB (47, 48, 50). Consistent with these reports, our results show that RANKL stimulation up-regulates blimp1 gene expression, whereas at the same time down-regulates the gene expression of bcl6, mafb, and especially irf8. In addition, LPS-PG inhibits blimp1 gene expression and suppresses RANKL-induced up-regulation of the blimp1 gene. Furthermore, LPS-PG up-regulates gene expression of bcl6, irf8, and mafb, and reverses RANKL-induced down-regulation of these genes. These results demonstrate that LPS-PG inhibits RANKL-induced osteoclastogenesis at least in part by suppressing the blimp1 gene and augmenting gene expression of bcl6, mafb, and especially that of irf8. On the other hand, IL-1 had a minimal effect on the gene expression of blimp1, bcl6, irf8, and mafb. However, IL-1 was able to potentiate RANKL-induced blimp1 gene expression. To our knowledge, this is the first study demonstrating that TLR2 and IL-1R signaling differentially regulate RANKL-induced blimp1, as well as bcl6, irf8, and mafb gene expression.

Interestingly, our results demonstrate that TLR2 and IL-1R divergently regulate RANKL-induced osteoclastogenesis via MyD88. In this regard, we show that MyD88 is involved in LPS-PG and IL-1-mediated differential regulation of NFATc1, Blimp1, Ca\(^{2+}\) oscillation, as well as osteoclastogenic and anti-osteoclastogenic gene expression. The mechanism(s) underlying the divergent role of MyD88 in TLR2 and IL-1R-mediated regulation of osteoclast differentiation is not clear. MyD88 is composed of an N-terminal death domain and a C-terminal TIR domain. Upon ligand binding, TLR/IL-1Rs hetero- or homodimerize and recruit MyD88 through their respective TIR domains, which in turn dimerizes via its death domain and TIR domain and interacts with the interleukin-1 receptor-associated kinases to form a macromolecular complex named Mydosome (77). The TIR domain is typically composed of 135–140 residues, with sequence conservation between 20 and 30% in different proteins. The sequence and structure of different TIR domains determine the specificities of receptor and adaptor protein TIR-TIR interactions (77). Given the plasticity and heterogeneity of complexes formed by this domain in MyD88,
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it is likely that different regions of TIR are involved in the interactions with TLR2 or IL-1R. Furthermore, although both TLR2 and IL-1R utilize MyD88 signaling, there are key differences between these receptors (7, 78). For example, TLR2 signaling via MyD88 is dependent on the interaction of TIRAP/Mal, another MyD88 adaptor family member, whereas IL-1R signaling requires only MyD88 (78). Thus, differences between TLR2/MyD88- and IL-1R/MyD88-signaling complexes could influence downstream signaling resulting in a differential gene activation (7). Future structure studies and site-directed mutational analyses should help better understanding the molecular details of the MyD88-mediated interactions in IL-1R and TLR signaling.

In summary, our study reveals the involvement of multiple signaling cascades compromising OSCAR, calcium signaling, NFATc1, and Blimp1 in differential regulation of RANKL-induced osteoclastogenic and anti-osteoclastogenic genes by LPS-PG and IL-1 through MyD88. It is likely that TLR2 and IL-1R signaling work in a complementary and cooperative manner to fine-tune the extent of osteoclastogenesis in an inflammatory setting. Understanding the signaling cross-talk among TLR, IL-1R, and RANK is critical for the identification of therapeutic targets to ameliorate/prevent bacteria-mediated bone loss.

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References
1. Lamont, R. J., and Jenkinson, H. F. (1998) Life below the gum line: pathogenic mechanisms of Porphyromonas gingivalis. Microbiol. Mol. Biol. Rev. 62, 1244–1263
2. Cochran, D. L. (2008) Inflammation and bone loss in periodontal disease. J. Periodontol. 79, 1569–1576
3. Goldring, S. R., and Gravallese, E. M. (2000) Pathogenesis of bone erosions in rheumatoid arthritis. Curr. Opin. Rheumatol. 12, 195–199
4. Teitelbaum, S. L., and Ross, F. P. (2003) Genetic regulation of osteoclast development and function. Nat. Rev. Genet. 4, 638–649
5. Asagiri, M., and Takayanagi, H. (2007) The molecular understanding of osteoclast differentiation. Bone 40, 251–264
6. Feng, X. (2005) RANKing intracellular signaling in osteoclasts. IUBMB Life 57, 389–395
7. Miller, L. S., O’Connell, R. M., Gutierrez, M. A., Pietras, E. M., Shahgian, A., Gross, C. E., Thirumala, A., Cheung, A. L., Cheng, G., and Medlin, R. L. (2006) MyD88 mediates neutrophil recruitment initiated by IL-1R but not TLR2 activation in immunity against Staphylococcus aureus. Immunology 24, 79–91
8. Cappellen, D., Luong-Nguyen, N. H., Bongiovanni, S., Grenet, O., Wanke, C., and Susa, M. (2002) Transcriptional program of mouse osteoclast differentiation governed by the macrophage colony-stimulating factor and the ligand for the receptor activator of NF-κB. J. Biol. Chem. 277, 21971–21982
9. Socransky, S. S., Haffajee, A. D., Cuqini, M. A., Smith, C., and Kent, R. L., Jr. (1998) Microbial complexes in subgingival plaque. J. Clin. Periodontol. 25, 134–144
10. Socransky, S. S., and Haffajee, A. D. (1992) The bacterial etiology of destructive periodontal disease: current concepts. J. Periodontol. 63, 322–331
11. Zhang, P., Liu, J., Xu, Q., Harber, G., Feng, X., Michalek, S. M., and Katz, J. (2011) TLR2-dependent modulation of osteoclastogenesis by Porphyromonas gingivalis through differential induction of NFATc1 and NF-κB. J. Biol. Chem. 286, 24159–24169
12. Akira, S., and Takeda, K. (2004) Toll-like receptor signaling. Nat. Rev. Immunol. 4, 499–511
13. Kopp, E., and Medzhitov, R. (2003) Recognition of microbial infection by Toll-like receptors. Curr. Opin. Immunol. 15, 396–401
14. Burns, E., Bachrach, G., Shapira, L., and Nussbaum, G. (2006) Cutting edge: TLR2 is required for the innate response to Porphyromonas gingivalis: activation leads to bacterial persistence and TLR2 deficiency attenuates induced alveolar bone resorption. J. Immunol. 177, 8296–8300
15. Jimi, E., Nakamura, I., Duong, L. T., Ibeke, T., Takahashi, N., Rodan, G. A., and Suda, T. (1999) Interleukin 1 induces multinucleation and bone-resorbing activity on osteoclasts in the absence of osteoblasts/stromal cells. Exp. Cell Res. 247, 84–93
16. Lee, Y. M., Fujikado, N., Manaka, H., Yasuda, H., and Iwakura, Y. (2010) IL-1β plays an important role in the bone metabolism under physiological conditions. Int. Immunol. 22, 805–816
17. Dinarello, C. A. (2009) Immunological and inflammatory functions of the interleukin-1 family. Annu. Rev. Immunol. 27, 519–550
18. Graves, D. T., and Cochran, D. (2003) The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. J. Periodontol. 74, 391–401
19. Strand, V., and Kavanagh, A. F. (2004) The role of interleukin-1 in bone resorption in rheumatoid arthritis. Rheumatology 43, iii10–iii16
20. Assuma, R., Oates, T., Cochran, D., Amar, S., and Graves, D. T. (1998) IL-1 and TNF-α antagonists inhibit the inflammatory response and bone loss in experimental periodontitis. J. Immunol. 160, 403–409
21. Jules, J., Zhang, P., Ashley, J. W., Wei, S., Shi, Z., Liu, J., Michalek, S. M., and Feng, X. (2012) Molecular basis of requirement of receptor activator of nuclear factor κB signaling for interleukin 1-mediated osteoclastogenesis. J. Biol. Chem. 287, 15728–15738
22. Verstrepen, L., Bekaert, T., Chau, T. L., Tavernier, J., Chariot, A., and Beyaert, R. (2008) TLR-4, IL-1R and TNF-R signaling to NF-κB variations on a common theme. Cell. Mol. Life Sci. 65, 2964–2978
23. O’Neill, L. A. (2008) The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. Immunol. Rev. 226, 10–18
24. Holt, S. C., Kesavalu, L., Walker, S., and Genco, C. A. (1999) Virulence factors of Porphyromonas gingivalis. Periodontology 20, 168–238
25. Kato, H., Taguchi, Y., Tominaga, K., Umeda, M., and Tanaka, A. (2014) Porphyromonas gingivalis LPS inhibits osteoblastic differentiation and promotes pro-inflammatory cytokine production in human periodontal ligament stem cells. Arch. Oral. Biol. 59, 167–175
26. Seo, T., Cha, S., Kim, T. I., Lee, J. S., and Woo, K. M. (2012) Porphyromonas gingivalis-derived lipopolysaccharide-activated mediation of MAPK signaling regulates inflammatory response and differentiation in human periodontal ligament fibroblasts. J. Microbiol. 50, 311–319
27. Holden, J. A., Attard, T. J., Laughton, K. M., Mansell, A., O’Brien-Simpson, N. M., and Reynolds, E. C. (2014) Porphyromonas gingivalis lipopolysaccharide weakly activates M1 and M2 polarized mouse macrophages but induces Inflammatory cytokines. Infect. Immun. 82, 4190–4203
28. Scohm, C., Coats, S. R., Hua, N., Kramer, C., Papadopoulos, G., Weinberg, E. O., Gudino, C. V., Hamilton, J. J., Darveau, R. P., and Genco, C. A. (2014) Distinct lipid A moieties contribute to pathogen-induced site-specific vascular inflammation. PLoS Pathol. 10, e1004215
29. Bainbridge, B. W., Coats, S. R., Pham, T. T., Reife, R. A., and Darveau, R. P. (2006) Expression of a Porphyromonas gingivalis lipid A palmitoyltransferase in Escherichia coli yields a chimeric lipid A with altered ability to stimulate interleukin-1 secretion. Cell. Microbiol. 8, 120–129
30. Zhang, D., Chen, L., Li, S., Gu, Z., and Yan, J. (2008) Lipopolysaccharide (LPS) of Porphyromonas gingivalis induces IL-1β, TNF-α and IL-6 production by THP-1 cells in a way different from that of Escherichia coli LPS. Innate Immun. 14, 99–107
31. Zhang, P., Martin, M., Michalek, S. M., and Katz, J. (2005) Role of mitogen-activated protein kinases and NF-κB in the regulation of proinflammatory
and anti-inflammatory cytokines by *Porphyromonas gingivalis* hemagglutinin *B. Insect. Immun.* 73, 3990–3998
32. Barreto-Chang, O. L., and Dolmetsch, R. E. (2009) Calcium imaging of corticosteroid neurons using Fura-2 AM. *J. Vis. Exp.* 23, 1067
33. Trebak, M., St. J. Bird, G., McKay, R. R., Birnbaumer, L., and Putney, J. W., Jr. (2003) Signaling mechanism for receptor-activated canonical transient receptor potential 3 (TRPC3) channels. *J. Biol. Chem.* 278, 16244–16252
34. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) A new generation of Ca$^{2+}$ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260, 3440–3450
35. Narayanan, K. B., and Park-Min, K. H., Antioniv, T., Reid, A. C., Silver, R. B., Humphrey, M. B., Nakamura, M., and Ivashkiv, L. B. (2009) Impaired osteoclastogenesis by TLRs and IFN-γ in human osteoclats. *J. Immuno.* 183, 7223–7233
36. Ma, T., Miyashita, K., Suen, A., Epstein, N. J., Tomita, T., Smith, R. L., and Goodman, S. B. (2004) Human interleukin-1-induced murine osteoclastogenesis is dependent on RANKL, but independent of TNF-α. *Cytokeine* 26, 138–144
37. Wei, S., Kitaura, H., Zhou, P., Ross, F. P., and Teitelbaum, S. L. (2005) IL-1 mediates TNF-induced osteoclastogenesis. *J. Clin. Invest.* 115, 282–290
38. Moon, J. B., Kim, J. H., Kim, K., Youn, B. U., Ko., A. Lee, S. Y., and Kim, N. (2012) Akt induces osteoclast differentiation through regulating the GSK3/β/NFAcTc1 signaling cascade. *J. Immunol.* 188, 163–169
39. Leite, F. R., de Aquino, S. G., Guimaraes, M. R., Cirelli, J. A., Zamboni, D. S., Silva, J. S., and Junior, C. R. (2015) Relevance of the myeloid differentiation factor 88 (MyD88) on RANKL, OPG, and Nod expressions induced by TLR and IL-1R signaling in bone marrow stromal cells. *Inflammation* 38, 1–8
40. Yang, J., Ruy, Y. H., Yun, C. H., and Han, S. H. (2009) Impaired osteoclastogenesis by staphylococcal lipoteichoic acid through Toll-like receptor 2 with partial involvement of MyD88. *J. Leukocyte Biol.* 86, 823–831
41. Amcheslavsky, A., and Bar-Shavit, Z. (2007) Toll-like receptor 9 ligand blocks osteoclast differentiation through induction of phosphatase. *J. Bone Miner. Res.* 22, 1301–1310
42. Wei, S., Wang, M. W., Teitelbaum, S. L., and Ross, F. P. (2002) Interleukin-4 reversibly inhibits osteoclastogenesis via inhibition of NF-κB and mitogen-activated protein kinases signaling. *J. Biol. Chem.* 277, 6622–6630
43. Ozu, W., and Bar-Shavit, Z. (2002) Dual modulation of osteoclast differentiation by lipopolysaccharide. *J. Bone Miner. Res.* 17, 1211–1218
44. Arai, F., Miyamoto, T., Ohneda, O., Inada, T., Sudo, T., Brasel, K., Miyata, T., Anderson, D. M., and Suda, T. (1999) Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor κB (RANK) receptors. *J. Exp. Med.* 190, 1741–1754
45. Takayanagi, H. (2005) Mechanistic insight into osteoclast differentiation in osteoimmunology: *J. Mol. Med.* 83, 170–179
46. Cella, M., Buonsanti, C., Strader, C., Kondo, T., Salmaggi, A., and Colonna, M. (2003) Impaired differentiation of osteoclasts in TREM2-deficient individuals. *J. Exp. Med.* 198, 645–651
47. Paloneva, J., Kestilä, M., Wu, J., Salminen, A., Bühling, T., Ruotsalainen, V., Hakola, P., Bakker, A. B., Phillips, J. H., Pekkarinen, P., Lanier, L. L., Ti monen, T., and Peltonen, L. (2000) Loss-of-function mutations in TYROBP (DAP12) result in a presenile dementia with bone cysts. *Nat. Genet.* 25, 357–361
48. Klesney-Tait, J., Turnbull, I. R., and Colonna, M. (2006) The TREM receptor family and signal integration. *Nat. Immunol.* 7, 1266–1273
49. Colonna, M., Turnbull, I., and Klesney-Tait, J. (2007) The enigmatic function of TREM-2 in osteoclastogenesis. *Adv. Exp. Med. Biol.* 602, 97–105
50. Park-Min, K. H., Ji, J. D., Antoniv, T., Reid, A. C., Silver, R. B., Humphrey, M. B., Nakamura, M., and Ivashkiv, L. B. (2009) IL-10 suppresses calcium-mediated costimulation of receptor activator NF-κB signaling during human osteoclast differentiation by inhibiting TREM-2 expression. *J. Immunol.* 183, 2444–2455
51. Kim, N., Takami, M., Rho, J., Josien, R., and Choi, Y. (2002) A novel member of the leucocyte receptor complex regulates osteoclast differentiation. *J. Exp. Med.* 195, 201–209
52. Herman, S., Müller, R. B., Krönke, G., Zwerina, J., Redlich, K., Hube, A., Gelse, H., Neumann, E., Müller-Ladner, U., and Schett, G. (2008) Induction of osteoclast-associated receptor, a key osteoclast stimulation molecule, in rheumatoid arthritis. *Arthritis Rheum.* 58, 3041–3050
53. Kim, G. S., Koh, J. M., Chang, J. S., Park, B. L., Kim, L. H., Park, E. K., Kim, S. Y., and Shin, H. D. (2005) Association of the OSCAR promoter polymorphism with BMD in postmenopausal women. *J. Bone Miner. Res.* 20, 1342–1348
54. Li, S., Miller, C. H., Giannopoulou, E., Hux, X., Ivashkiv, L. B., and Zhao, B. (2014) RBP-I imposes a requirement for ITAM-mediated costimulation of osteoclastogenesis. *J. Clin. Invest.* 124, 5057–5073
55. Zhao, B., Takami, M., Yamada, A., Wang, X., Koga, T., Hu, X., Tanura, T.,
Ozato, K., Choi, Y., Ivashkiv, L. B., Takayanagi, H., and Kamijo, R. (2009) Interferon regulatory factor-8 regulates bone metabolism by suppressing osteoclastogenesis. *Nat. Med.* **15**, 1066–1071

Ivashkiv, L. B., Zhao, B., Park-Min, K. H., and Takami, M. (2011) Feedback inhibition of osteoclastogenesis during inflammation by IL-10, M-CSF receptor shedding, and induction of IRF8. *Ann. N.Y. Acad. Sci.* **1237**, 88–94

Loiarro, M., Volpe, E., Ruggiero, V., Gallo, G., Furlan, R., Maiorino, C., Battistini, L., and Sette, C. (2013) Mutational analysis identifies residues crucial for homodimerization of Myeloid differentiation factor 88 (MyD88) and for its function in immune cells. *J. Biol. Chem.* **288**, 30210–30222

Horng, T., Barton, G. M., Flavell, R. A., and Medzhitov, R. (2002) The adaptor molecular TIRAP provides signaling specificity for Toll-like receptors. *Nature* **420**, 329–333