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Endosomal TLR3 signaling in stromal osteoblasts induces prostaglandin E_2–mediated inflammatory periodontal bone resorption

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Tsukasa Tominari[1], Miyuki Akita[1], Chiho Matsumoto[1], Michiko Hirata[1], Shosei Yoshinouchi[2], Yuki Tanaka[1], Kento Karouji[1], Yoshifumi Itoh[3,4], Takayuki Maruyama[5], Chisato Miyaura[1,2,3], Yukihiro Numabe[6], and Masaki Inada[1,2,3,*]

From the [1]Department of Biotechnology and Life Science, [2]Cooperative Major of Advanced Health Science, and [3]Institute of Global Innovation Research, Tokyo University of Agriculture and Technology, Koganei, Tokyo, Japan; [4]Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, Kennedy Institute of Rheumatology, University of Oxford, Oxford, UK; [5]Minase Research Institutes, Ono Pharmaceutical Co Ltd, Osaka, Japan; [6]Department of Periodontology, School of Dentistry, The Nippon Dental University, Chiyoda-ku, Tokyo, Japan

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Toll-like receptors (TLRs) are pattern recognition receptors that play a critical role in innate immune diseases. TLR3, which is localized in the endosomal compartments of hematopoietic immune cells, is able to recognize double-stranded RNA (dsRNA) derived from viruses and bacteria and thereby induce innate immune responses. Inflammatory periodontal bone resorption is caused by bacterial infections, which initially is regulated by innate immunity; however, the roles of TLR3 signaling in bone resorption are still not known. We examined the roles of TLR3 signaling in bone resorption using poly(I:C), a synthetic dsRNA analog. In cocultures of mouse bone marrow cells and stromal osteoblasts, poly(I:C) clearly induced osteoclast differentiation. In osteoblasts, poly(I:C) increased PGE_2 production and upregulated the mRNA expression of PGE_2-related genes, *Ptgs2* and *Ptges*, as well as that of a gene related to osteoclast differentiation, *Tnfsf11*. In addition, we found that indomethacin (a COX-2 inhibitor) or an antagonist of the PGE_2 receptor EP4 attenuated the poly(I:C)-induced PGE_2 production and subsequent *Tnfsf11* expression. Poly(I:C) also prolonged the survival of the mature osteoclasts associated with the increased mRNA expression of osteoclast marker genes, *Nfatc1* and *Ctsk*. In *ex vivo* organ cultures of periodontal alveolar bone, poly(I:C) induced bone-resorbing activity in a dose-dependent manner, which was attenuated by the simultaneous administration of either indomethacin or an EP4 antagonist. These data suggest that TLR3 signaling in osteoblasts controls PGE_2 production and induces the subsequent differentiation and survival of mature osteoclasts. Endogenous TLR3 in stromal osteoblasts and osteoclasts synergistically induces inflammatory alveolar bone resorption in periodontitis.

Periodontal disease is a local inflammatory disease associated with bacterial infection that results in alveolar bone resorption and tooth loss. The accumulation of bacterial plaque in the periodontal pocket leads to the pathogenesis and progress of periodontal disease. Lipopolysaccharide (LPS), a membrane component of gram-negative bacteria, is a well-known microbe-associated molecular pattern that causes inflammatory periodontal disease (1). In our previous study, which reported an experimental model of periodontal disease, the production of prostaglandin (PG) E_2 mediated severe bone loss (2). Mice lacking membrane-bound PGE synthase (mPGES)-1, an inducible enzyme for PGE synthesis, failed to develop alveolar bone loss by LPS injection, suggesting that mPGES-1-induced PGE_2 synthesis is essential for the LPS-mediated development of periodontal disease.

The balance between osteoclastic bone resorption and osteoblastic bone formation maintains bone mass. For osteoclast differentiation, receptor activator of NF-κB (RANK) ligand (RANKL), which belongs to the tumor necrosis factor superfamily, is an essential molecule. RANKL expression is induced by several factors, including inflammatory cytokines on the membrane of osteoblasts (3–5). RANK-expressing hematopoietic macrophages as osteoclast precursor cells interact with RANKL-expressing stromal osteoblasts. RANK-RANKL interaction activates several signaling pathways, such as the MAPK/AP-1 pathway, NF-κB pathway, and calcium pathway, resulting in the activation of nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), a master transcription factor for osteoclast differentiation. NFATc1 then induces various osteoclast marker proteins, such as cellular fusion factors including osteoclast stimulatory transmembrane protein and dendritic cell-specific transmembrane protein, as well as proteases, cathepsin K and tartrate-resistant acid phosphatase (TRAP). The osteoclast precursor cells then fuse with each other and differentiate into bone-resorbing osteoclasts (6–8).

PGE_2 is considered a major inflammatory mediator *via* the upregulation of RANKL in periodontal disease (2). The inflammatory response upregulates the synthesis of the enzymes associated with the production of PGE_2, namely, cytosolic phospholipase A_2, cyclooxygenase (COX)-2, and mPGES-1.

* For correspondence: Masaki Inada, m-inada@cc.tuat.ac.jp.

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Arachidonic acids are released from the phospholipid membrane by cytosolic phospholipase A_2, then COX-2 converts arachidonic acid into PGH_2, and mPGES-1 mediates the synthesis of PGE_2 from PGH_2. EP receptors (EP1, EP2, EP3, and EP4) are identified as PGE_2 receptors. We have reported that EP2 and EP4 are involved in PGE_2-mediated bone resorption and EP4 is reported to be the major receptor associated with PGE_2-mediated bone resorption (9–11). Previous studies have shown that inflammatory molecules, such as LPS and inflammatory interleukins, increased the PGE_2 production *via* the NF-κB pathway. PGE_2 bound to EP4 then enhances the RANKL expression *via* the activation of CREB transcription factor in osteoblasts, leading to osteoclast differentiation (10–14).

Toll-like receptors (TLRs) play essential roles in the innate immune response, recognizing various microbial components (15). In mammals, TLR1, 2, 4, 5, and 6 are expressed on the cell surface, whereas TLR3, 7, 8, and 9 localize at the endosomal membrane. LPS, an outer membrane component of gram-negative bacteria, is a potent inducer of periodontal disease. LPS and TLR4 signaling promotes proinflammatory cytokine production in periodontal tissues (16), stimulates PGE_2-mediated RANKL expression in stromal osteoblasts, and inhibits osteogenic differentiation in periodontal ligament stem cells (17), leading to inflammatory alveolar bone resorption. Synthetic ligands for TLR2/1 and TLR2/6, Pam3CSK4 and Pam2CSK4, were demonstrated to induce PGE_2-mediated osteoclast differentiation and alveolar bone destruction in a mouse model of periodontal disease (18). The expression of TLR1 is also reported in periodontal tissues (19, 20) and TLR5, which recognized bacterial flagellin, has been reported to induce osteoclast differentiation and bone loss (21). These reports indicate that TLRs located on the cell surface are involved in inflammatory alveolar bone loss; however, there is a little evidence of the roles of endosomal TLRs in inflammatory bone resorption, such as periodontal disease. One of the endosomal TLRs, TLR3 in hematopoietic immune cells, recognizes double-stranded RNA (dsRNA) derived from viruses, bacteria, and dead cells (22–26). TLR3-dsRNA signaling activates NF-κB and type I interferon signaling, leading to innate immune responses. Recently, polyinosinic-polyinocytidylic acid [poly(I:C)] has been widely used as a TLR3 ligand for the investigation of TLR3 signaling. Poly(I:C) promoted the development of arthritis by inducing the production of proinflammatory cytokines *via* TLR3 signaling (27, 28); however, it is not clear if TLR3 signaling plays a role in inflammatory diseases, such as periodontal bone resorption in periodontitis.

In the present study, we examined the roles of TLR3 and the ligand in inflammatory bone resorption. Poly(I:C) internalized into the endosome and induced the PGE_2-mediated expression of RANKL *via* the NF-κB signaling pathway in stromal osteoblasts, leading to osteoclast differentiation. Poly(I:C) also prolonged the survival of differentiated mature osteoclasts.

Our data indicate that poly(I:C)-mediated TLR3 signaling in stromal osteoblasts and osteoclasts collaboratively contributes to the development of alveolar bone loss in periodontal disease.

Results

TLR3 and the ligand [poly(I:C)] induced osteoclast differentiation and bone resorption

A quantitative PCR (qPCR) analysis and Western blotting were performed to examine the mRNA and protein expression of TLR3 in mouse primary osteoblasts (POBs) and Raw264.7 cells with or without treatment of sRANKL, and these cells were shown to express TLR3 mRNA and protein (Fig. 1, *A* and *B*). These data revealed that all of these bone cells expressed TLR3. Next, we examined the effects of TLR3 signaling in the cocultures of bone marrow cells (BMCs) and POBs using the ligand poly(I:C). As shown in Figure 1, *B* and *C*, poly(I:C) significantly induced osteoclast differentiation in a dose-dependent manner. To test whether TLR3 activates bone resorption, mouse calvariae as a cortical bone were cultured with poly(I:C). As shown in Figure 1*D*, poly(I:C) promoted bone-resorbing activity in a dose-dependent manner. These data suggested that TLR3 activation initiated osteoclast differentiation and bone resorption.

Poly(I:C) stimulated PGE_2 production via the upregulation of PGE_2 synthesis–related genes and Tnfsf11 gene in NF-κB signaling in osteoblasts

To explore the mechanism of poly(I:C)-induced osteoclast differentiation, we examined the mRNA expression of *Ptgs2*, *Ptges*, and *Tnfsf11* in osteoblasts using qPCR. As shown in Figure 2*A*, poly(I:C) treatment upregulated the mRNA expression of *Ptgs2*, *Ptges*, and *Tnfsf11* gene in osteoblasts in a dose-dependent manner. We also confirmed that poly(I:C) induced the production of PGE_2 in osteoblasts (Fig. 2*B*). To examine the roles of poly(I:C) in the NF-κB pathway, IκBα (native NF-κB inhibitor) was analyzed by Western blotting. As shown in Figure 2, *C* and *D*, poly(I:C) decreased IκBα protein, suggesting that poly(I:C) treatment increases NF-κB activity by suppressing IκBα inhibitory activity of NF-κB. To further analyze the NF-κB transcriptional activity, we performed a dual-luciferase reporter gene assay using the pNFκB-TA system. As shown in Figure 2*E*, poly(I:C) treatment promoted the transcriptional activity of NF-κB in a dose-dependent manner. These results suggest that poly(I:C) promotes *Cox2*- and *Ptges*-mediated PGE_2 production *via* NF-κB signaling, leading to osteoclast differentiation.

Indomethacin and EP4 antagonist blocked poly(I:C)-induced bone resorption

To test whether poly(I:C)-induced osteoclast differentiation was mediated by PGE_2, BMCs and POBs were cocultured with or without poly(I:C) and indomethacin, a nonsteroidal anti-inflammatory drug, and/or antagonist of EP4 PGE_2 receptor. Our previous studies showed that EP4 receptor mainly mediated PGE_2-induced inflammatory bone resorption (10, 12). As shown in Figure 3, *A* and *B*, both indomethacin and EP4 antagonist attenuated poly(I:C)-induced osteoclast differentiation. Treatment with indomethacin and EP4 antagonist also suppressed poly(I:C)-induced bone-resorbing activity in

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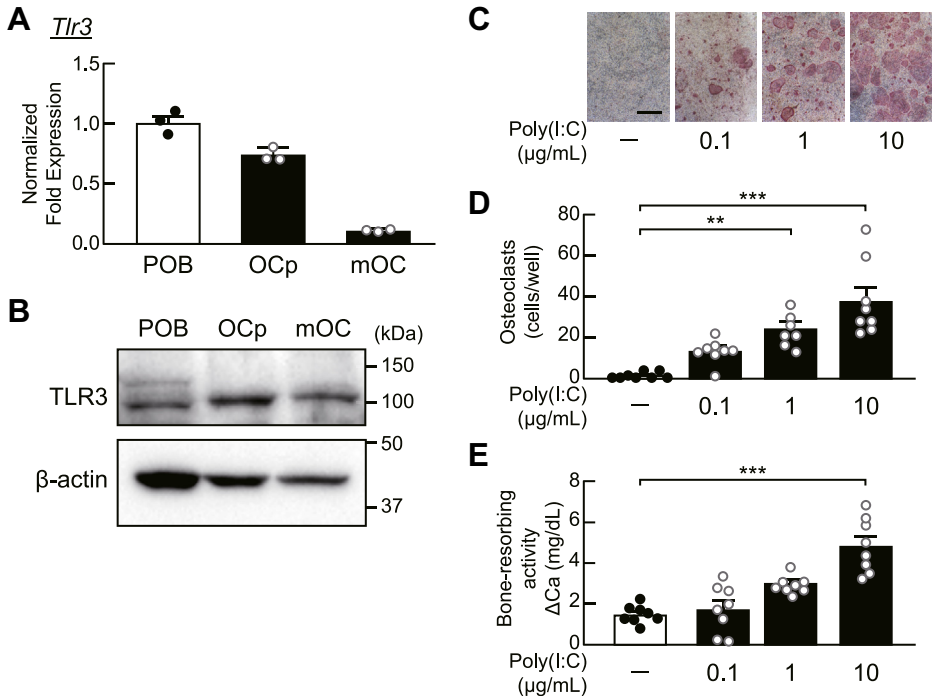

Figure 1. The activation of TLR3 by poly(I:C) induced osteoclast differentiation and bone resorption. *A*, the TLR3 mRNA expression in mouse primary osteoblastic cells (POBs), Raw264.7 cells (osteoclast precursor cells; OCp), and sRANKL-treated Raw264.7 cells (mature osteoclasts; mOC). The data are expressed as the mean ± SEM of triplicate data from three representative independent experiments. *Actb* was used as a normalized gene. *B*, the TLR3 protein expression in POBs, OCp, and mOC. β-Actin was used as a normalized protein. *C*, mouse POB and BMCs were cocultured with poly(I:C) for 7 days. The images showed TRAP-positive multinuclear osteoclasts. The scale bar represents 500 μm. *D*, TRAP-positive osteoclasts were counted. The data are expressed as the mean ± SEM of eight wells from three representative independent experiments. *E*, mouse calvariae from newborn mice were cultured with poly(I:C) for 5 days. The Ca^{2+} concentration was measured using *o*-cresolphthalein complexone methods to elucidate bone-resorbing activity. The data are expressed as the mean ± SEM of eight bones from three representative independent experiments. *Asterisks* indicate a significant difference between two groups: $^{**}p < 0.01$ and $^{***}p < 0.001$.

ex vivo calvarial bone organ cultures (Fig. 3*C*). In osteoblasts, the poly(I:C)-induced *Tnfsf11* gene induction was also attenuated by treatment with indomethacin and EP4 antagonist (Fig. 3*D*). These results indicated that poly(I:C) upregulated the *Tnfsf11* expression *via* the PGE_2-EP4 signaling pathway and induced osteoclast differentiation and bone resorption.

Endocytosis inhibitors suppressed the poly(I:C)-induced mRNA expression of Tnfsf11 in stromal osteoblasts

A previous report suggested that extracellular poly(I:C) was incorporated into macrophages *via* cell surface receptor Mac-1 (macrophage antigen 1, also known as CD11b)-mediated endocytosis, to bind to TLR3 at endosomal compartment (29). We examined whether externally applied poly(I:C) endocytosed into stromal osteoblasts. For the detection of poly(I:C) and TLR3 in osteoblasts, GFP-linked endosome marker RhoB-GTPase-coded expression plasmid vector (GFP-endosome)-transfected POB was treated with Rhodamine-labeled poly(I:C) (poly[I:C]-Rhodamine). Both GFP–endosome and poly(I:C)–Rhodamine were visualized using a confocal microscope. The localization of the endosomal marker (green) and poly(I:C) (red) was detected by confocal microscopy. Poly(I:C) was colocalized with endosomes, whereas the endocytosis inhibitors Pitstop2 (a clathrin inhibitor) and Dynasore (a dynamin inhibitor) failed to detect the signal of poly(I:C) (Fig. 4*A*), indicating that poly(I:C) was internalized into osteoblasts by endocytosis. Treatment of the cells with the endocytosis inhibitors attenuated the poly(I:C)-induced *Tnfsf11* mRNA expression (Fig. 4*B*). These data indicated that poly(I:C) was endocytosed into stromal osteoblasts and that it upregulated the *Tnfsf11* expression *via* the PGE_2–EP4 signaling.

Poly(I:C) prolonged the survival of mature osteoclasts

We examined the effect of poly(I:C) on osteoclast differentiation and survival. First, to examine the effect of poly(I:C) on osteoclast differentiation, Raw264.7 cells were cultured with sRANKL with various concentrations of poly(I:C) (0.1–10 μg/ml). As shown in Figure 5, *A* and *B*, poly(I:C) did not alter the sRANKL-induced osteoclast differentiation. Next, we examined the effect of poly(I:C) on differentiated osteoclasts. To examine the effect of poly(I:C) on osteoclast survival, Raw264.7 cells were cultured with sRANKL for 3 days (Raw264.7 cells differentiated into mature osteoclasts), and mature osteoclasts were cultured without sRANKL for 1 day in the presence of poly(I:C). As shown in Figure 5, *C* and *D*, the removal of sRANKL from mature osteoclasts significantly decreased the number of surviving osteoclasts due to the absence of cell survival signaling from sRANKL. In addition, treatment with poly(I:C) instead of sRANKL prolonged the survival of mature osteoclasts in a dose-dependent manner. To examine the mechanism of poly(I:C)-induced osteoclast

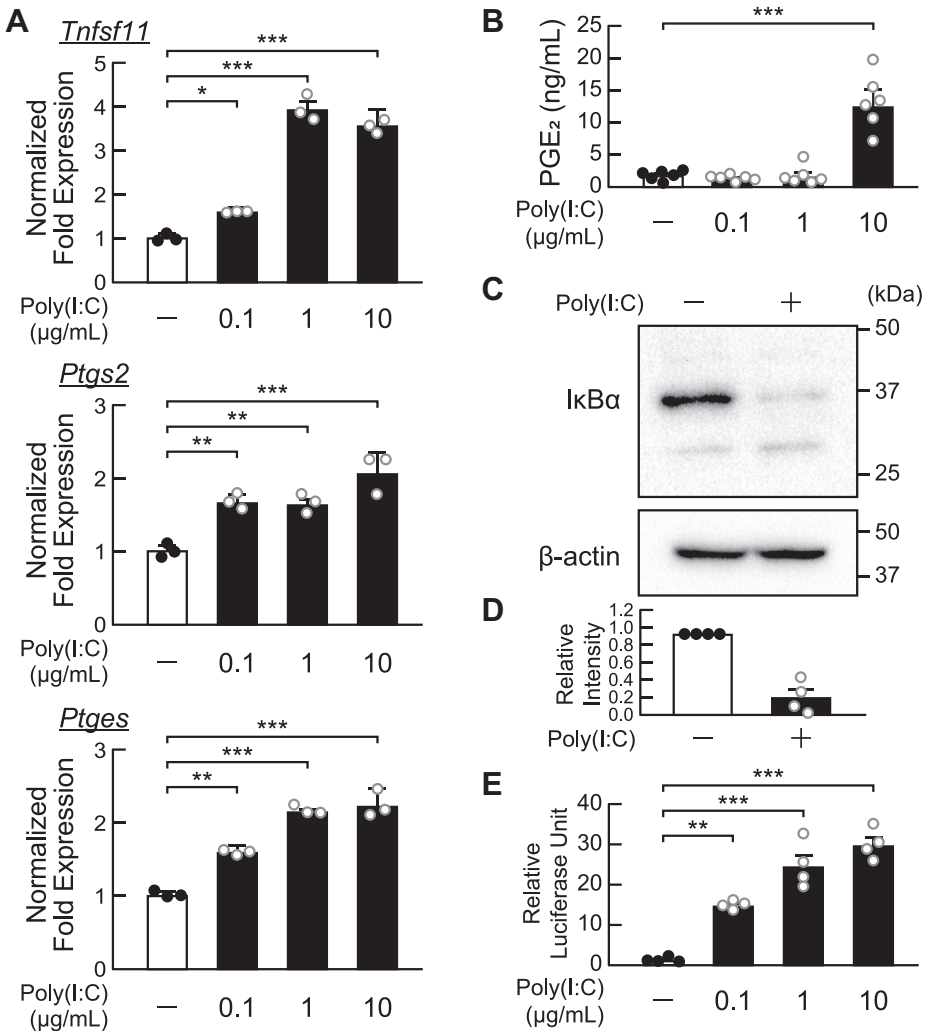

Figure 2. Poly(I:C) stimulated PGE_2 production *via* the upregulation of PGE_2-related genes in osteoblasts. *A*, mouse POBs were cultured with poly(I:C) for 24 h, and total RNA was extracted and the *Rankl*, *Cox2*, and *Ptges* mRNA expression was analyzed by real-time PCR. The data are expressed as the mean ± SEM of triplicate data from a representative experiment of three independent experiments. *Actb* was used as a normalized gene. *B*, mouse POBs were cultured with poly(I:C) for 24 h, and the PGE_2 in the conditioned medium was measured. The data are expressed as the mean ± SEM of six wells from three representative independent experiments. *C*, mouse POBs were treated with 10 μg/ml of poly(I:C) for 15 min. Whole lysates were collected, and IκBα and β-actin were detected by Western blotting. The blot images were representative of four independent experiments. *D*, relative intensity of IκB expression in (*C*) was analyzed. The data are expressed as the mean ± SEM of four independent experiments. *E*, the transcription activity of NF-κB was measured. The pNFκB-TA-Luc plasmid (0.4 μg) and pGL4.74[hLuc/TK] plasmid (40 ng) were transfected into POBs followed by 10 μg/ml of poly(I:C) treatment, and the luciferase activity was measured with a Dual-luciferase Reporter Assay system. The data are expressed as the mean ± SEM of four wells from three representative independent experiments. Asterisks indicate a significant difference between two groups: $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

survival, we analyzed the genes associated with osteoclast differentiation (*Nfatc1* and *Ctsk*) by RT-qPCR. As shown in Figure 5*E*, the mRNA expression of both *Nfatc1* and *Ctsk* was significantly increased by the addition of poly(I:C).

Poly(I:C) induced periodontal alveolar bone resorption in ex vivo *organ culture system*

We previously established an *ex vivo* experimental model of periodontal bone resorption (2). Mouse mandibular alveolar bone consisting of cortical and cancerous bones (Fig. 6*A*) was cultured in the *ex vivo* organ culture system with poly(I:C) for 5 days. Poly(I:C) treatment increased the bone-resorbing activity in the alveolar bone in a dose-dependent manner (Fig. 6*B*). Treatment with an inhibitor of PGE_2 production (indomethacin) and antagonist of EP4 receptor significantly suppressed the poly(I:C)-induced bone-resorbing activity (Fig. 6*C*). These results showed that poly(I:C)-mediated TLR3 activation induced the production of PGE_2, which was attenuated by treatment with indomethacin and a PGE_2 receptor antagonist.

Discussion

In the present study, we examined TLR3 and poly(I:C)-induced PGE_2-mediated inflammatory bone resorption (Figs. 1–3). The internalization of poly(I:C) into the endosomes of stromal osteoblasts induced PGE_2 production, and subsequent *Tnfsf11* expression in stromal osteoblast enhanced osteoclast differentiation (Fig. 4). Treatment with TLR3 and poly(I:C), instead of sRANKL, also enhanced cell survival of mature osteoclasts (Fig. 5). Finally, poly(I:C) induced

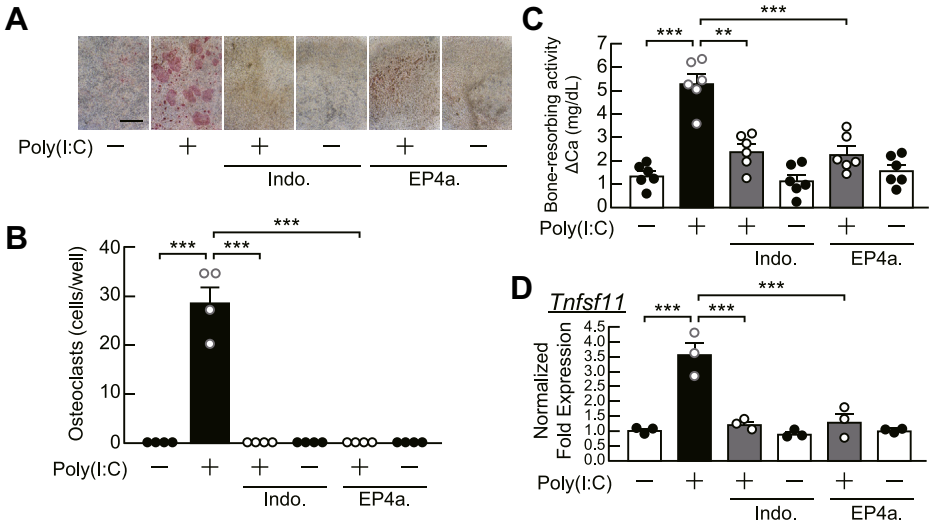

Figure 3. Indomethacin and EP4 antagonist inhibited poly(I:C)-induced bone resorption. *A*, mouse POB and BMCs were cocultured with 10 μg/ml of poly(I:C) and 10 μM of indomethacin (Indo.) or 10 μM of EP4 antagonist (EP4a) for 7 days. The images showed TRAP-positive multinuclear osteoclasts. The scale bar represents 500 μm. *B*, TRAP-positive osteoclasts were counted. The data are expressed as the mean ± SEM of four wells from three representative independent experiments. *C*, mouse calvariae from newborn mice were cultured with poly(I:C) and indomethacin or EP4a for 5 days. The Ca^{2+} concentration was measured using the *o*-cresolphthalein complexone method to elucidate bone-resorbing activity. The data are expressed as the mean ± SEM of six bones from three representative independent experiments. *D*, mouse POBs were cultured with poly(I:C) and indomethacin or EP4a for 24 h, and total RNA was extracted and the *Rankl* mRNA expression was analyzed by real-time PCR. The data are expressed as the mean ± SEM of triplicate data from a representative experiment of three independent experiments. *Actb* was used as a normalized gene. *Asterisks* indicate a significant difference between two groups: $^{**}p < 0.01$ and $^{***}p < 0.001$.

bone-resorbing activity in *ex vivo* organ cultures of periodontal bone, and the activity was attenuated by treatment with an inhibitor of PGE_2 production, indomethacin, and a PGE_2 receptor antagonist, EP4 antagonist (Fig. 6). These data suggest that TLR3 and the ligand-induced PGE_2 production enhanced inflammatory periodontal bone resorption. The signaling stream of TLR3 and poly(I:C)-mediated bone resorption is illustrated in Figure 7.

In inflammatory bone diseases, such as periodontal disease, infection with mixed gram-negative bacteria induces alveolar bone resorption and subsequent tooth loss (30). It is well known that the major microbe-associated molecular patterns of periodontal disease are gram-negative bacteria, including *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and LPS, an outer membrane component of gram-negative bacteria, which causes inflammatory bone resorption *via* TLR4 signaling (2, 31, 32). Gram-positive bacteria are also involved in inflammatory bone resorption *via* TLR2 signaling in periodontal disease (18, 32–34). Endosomal TLR9, which is a sensor of bacterial DNA, contributed to the periodontal bone resorption induced by *P. gingivalis* infection (35). Cell surface TLRs activate the myeloid differentiation primary response 88 (MyD88)-dependent NF-κB pathway to upregulate proinflammatory cytokines (15). TLR4 activates both MyD88-dependent and MyD88-independent pathways, since TLR4 relocates from the cell surface into the endosomal compartment *via* endocytosis (15, 36). Endosomal TLRs, such as TLR3, TLR7, TLR8, and TLR9, recognize nucleic acids, including dsRNA, single-stranded RNA (ssRNA), and DNA. In humans, TLR8 senses ssRNA to activate NF-κB signaling; on the other hand, the ligand for mouse TLR8 is unidentified and TLR8 signaling could suppress the expression and signaling of TLR7 in dendritic cells (37). TLR7 and TLR9 have also been reported to activate the MyD88-dependent NF-κB pathway similarly to cell surface TLRs. On the other hand, TLR3 activates the MyD88-independent (TRIF-dependent) NF-κB pathway (15).

We previously reported that gram-negative bacteria–derived LPS (TLR4 ligand), Pam3CSK4 (synthetic TLR2/1 ligand), and Pam2CSK4 (synthetic TLR2/6 ligand) contributed to PGE_2-mediated bone resorption in periodontal disease (2, 18). Recent studies have shown that bacterial flagellin stimulated osteoclastogenesis *via* TLR5 signaling *in vitro* and calvarial bone resorption *in vivo* (21, 38). Thus, cell surface TLRs, including TLR2/1, TLR2/6, TLR4, and TLR5, activated by a bacterial component, are able to induce osteoclast differentiation and bone resorption.

All of the above reports mentioned TLRs are found on the cellular surface; however, the roles of endosomal TLR3 in bone resorption is unknown. TLR3 has been reported to recognize RNA from viruses, bacteria, and dead cells to initiate inflammatory responses (22–26). A previous study reported that the mRNA expression of TLR3 was detected on osteoclast precursor cells but not mature osteoclasts using Southern blotting (39). We confirmed that TLR3 mRNA and protein were expressed in stromal osteoblasts, hematopoietic precursor cells of osteoclasts and mature osteoclasts; however, the expression of TLR3 in mature osteoclast was only diminished (Fig. 1*A*). We therefore examined the effect of poly(I:C) on those stromal and hematopoietic bone cells, using dsRNA analog as a TLR3 ligand. Poly(I:C) promoted both osteoclast differentiation and bone resorption (Fig. 1, *B–D*). The bacterial RNA from periodontal gram-negative bacteria was difficult to be purified because it is challenging to perfectly delete LPS contamination.

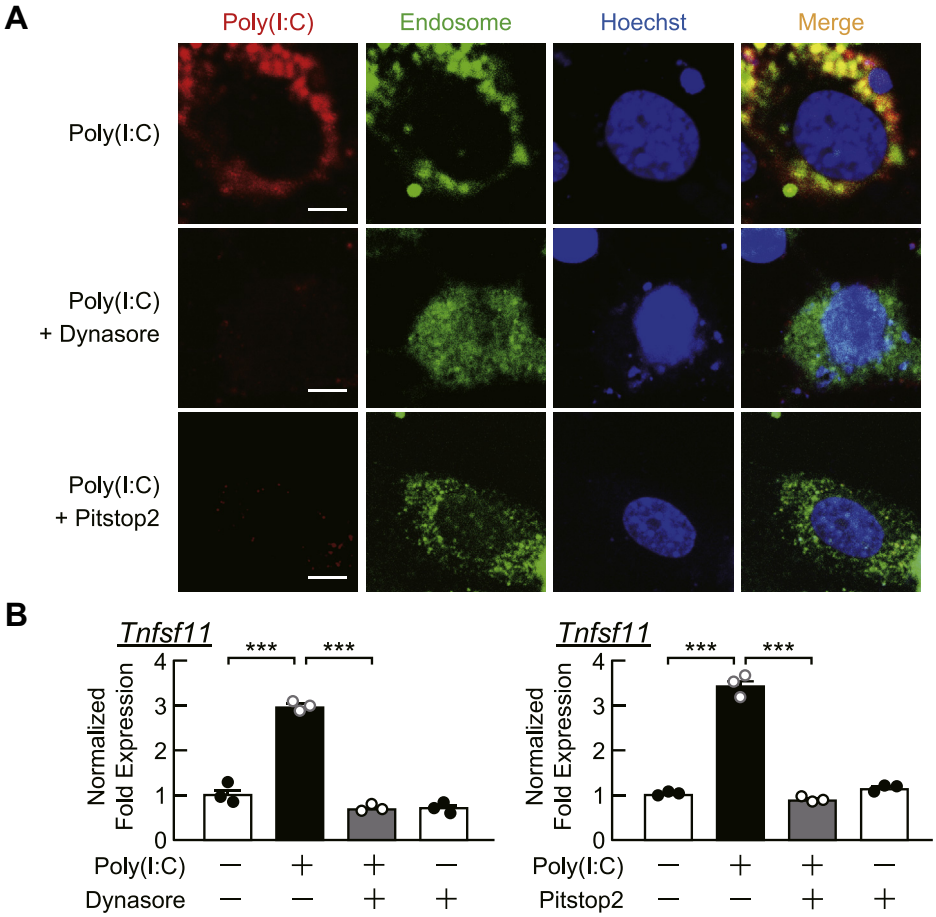

Figure 4. Poly(I:C) was endocytosed by osteoblasts and upregulated the expression of RANKL. *A*, colocalization of endosomes and poly(I:C) was detected by confocal microscopy. The pAcGFP1-RhoB vector, an endosome marker, was transfected into POBs. POBs were cultured in the presence of 10 μg/ml of Rhodamine-labeled poly(I:C) with or without 30 μM of dynasore or 25 μM of Pitstop2 for 24 h. Nuclei were stained with Hoechst. The scale bar represents 10 μm. *B*, POBs were cultured with 10 μg/ml of poly(I:C) and 25 μM of Pitstop2, a clathrin inhibitor, or 30 μM of Dynasore, a dynamin inhibitor for 24 h. Total RNA was extracted, and the mRNA expression of *Rankl* was analyzed by real-time PCR. The data are expressed as the mean ± SEM of triplicate data from a representative experiment of three independent experiments. *Actb* was used as a normalized gene. *Asterisks* indicate a significant difference between two groups: ***$p < 0.001$.

We previously reported that PGE_2 was an inevitable mediator of inflammatory bone resorption induced by Pam3CSK4 or Pam2CSK4 that activated TLR2 signaling. The other inducer of LPS, TLR4 signaling, induced the production of PGE_2 *via* EP4 receptor signaling (2, 10, 18). In the present study, we indicated that stromal lineage of osteoblasts endocytosed poly(I:C) as a TLR3 ligand, which subsequently induced the production of PGE_2 *via* the COX-2 and mPGES-1 pathway (Figs. 2 and 4). We also showed that the blockage of the PGE_2 production and function using indomethacin and EP4 attenuated the poly(I:C)-induced inflammatory effects (Fig. 3). Furthermore, treatment with the endocytosis inhibitors pitstop2 and dynasore reduced the expression of poly(I:C)-induced *Tnfsf11* (Fig. 4*B*). These data suggested stromal osteoblasts sensed the TLR3 ligand in endosomes and controlled innate immunity through PGE_2 production, which induced inflammatory periodontal bone resorption through the PGE_2-EP4 pathway. In other aspects of poly(I:C)-mediated TLR3 signaling, Park *et al.* (40) reported that TLR3 activated by poly(I:C) promoted the Ca^{2+}-dependent expression of inflammatory cytokines, including IL-6 and IL-8, in human mesenchymal stem cells. These inflammatory cytokines also induced RANKL expression in osteoblasts (41, 42). Mori *et al.* (43) reported that TLR3 activation increased TLR2 expression in human gingival fibroblasts and epithelial cells, suggesting that TLR3 may enhance TLR2 and its ligands in bacterial pathogen-mediated periodontal disease. The findings of these reports suggest that poly(I:C) *via* the TLR3 signaling in stromal osteoblasts supported osteoclast differentiation by inducing the pathogen-mediated production of PGE_2.

Regarding the other action of poly(I:C) on osteoclasts, Takami *et al.* (39) reported that poly(I:C) inhibited osteoclast differentiation at >10 μg/ml in Raw264.7 cultures and poly(I:C) ≤10 μg/ml failed to affect osteoclast differentiation. Similarly, our present study showed that poly(I:C) ≤10 μg/ml had no effect on osteoclast differentiation (Fig. 5, *A* and *B*). We further demonstrated that poly(I:C) prolonged the survival of mature osteoclasts associated with the upregulation of the expression of *Nfatc1* and *Ctsk* mRNA, suggesting that poly(I:C)-TLR3 signaling has compensatory effects on the survival of mature osteoclasts in the absence of RANKL. Takami *et al.* also reported that the survival of mature

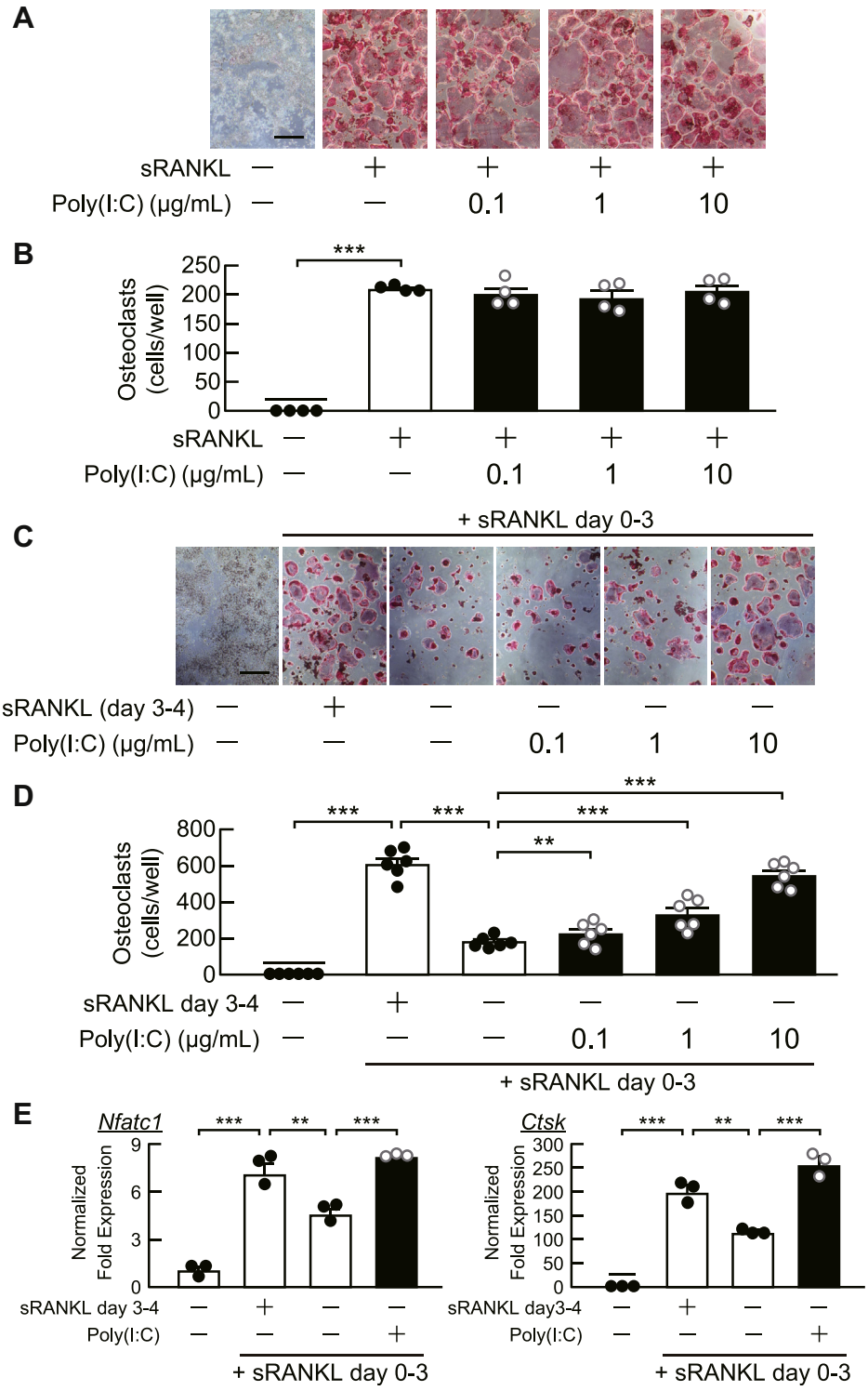

Figure 5. Poly(I:C) prolonged the lifespan of mature osteoclasts. *A*, Raw264.7 cells were cultured in the presence of sRANKL (100 ng/ml) with poly(I:C) for 4 days. The images show TRAP-positive multinuclear osteoclasts. The scale bar represents 200 μm. *B*, TRAP-positive osteoclasts were counted. The data are expressed as the mean ± SEM of four wells from three representative independent experiments. *C*, Raw264.7 cells were cultured with sRANKL (100 ng/ml) for 3 days. After mature osteoclasts were formed, osteoclasts were treated with poly(I:C) in the absence of sRANKL for 1 day. The images show TRAP-positive multinuclear osteoclasts. The scale bar represents 200 μm. *D*, TRAP-positive osteoclasts were counted. The data are expressed as the mean ± SEM of six wells from three representative independent experiments. *E*, the mRNA expression of *Nfatc1* and *Ctsk* was analyzed by real-time PCR. The data are expressed as the mean ± SEM of triplicate data from a representative experiment of three independent experiments. *Actb* was used as a normalized gene. *Asterisks* indicate a significant difference between two groups: $^{**}p < 0.01$ and $^{***}p < 0.001$.

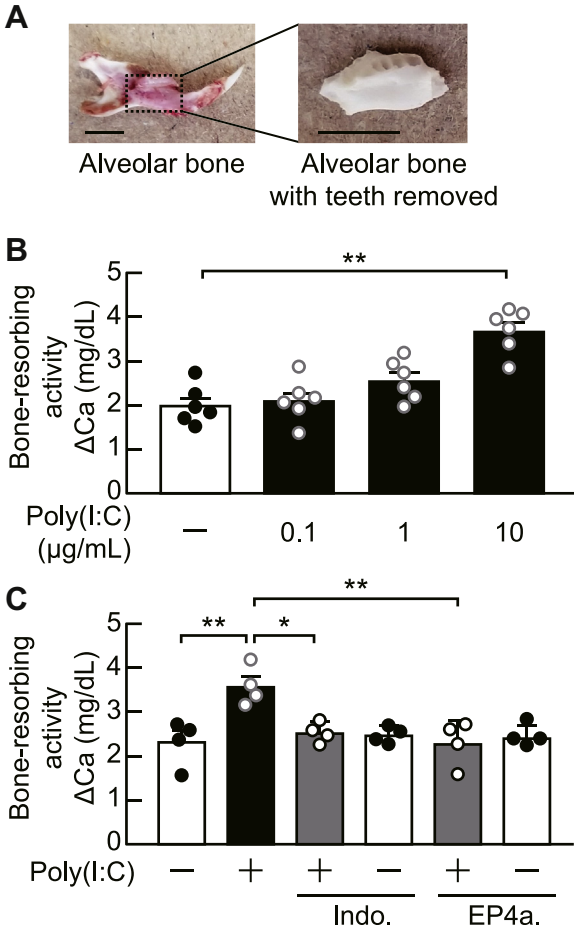

Figure 6. Poly(I:C) induced alveolar bone resorption in an *ex vivo* model of periodontal bone resorption. *A*, mouse alveolar bones were collected and teeth were removed to prepare organ cultures of alveolar bone. The scale bar represents 5 mm. *B* and *C*, mouse alveolar bones were cultured with 10 μg/ml of poly(I:C) in the presence or absence of 10 μM of indomethacin or 10 μM of EP4 antagonist for 5 days. The Ca^{2+} concentration was measured using the *o*-cresolphthalein complexone method to elucidate the bone-resorbing activity. The data are expressed as the mean ± SEM of four or six bones from three representative independent experiments. *Asterisks* indicate a significant difference between two groups: *$p < 0.05$ and **$p < 0.01$.

osteoclasts was not changed at ≤1000 μg/ml of poly(I:C) in different culture systems of mouse bone marrow–induced purified osteoclast (44). These results are interesting. Continuous MCSF treatment–induced osteoclast precursor may have different signaling effects on poly(I:C) treatment. Further studies will be needed to clarify the molecular mechanism underlying the poly(I:C)-induced osteoclast survival of different osteoclast precursor cells.

Regarding the poly(I:C) endocytic pathway in cells, Itoh *et al.* (45) reported that poly(I:C) was involved in endocytosis *via* the clathrin-mediated pathway in myeloid dendritic cells. Lee *et al.* (46) showed that poly(I:C) directly bound to cell surface–located CD14 and was incorporated into bone marrow–derived macrophages. Several reports have indicated that macrophages endocytosed extracellular dsRNA *via* cell surface receptor Mac-1, followed by initiating TLR3-mediated inflammatory responses in endosome (29, 47). These reports indicated that the macrophage lineages of the investigated cells endocytosed poly (IC) using cellular surface receptors that mediate the intracellular uptake to endosome. Therefore, the macrophage lineage of osteoclasts possibly endocytosed poly (IC) that resulted in the prolonged osteoclast survival *via* the upregulation of the expression of *Nfatc1* through TLR3/NF-κB signaling. Further analysis is need to confirm the endocytic pathways of TLR3 in mature osteoclasts.

In studies of rheumatoid arthritis, the activation of TLR3 and TLR7 has been reported to induce joint destruction through the upregulation of RANKL expression in synovial fibroblasts and directly affect monocytes differentiated into osteoclasts in the presence of M-CSF (26, 28, 48). Further studies are needed to verify whether stromal/fibroblast lineage cells respond to TLR3 and whether the ligand internalization induces the production of PGE_2 with the progression of other immune diseases.

Recently, TLR3 has also been reported to induce neutrophil extracellular traps in hematopoietic immune cells, such as neutrophils, which are antimicrobial web-like structures abundantly containing self-RNA (49, 50), and self-RNA in neutrophil extracellular traps may also activate TLR3 signaling. Since RNAs derived from infected bacteria and dead cells can accumulate in inflammatory periodontal tissues, TLR3 signaling may at least partially contribute to the development of periodontal bone resorption.

In the present study, we discovered the potential role of TLR3 signaling in periodontal bone resorption. TLR3 signaling activated by poly(I:C) or dsRNAs derived from bacteria and/or dead cells promotes inflammatory bone resorption. The internalization of the TLR3 ligand in the endosomes of stromal osteoblasts induced the production of PGE_2 and the subsequent osteoclast differentiation and survival. TLR3 and the ligand-induced bone-resorbing activity in periodontal bone were attenuated by the treatment of COX inhibitor and EP4 antagonist. EP4 antagonist is therefore a potentially effective treatment for TLR3-ligand–induced periodontal bone resorption.

In conclusion, TLR3 signaling produced PGE_2 in stromal osteoblasts and induced the subsequent osteoclast differentiation and survival, which promoted inflammatory periodontal alveolar bone resorption.

Experimental procedures

Animals and reagents

Newborn and 6-week-old *ddY* mice were obtained from Japan SLC Inc. All procedures were performed in accordance with institutional guidelines for animal research committee at the Tokyo University of Agriculture and Technology (protocol number 29-89). Poly(I:C) was obtained from InvivoGen Co Ltd. Indomethacin was purchased from Fujifilm Wako Pure Chemical Corp. EP4 antagonist ONO-AE3-208 was supplied from ONO Pharmaceutical Co, Ltd. Dynasore and Pitstop2 were purchased from Merck KGaA. Soluble RANKL recombinant protein (sRANKL) was purchased from PeproTech Inc.

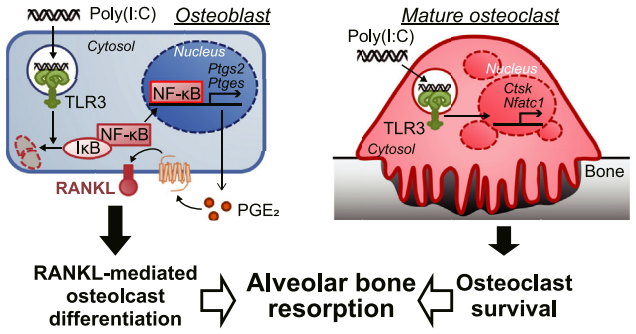

Figure 7. Model illustrating the roles of TLR3 signaling in osteoblasts and osteoclasts in alveolar bone resorption. TLR3 signaling activated by poly(I:C) induced the PGE_2-mediated expression of RANKL in osteoblasts and directly prolonged the life span of mature osteoclasts, leading to severe alveolar bone resorption.

Cultures of primary mouse osteoblastic cells

Mouse primary osteoblasts (POBs) were isolated from the calvariae of newborn mice after five routine sequential digestions with 0.1% bacterial collagenase (FUJIFILM Wako Pure Chemical Corp) and 0.2% dispase (Roche Molecular Systems Inc), as described (2, 18). POBs were cultured in αMEM (Thermo Fisher Scientific Inc) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences Inc) and penicillin–streptomycin at 37 °C under 5% CO_2.

Osteoclast differentiation in cocultures of BMCs and POBs

Bone marrow cells (BMCs) were collected from the tibiae of 6-week-old male mice. BMCs and POBs were cocultured in the presence or absence of poly(I:C) (0.1, 1, and 10 μg/ml), indomethacin (10 μM), or EP4 antagonist (10 μM). Multinuclear cells were stained for TRAP after 7 days. TRAP-positive multinuclear cells containing three or more nuclei per cell were counted as osteoclasts.

Osteoclast differentiation in cultures of Raw264.7 cells

In the osteoclast differentiation experiment, Raw264.7 cells, a murine macrophage cell line, were cultured in the presence of sRANKL (100 ng/ml) with poly(I:C) (0.1, 1, and 10 μg/ml) for 4 days. In the osteoclast survival experiment, Raw264.7 cells were cultured in the presence of sRANKL (100 ng/ml) for 3 days. Upon osteoclast formation, cells were further stimulated with poly(I:C) (0.1, 1, and 10 μg/ml) without sRANKL for 1 day. Osteoclasts were stained for TRAP, and TRAP-positive multinuclear cells with three or more nuclei per cell were counted as osteoclasts.

Measurement of PGE_2 production

The PGE_2 concentration in the conditioned media was measured using an enzyme immunoassay (GE healthcare UK Ltd). The antibody had the following cross-reactivity determined by the bound to free ratio: PGE_2, 100%; PGE_1, 7.0%; 6-keto-$PGF_{1\alpha}$, 5.4%; $PGF_{2\alpha}$, 4.3%; and PGD_2, 1.0%.

Western blotting

POBs were treated with poly(I:C) (10 μg/ml) for 1 h and lysed in a lysis buffer containing PhosSTOP (Roche) and cOmplete protease inhibitor cocktail EASYPack (Roche). The cell lysates were centrifuged at 12,000*g* for 10 min, and the supernatant was collected. The protein concentration of the supernatant was determined by BCA protein assay kit (Thermo Fisher Scientific Inc). Samples (10 μg of protein) were applied to SDS-PAGE (10% polyacrylamide gel) and transferred onto polyvinylidene difluoride membranes (Merck KGaA). Membranes were blocked with 5% dry milk in PBS-T (PBS with 0.05% Tween-20) and incubated with primary antibodies at 4 °C. Membranes were incubated with the corresponding secondary antibody in 1% skim milk in PBS-T and developed with ECL prime Western blotting detection reagent (GE Healthcare Japan Corp) by ChemiDoc XRS+ (Bio-Rad Laboratories Inc). Primary antibodies against TLR3 (104 kDa; Novus Biologicals LLC), IκBα (35–41 kDa; Santa Cruz Biotechnology Inc), and β-actin (43 kDa; Santa Cruz Biotechnology) were used.

Analysis of mRNA expression by quantitative PCR

Total RNA was extracted from POBs and Raw264.7 cells, and cDNA was synthesized from 5 μg of total RNA by reverse transcriptase (Superscript II Preamplification System; Thermo Fisher Scientific Inc). The cDNA was amplified by qPCR. The sequences of mouse PCR primer pair were as follows: *Actb* (β-actin): (forward) 5′-ccccattgaacatggcattg-3′ and (reverse) 5′-acgaccagaggcatacagg-3′; *Tlr3* (TLR3): (forward) 5′-atttagagt ccaacggcttagatg-3′ and (reverse) 5′-ttccagtaaaaagagatcctccag-3′; *Tnfsf11* (RANKL): (forward) 5′-gagaacttgggattttgatgc-3′ and (reverse) 5′-gactccactctggagagt-3′; *Ptgs2* (COX-2): (forward) 5′-tcagccaggcagcaaatccttg-3′ and (reverse) 5′-tagtctctcctatgag-tatgagtc-3′; *Ptges* (mPGES-1): (forward) 5′-atgccttccccgggcctg-3′ and (reverse) 5′-tcacagatggtgggccac-3′; *Nfatc1* (NFATc1): (forward) 5′- agtctctttccccgacatca-3′ and (reverse) 5′- cacctc-gatccgaagctc-3′; *Ctsk* (Cathepsin K): (forward) 5′-cattctcagaca cacaatccac-3′ and (reverse) 5′-gatactggacaccactggga-3′. qPCR was performed with SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories Inc). The relative normalized gene expression was quantified by the ΔΔCq method, and β-actin was used as a normalized gene.

Dual luciferase reporter gene assay

The pNFκB-TA plasmid (0.4 μg) contains four tandem copies of the NF-κB consensus sequence with the firefly luciferase reporter gene (Clontech Laboratories, Inc). The pGL4.74[hLuc/TK] plasmid (40 ng), which contains the renilla luciferase reporter gene (Promega Corp), was used as an internal control reporter vector. Both plasmids (0.4 μg and 40 ng, respectively) were transfected into POBs using Lipofectamine 3000 (Thermo Fisher Scientific Inc) and cultured for 24 h with or without poly(I:C). The luciferase activity was measured using a Dual-luciferase Reporter Assay System (Promega Corp) with an ARVO MX multilabel/luminescence counter (PerkinElmer Corp). This assay was performed under the conditions described in our previous report (18).

Bone-resorbing activity in organ cultures of calvaria

To measure the bone-resorbing activity in organ cultures of the calvaria, calvariae from newborn mice were cultured for

24 h in BGJb supplemented with 0.1% bovine serum albumin. The calvariae were transferred to new media in the presence or absence of poly(I:C) and cultured for another 5 days. The Ca^{2+} concentration in the conditioned medium was measured by the *o*-cresolphthalein complexone method to determine the bone-resorbing activity.

The detection of colocalization of poly(I:C) and endosomes by confocal microscopy

The pAcGFP1-Endo vector (Takara Bio Inc), which encodes a fusion protein consisting of RhoB GTPase with an N-terminal c-Myc epitope tag for endosome imaging, was transfected into POBs using Lipofectamine 3000 for 24 h. Then, POBs were treated with 10 μg/ml of Rhodamine-labeled poly(I:C) (InvivoGen Co Ltd) with or without 30 μM dynasore or 25 μM Pitstop2 and cultured for 24 h. POBs were fixed with 10% formalin. The nuclei were stained with Hoechst 33342 using NucBlue Live Ready Probes Reagent (Thermo Fisher Scientific Inc). Fluorescence imaging was analyzed using Olympus Fluoview FV1000 (Olympus Corp). Poly(I:C)-Rhodamine was observed at excitation (559 nm) and emission (575–675 nm); pAcGFP-Endo was observed at excitation (473 nm) and emission (490–540 nm); Hoechst 33342 was observed at excitation (405 nm) and emission (430–455 nm).

Ex vivo *model of periodontal bone resorption*

Mouse mandibular alveolar bones were cultured for 24 h in BGJb containing 0.1% bovine serum albumin. After 24 h, alveolar bones were transferred to new media with poly(I:C) and cultured for another 5 days. The Ca^{2+} concentration in the conditioned medium was measured by the *o*-cresolphthalein complexone method to determine bone-resorbing activity.

Statistical analysis

All data were expressed as the mean ± SEM. A one-way ANOVA followed by Tukey's test, as a post hoc analysis, was used for comparisons among three or more groups. All statistical analyses were performed using the IBM SPSS Statistics software program Ver. 25 (Armonk).

Data availability

All data produced in this investigation are provided in the article.

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Abbreviations—The abbreviations used are: BMC, bone marrow cell; COX, cyclooxygenase; LPS, Lipopolysaccharide; mPGES, membrane-bound PGE synthase; PG, prostaglandin; POB, primary osteoblast; qPCR, quantitative PCR; RANK, receptor activator of NF-κB; RANKL, RANK ligand; TLR, Toll-like receptor.

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