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Lipopolysaccharide of *Aggregatibacter actinomycetemcomitans* up-regulates inflammatory cytokines, prostaglandin E_2 synthesis and osteoclast formation in interleukin-1 receptor antagonist-deficient mice

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Background and Objective: The interleukin (IL)-1 receptor antagonist (Ra) binds to IL-1 receptors and inhibits IL-1 activity. However, it is unclear whether the IL-1Ra plays a protective role in periodontal disease. The purpose of this study was to compare IL-1Ra knockout (KO) and wild-type (WT) mice in regard to proinflammatory cytokine production, osteoclast formation and bone resorption in response to periodontal bacterial lipopolysaccharide (LPS).

Material and Methods: Peritoneal macrophages (Mϕs) were obtained from 13-wk-old IL-1Ra KO and WT mice. Peritoneal Mϕs were cultured with or without 10 μg/mL of *Aggregatibacter actinomycetemcomitans* LPS for 24 h. The levels of IL-1alpha (IL-1α), IL-1beta (IL-1β), tumor necrosis factor-α (TNF-α) and IL-6 were measured in periotoneal Mϕs supernatant fluid (PM-SF) using an ELISA. Bone marrow cells were obtained from the mice and stimulated with PM-SF for 9 d, then stained with TRAP. The frequency of TRAP-positive multinucleated giant cell formation was calculated based on a fusion index. PM-SF-stimulated calvarial bone resorption was analyzed using micro-computed tomography, and calvarial histological analysis was performed using hematoxylin and eosin and TRAP staining. The expression of cyclooxygenase-2 (*Cox2*), prostanoid receptor EP4 (*Ep4*) and *Rank* mRNAs in bone marrow cells were

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measured using real-time quantitative PCR, while prostaglandin E_2 (PGE_2) production was determined by ELISA.

Results: The levels of IL-1α, IL-1β, TNF-α and IL-6 in IL-1Ra KO mice PM-SF stimulated with *A. actinomycetemcomitans* LPS were significantly increased by approximately 4- ($p < 0.05$), 5- ($p < 0.05$), 1.3- ($p < 0.05$) and 6- ($p < 0.05$) fold, respectively, compared with the levels in WT mice. Moreover, osteoclast formation, expression of *Rank*, *Ep4* and *Cox2* mRNAs and production of PGE_2 were significantly increased by approximately 2- ($p < 0.05$), 1.6- ($p < 0.05$), 2.5- ($p < 0.05$), 1.6- ($p < 0.05$) and 1.9- ($p < 0.05$) fold, respectively, in IL-1Ra KO mice stimulated with *A. actinomycetemcomitans* LPS compared with WT mice.

Conclusion: IL-1Ra regulates IL-1 activity and appears to reduce the levels of other inflammatory cytokines, including TNF-α and IL-6, while it also reduces expression of the EP4 receptor related to prostanoid sensitivity and osteoclast formation. These results suggest that IL-1Ra is an important molecule for inhibition of inflammatory periodontal bone resorption.

Subgingival biofilm plays a major role in the pathogenesis of periodontal disease by stimulating an immune response that can lead to periodontal breakdown (1,2). In addition to genetic and acquired risk factors that can modify the host response, susceptibility to periodontal disease, as well as its severity and progression, are influenced by environmental factors (3–5). Microbial challenge consisting of antigens, lipopolysaccharide (LPS) and other virulence factors stimulates host responses (6). *Aggregatibacter actinomycetemcomitans* LPS is thought to be involved in the pathogenesis of alveolar bone loss in periodontitis because it has been shown to induce bone resorption *in vivo* and *in vitro* (7,8). Ishihara *et al.* reported that *A. actinomycetemcomitans* LPS enhanced bone resorption in an organ culture system in which Ca^{2+} release from bone was measured (9). Also, *A. actinomycetemcomitans* LPS promoted osteoclastic differentiation in cultures of mouse bone-marrow cells in the presence of 1,25-dihydroxyvitamin D3 and glucocorticoids (10). These findings indicate that *A. actinomycetemcomitans* LPS is an important bacterial component for stimulating alveolar bone loss in periodontal disease. In addition, host interactions with infectious agents stimulate cells of the immune system to release inflammatory mediators, including proinflammatory cytokines, which can promote extracellular matrix destruction in the gingivae and stimulate bone resorption (11). Interleukin-1 (IL-1) is a well-known major proinflammatory cytokine that directly activates osteoclast formation and activation (12,13). Three of its gene products, interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β) and interleukin-1 receptor antagonist (IL-1Ra), bind to IL-1 receptors. Of those, IL-1α and IL-1β exert similar biologic activities via IL-1 receptor type I, whereas IL-1Ra is a naturally occurring inhibitor of IL-1 that acts by competitively binding to the receptor. IL-1Ra levels in gingival crevicular fluid have been shown to be correlated with the inflammatory condition of gingival tissue and disease severity in periodontitis (14). Although periodontitis and rheumatoid arthritis (RA) are etiologically different, these diseases share similar clinical and pathogenic characteristics. Clinically, both are characterized by local destruction of hard and soft tissues as a consequence of inflammation (15). Previous studies have shown that the levels of IL-1Ra are decreased in patients with autoimmune disease, such as in RA synovial fluid (16), and in those with inflammatory disease, including periodontitis gingival crevicular fluid (14), suggesting that this cytokine may play a regulatory role in RA. In addition, IL-1Ra knockout (KO) mice with a BALB/cA background have been shown to spontaneously develop chronic inflammatory polyarthropathy that closely resembles RA in humans, while histopathologic analysis has demonstrated marked synovial and periarticular inflammation, accompanied by articular erosion, caused by invasion of granulation tissue in those mice (17,18).

Nevertheless, the effects of IL-1Ra on inflammatory cytokine production and bone resorption in IL-1Ra KO mice stimulated with periodontal disease pathogens have not been investigated. Therefore, the aim of this study was to evaluate osteoclast formation and bone-resorbing activity in supernatant fluids from cultured peritoneal macrophages (Mϕs) obtained from IL-1Ra KO mice stimulated with LPS of *A. actinomycetemcomitans*.

Material and methods

Animals

IL-1Ra KO mice with a BALB/cA background, kindly provided by

Dr Y. Iwakura (Tokyo University of Science, Chiba, Japan), and control wild-type (WT) BALB/cA mice, purchased from CLEA Japan, Inc. (Tokyo, Japan), were used. All mice were maintained under specific pathogen-free conditions at Aichi-Gakuin University of Japan, and were given standard mouse chow and water *ad libitum*. All experimental procedures were examined and approved by the Animal Research Ethics Committee of Aichi-Gakuin University of Japan.

Mouse peritoneal Mϕ isolation and culture protocol

Thirteen-week-old IL-1Ra KO and WT mice were injected with 2 mL of sterile thioglycollate medium (Nissui, Tokyo, Japan) into the peritoneal cavity. Three days later, thioglycollate-elicited peritoneal Mϕs were harvested from peritoneal lavage samples with 3 mL of RPMI-1640 (Invitrogen, Grand Island, NY, USA) (19). Isolated Mϕs were plated in six-well plates, at a concentration of 2×10^6 cells/well, in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and standard penicillin/streptomycin. After 3 h of incubation at 37°C in an atmosphere containing 5% CO_2, nonadherent cells were washed out with warm phosphate-buffered saline. Adherent Mϕs were cultured for 2 d, then the medium was changed to FBS-free medium 1 h before the assays. *A. actinomycetemcomitans* LPS was extracted using a hot phenol procedure and purified as previously described (20,21). Mϕs were cultured with or without 10 μg/mL of *A. actinomycetemcomitans* LPS for 24 h, then 2 mL of periotoneal Mϕs supernatant fluid (PM-SF) was collected and stored at −80°C before the assays.

Measurement of cytokine levels in PM-SF

We used Quantikine mouse IL-1α, IL-1β, tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions, to detect the levels of IL-1α, IL-1β, TNF-α and IL-6, respectively. The minimum detectable doses were found to be 2.5, 3.0, 5.1 and 1.1 pg/mL, respectively. Cytokine determinations were performed in triplicate for each sample.

Analysis of mice calvarial bone resorption

Bone resorption of mice calvaria was induced by injection of 200 μL of PM-SF, which was administered with a 25-gauge needle at a point on the midline of the skull located between the ears and the eyes. On day 5 after subcutaneous injection of PM-SF, the mice were killed by cervical dislocation, the calvaria were removed and micro-computed tomography (micro-CT) analysis was performed to confirm the development of osteolysis. A Rigaku R_mCT scanning device (Rigaku, Tokyo, Japan) was used to perform the micro-CT scans, with the X-ray voltage set at 90 kV. Data obtained were analyzed using LaTheta software (Aloka, Tokyo, Japan).

Histological analysis of mice calvaria

Following micro-CT analysis, calvaria specimens were fixed in 4% paraformaldehyde overnight at 4°C, then decalcified with 10% EDTA solution (pH 7.6) for 5 d at 4°C. Each calvaria was then processed for paraffin embedding and 4-μm-thick sections were obtained for each specimen. The first section was stained with hematoxylin and eosin for histological observations, while the next serial section was stained with TRAP to reveal osteoclasts, which are considered to be TRAP-positive multinucleate cells. These cells were counted using a light microscope (Nikon, Tokyo, Japan).

Osteoclast formation of bone marrow cells *in vitro*

Bone marrow cells were obtained from the tibiae of 6-wk-old IL-1Ra KO and WT mice and suspended in α-minimal essential medium (Invitrogen), supplemented with 10% FBS, in 60-mm-diameter dishes. They were cultured for 16 h, then adherent cells, such as fibroblasts and stromal cells, were discarded. Nonadhered cells were used in the following experiments. Next, nonadhered cells were incubated for 16 h in the presence of Mϕ colony-stimulating factor (M-CSF) (50 ng/mL) (R&D Systems), and adherent cells were harvested and further cultured for 3 d with M-CSF, while nonadherent cells were completely removed from the cultures by pipetting. Adherent cells were used as bone marrow cells and further cultured with PM-SF, M-CSF (50 ng/mL) and RANKL (10 ng/mL) (PeproTech, Rocky Hill, NJ, USA) in 48-well plates for 3 d. Cells were fixed and stained for TRAP, as previously described (22). The frequency of multinucleated giant cell formation was calculated based on a fusion index, which has been described previously (23). Briefly, a minimum of 1000 nuclei within TRAP-positive multinucleated giant cells (> 4 nuclei/cell) were counted. The fusion indices of the cells were calculated according to the following formula: Fusion index (%) = [total number of nuclei within multinucleated (> 4 nuclei/cell) cells/total number of nuclei counted] × 100.

Expression and production of prostanoid synthesis gene and bone metabolism in bone marrow cells

Bone marrow cells were stimulated with PM-SF for 7 d, then the levels of expression of cyclooxygenase-2 (*Cox2*), prostanoid receptor EP4 (*Ep4*) and *Rank* were measured using quantitative PCR assays, while the levels of prostaglandin E_2 (PGE_2) were measured using an ELISA (R&D Systems). The lowest detectable dose of PGE_2 was found to be 13.4 pg/mL. *Cox2*, *Ep4* and *Rank* primers were purchased from Applied Biosystems (Foster, CA, USA).

Statistical analysis

Data were analyzed using PASW Statistics 18.0 software (SPSS Japan, Tokyo, Japan). Differences among groups were analyzed using one-factor ANOVA, the Bonferroni multiple comparison test and the Mann–Whitney *U*-test. Data are expressed as mean ± SD.

Results

Inflammatory cytokine levels in PM-SF of WT and IL-1Ra KO mice stimulated with *A. actinomycetemcomitans* LPS

ELISA findings for selected key pro-inflammatory cytokines showed significant differences between PM-SF obtained from WT and IL-1Ra KO mice (Fig. 1). *A. actinomycetemcomitans* LPS induced significantly higher levels of IL-1α, IL-1β, TNF-α and IL-6 in IL-1Ra KO mice PM-SF compared with WT mice (WT vs. KO: IL-1α, 60.2 ± 2.4 pg/mL vs. 246.4 ± 12.6 pg/mL; IL-1β, 16.2 ± 0.6 pg/mL vs. 83.2 ± 3.7 pg/mL; TNF-α, 1424.3 ± 3.9 pg/mL vs. 1819.2 ± 65.1 pg/mL; and IL-6, 214.6 ± 39.4 pg/mL vs. 1303.6 ± 195.1 pg/mL). Interestingly, significantly higher levels of IL-1β were found in PM-SF from IL-1Ra KO mice without *A. actinomycetemcomitans* LPS compared with PM-SF from WT mice administered *A. actinomycetemcomitans* LPS (KO vs. WT: 42.5 ± 2.5 pg/mL vs. 16.2 ± 0.6 pg/mL).

Surface morphology and histological analysis of calvaria from IL-1Ra KO and WT mice

Five days after injection of PM-SF, the calvaria surfaces of WT mice were not changed by phosphate-buffered saline stimulated with PM-SF [control PM-SF (C-PM-SF)]. In contrast, those of WT mice were slightly pitted when LPS stimulated with PM-SF (L-PM-SF) was injected, which was the same finding in KO mice when C-PM-SF was injected. Obvious calvarial surface pitting was observed around the coronal and sagittal suture crossing area in IL-1Ra KO mice when L-PM-SF was injected. (Fig. 2D-a). Calvarial histological specimens from IL-1Ra KO and WT mice were stained with hematoxylin and eosin and TRAP. WT mice calvaria injected with C-PM-SF did not show destroyed fibrous tissues below the suture, and no inflammation appeared (Fig. 2A-b), whereas those injected with L-PM-SF demonstrated inflammation cells above the calvaria (Fig. 2C-b). IL-1Ra KO mice injected with C-PM-SF and L-PM-SF showed fibrous tissue destruction along with inflammatory cell invasion, as well as bone resorption below the suture (Fig. 2B-b and 2D-b). In addition, those mice showed osteoclast formation around the suture area compared with WT mice (Fig. 2B-c and 2D-c).

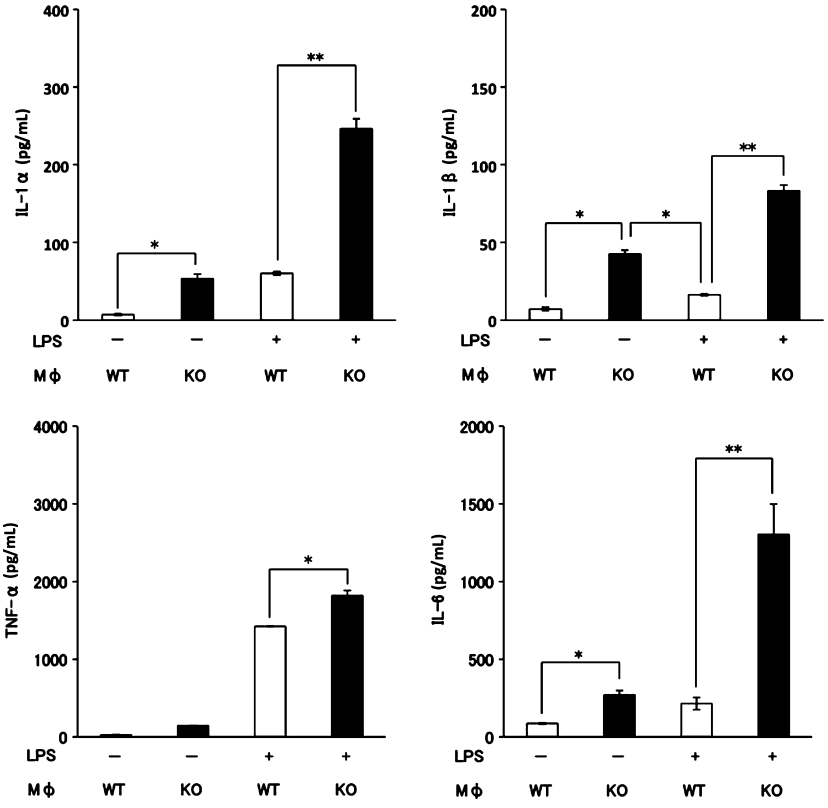

Fig. 1. Inflammatory cytokine levels in peritoneal macrophage supernatant fluid (PM-SF) from interleukin-1 receptor antagonist (IL-1Ra) knockout (KO) and wild-type (WT) mice stimulated with *Aggregatibacter actinomycetemcomitans* lipopolysaccharide (LPS). Cytokine analysis was performed using PM-SF from 13-wk-old male IL-1Ra KO ($n = 6$) and WT ($n = 6$) mice. The levels of interleukin-1alpha (IL-1α), interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) were measured by ELISA. Differences among groups were analyzed using one-factor ANOVA and the Bonferroni multiple comparison test. Data are expressed as mean ± SD. $*p < 0.05$, $**p < 0.01$.

Osteoclast formation by bone marrow cells stimulated with PM-SF from IL-1Ra KO and WT mice

To determine osteoclast formation, we used an osteoclast fusion index (Fig. 3). C-PM-SF scarcely induced osteoclast formation in IL-1Ra KO and WT mice bone marrow cells. In contrast, IL-1Ra KO mice bone marrow cells stimulated with L-PM-SF from IL-1Ra KO mice exhibited a significantly increased fusion index (about twofold higher) compared with WT mice bone marrow cells stimulated with WT mouse L-PM-SF (WT vs. KO: 30.2 ± 4.9% vs. 60.2 ±7.8%).

Expression of *Cox2*, *Ep4* and *Rank* mRNAs and production of PGE_2 in IL-1Ra KO and WT mice bone marrow cells stimulated with PM-SF

Cox2 mRNA expression and PGE_2 production were significantly increased in IL-1Ra KO and WT bone marrow cells stimulated with L-PM-SF compared with IL-1Ra KO and WT bone marrow cells stimulated with C-PM-SF (Fig. 4A and 4B). Also, the concentration of PGE_2 in WT bone

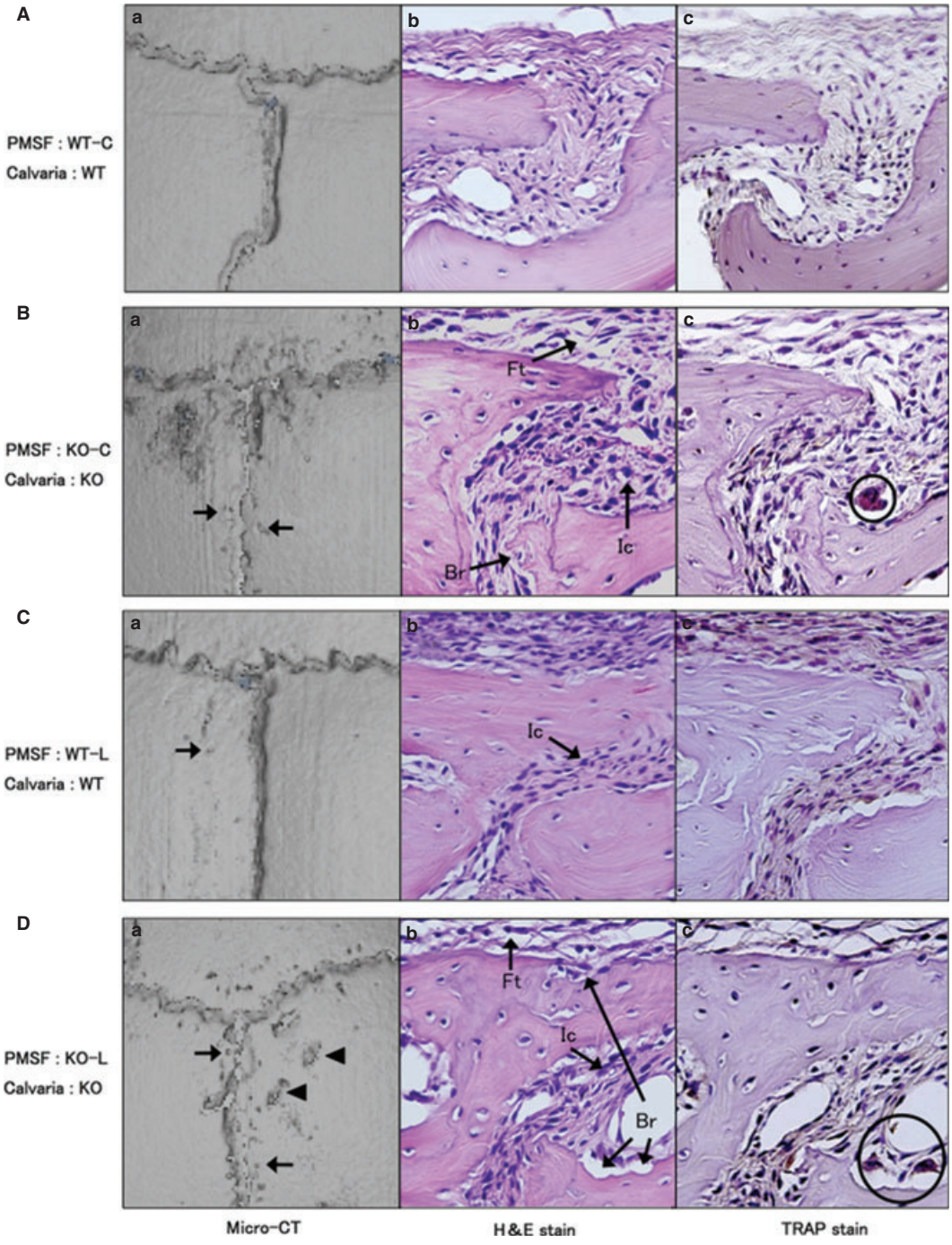

Fig. 2. Comparison of bone surface morphology and histological findings following stimulation with peritoneal macrophage supernatant fluid (PM-SF) from interleukin-1 receptor antagonist (IL-1Ra) knockout (KO) and wild-type (WT) mice. Following stimulation with LPS stimulated (L)- or control (C)-PM-SF, WT mice calvaria samples showed smooth surfaces without pitting (A-a), while those of IL-1Ra KO mice showed rough pitting (B-a, C-a, D-a). Calvarial sections were obtained from IL-1Ra KO (B,D) and WT (A,C) mice and histological sections of calvarial bone were stained with hematoxylin and eosin (H & E) (A-b, B-b, C-b, D-b) or with TRAP (A-c, B-c, C-c, D-c). Solid arrow indicates slight pitting and arrowhead indicates greater pitting. Cells stained brown (indicated by circle) were considered to be TRAP positive. Br, bone resorption; Ft, fibrotic tissue destruction; Ic, inflammatory cell; micro-CT, micro-computed tomography.

marrow cells stimulated with L-PM-SF was significantly higher than when stimulated with C-PM-SF (C-PM-SF vs. L-PM-SF in WT: 94.4 ± 3.2 pg/mL vs. 338.0 ± 7.7 pg/mL), the concentration of PGE_2 in IL-1Ra KO bone marrow cells stimulated with L-PM-SF was significantly higher than when stimulated with C-PM-SF (C-PM-SF vs. L-PM-SF in IL-1Ra KO: 119.3 ± 4.2 pg/mL vs. 685.0 ± 55.3 pg/mL) and the concentration of PGE_2 in

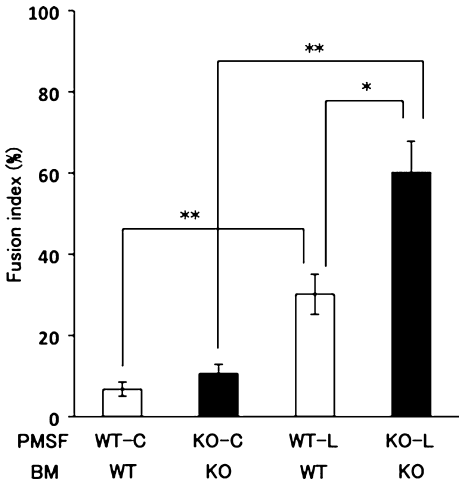

Fig. 3. Comparison of osteoclast fusion index following stimulation with lipopolysaccharide-stimulated (L) or control (C) peritoneal macrophage supernatant fluid (PM-SF) from interleukin-1 receptor antagonist (IL-1Ra) knockout (KO) and wild-type (WT) mice. The osteoclast fusion index was determined for each culture condition. Differences among groups were analyzed using one-factor ANOVA and the Bonferroni multiple comparison test. Data are expressed as mean ± SD. BM, bone marrow. $*p < 0.05$, $**p < 0.01$.

IL-1Ra KO bone marrow cells stimulated with L-PM-SF was significantly higher than that in WT bone marrow cells stimulated with L-PM-SF (WT vs. KO: 338.0 ± 7.7 pg/mL vs. 685.0 ± 55.3 pg/mL). Furthermore, expression of *Ep4* mRNA in IL-1Ra KO bone marrow cells stimulated with L-PM-SF was significantly higher than that in WT bone marrow cells stimulated with L-PM-SF (WT vs. KO: 1.4 ± 0.3-fold vs. 3.5 ± 0.6-fold) (Fig. 4C). In addition, expression of *Rank* mRNA was significantly increased in IL-1Ra KO and WT bone marrow cells stimulated with L-PM-SF compared with those stimulated with C-PM-SF (C-PM-SF vs. L-PM-SF; WT: 1.0 ± 0.1-fold vs. 10.7 ± 1.2-fold; KO: 2.9 ± 0.8-fold vs. 17.1 ± 2.9-fold) (Fig. 4D).

Effects of intracellular IL-1Ra on prostanoid synthesis, bone metabolism gene expression and osteoclast formation

Previous data indicate that the osteoclast fusion index and bone resorption are increased more in IL-1Ra KO mice compared with WT mice. However, it remains unclear whether this phenomenon is dependent on the concentration of inflammatory cytokines in PM-SF or on bone marrow cells with intercellular deficiency of the *Il1Ra* gene. In the present study, we added IL-1Ra KO L-PM-SF to both WT and IL-1Ra KO bone marrow cells, and found that the levels of *Cox2* and *Rank* mRNAs were not significantly increased in WT compared with IL-1Ra KO bone marrow cells. Interestingly, expression of *Ep4* mRNA and production of PGE_2 were significantly higher in IL-1Ra KO mice bone marrow cells than in WT mice bone marrow cells (WT vs. KO: *Ep4*, 1.0 ± 0.2-fold vs. 2.5 ± 0.2-fold; PGE_2, 554.0 ± 61.5 pg/mL vs. 682.8 ± 78.1 pg/mL). Furthermore, the osteoclast fusion index was significantly increased in IL-1Ra KO bone marrow cells stimulated with IL-1Ra KO L-PM-SF compared with WT bone marrow cells stimulated in the same manner (WT vs. KO: 35.8 ± 3.2% vs. 43.4 ± 5.5%) (Table 1).

Discussion

The response to periodontal pathogens by IL-1Ra KO mice has not been previously reported. In the present study, we compared biological reactions to LPS from periodontal bacteria in IL-1Ra KO mice and compared the results with those obtained in WT mice. Mϕs from IL-1Ra KO mice stimulated with *A. actinomycetemcomitans* LPS produced higher levels of IL-1, TNF-α and IL-6 compared with Mϕs from WT mice (Fig. 1). A previous study indicated that TNF-α production in IL-1Ra KO mice was increased when T-cells were stimulated with anti-CD3 IgG_1 (24), while others showed that TNF-α transgenic mice produced high levels of IL-1 and IL-6 compared with WT mice (25), and IL-1Ra reduced LPS-induced IL-1β and TNF-α release from alveolar macrophages (26). Thus, the production of proinflammatory cytokines, such as IL-1, TNF-α and IL-6, may be linked. Moreover, Ishibe *et al*. found that nuclear extracts of nuclear factor kappa B (NF-κB) p65 were higher in untreated hepatocytes from IL-1Ra KO mice (BALB/c background) than those from WT mice, though there was no difference in regard to cytosolic extracts of NF-κB p65 between them (27). In our study, we found elevated levels of inflammatory cytokine production in IL-1Ra KO mice Mϕ compared with those from WT mice, because the activity of NF-κB in IL-1Ra KO mice is greater than that in WT mice, both with and without stimulation with *A. actinomycetemcomitans* LPS.

Calvarial surface pitting and fibrous tissue destruction, along with inflammatory cell invasion below the suture, were increased in the IL-1Ra KO mice compared with WT mice following stimulation with L-PM-SF (Fig. 2), while osteoclast formation was also increased in the IL-1Ra KO mice (Fig. 3). It was previously reported that PGE_2 has a direct effect on osteoclast formation by hematopoietic precursors in spleen-cell cultures treated with RANKL and M-CSF (28). Also, induction of PGE_2 production following stimulation with IL-1 is known to be intimately involved in osteoclast formation (29). The COX-2 inhibitor, NS-398, as well as the COX-1 and COX-2 inhibitor, indomethacin, have been shown to inhibit osteoclast formation (30). PGE_2 exerts its effects via interactions with specific cell-surface receptors, with four PGE_2 subtype receptors, namely EP1, EP2, EP3 and EP4. Of those, EP4 was found to be

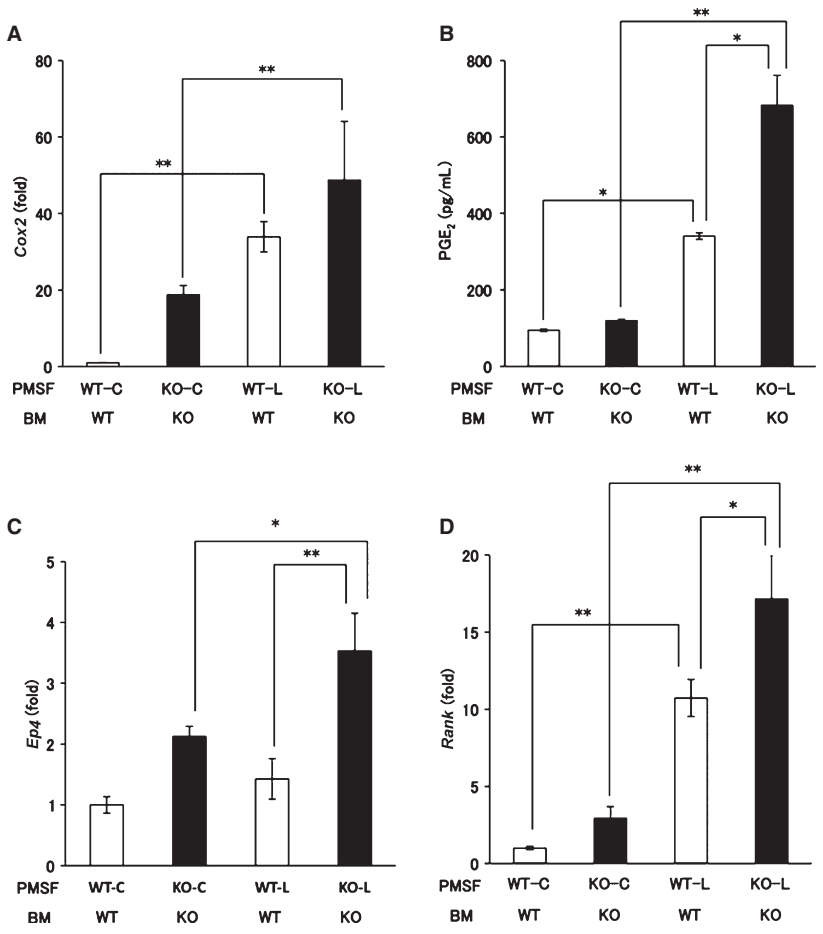

Fig. 4. Expression of cyclooxygenase-2 (*Cox2*), prostanoid receptor EP4 (*Ep4*) and *Rank* mRNAs and production of prostaglandin E2 (PGE_2) in interleukin-1 receptor antagonist (IL-1Ra) knockout (KO) and wild-type (WT) mice bone marrow cells following stimulation with lipopolysaccharide-stimulated (L) or control (C) peritoneal macrophage supernatant fluid (PM-SF). Expression of *Cox2* (A), *Ep4* (C) and *Rank* (D) mRNAs was measured using quantitative PCR (qPCR), and PGE_2 (B) production was measured using ELISA. Differences among groups were analyzed using one-factor ANOVA and the Bonferroni multiple comparison test. Data are expressed as mean ± SD. BM, bone marrow. $*p < 0.05$, $**p < 0.01$.

Table 1. Effects of intracellular interleukin-1 receptor antagonist (IL-1Ra) on prostanoid synthesis, bone metabolism gene expression and osteoclast formation

BM PM-SF	WT KO	KO KO	*p*-value
Cox2 (fold)	1.0 ± 0.1	1.1 ± 0.2	NS
PGE_2 (pg/mL)	554.0 ± 61.5	682.8 ± 78.1	0.045
Rank (fold)	1.0 ± 0.1	1.1 ± 0.1	NS
Ep4 (fold)	1.0 ± 0.2	2.5 ± 0.2	0.012
Fusion index (%)	35.8 ± 3.2	43.4 ± 5.5	0.029

Expression of cyclooxygenase-2 (*Cox2*), prostanoid receptor EP4 (*Ep4*) and *Rank* mRNAs were measured using quantitative PCR (qPCR), while production of prostaglandin E2 (PGE_2) was measured using ELISA. Osteoclast formation was determined using a fusion index. The expression of *Cox2*, *Ep4* and *Rank* mRNAs in IL-1Ra knockout (KO) bone marrow (BM) cells are expressed as the fold difference relative to expression in wild-type (WT) BM cells, which were determined as 1. Differences among groups were analyzed using the Mann–Whitney *U*-test. Data are expressed as mean ± SD. $p < 0.05$. NS, not significant.

the dominant receptor for osteoclast formation and bone resorption by PGE_2 in experiments using EP4 receptor KO mice and receptor agonists (31,32). On the other hand, PGE_2 and the EP4 agonist induce bone formation via expression of core-binding factor α1 (32). PGE_2 and EP4 appear to induce both osteoblastogenesis and osteoclastogenesis, and temporarily and spatially integrate those two actions *in situ* during bone remodeling. In this respect, the action of PGE_2 may be similar to that of Parathyroid hormone, which also promotes both bone formation and resorption (33–35). PGE_2 was reported to be a potent inducer of bone resorption in bone organ cultures, whereas repeated injection of the compound *in vivo* was shown to induce bone formation in a variety of animals, including humans (36). In the present study, the levels of expression of *Cox2* and *Ep4* mRNAs and production of PGE_2 in both types of mice tested seemed to be related to bone resorption. The mechanism of such osteoclast formation and bone resorption caused by *A. actinomycetemcomitans* LPS induces various inflammatory cytokines, and those cytokines induce PGE_2 via the EP4 receptor (Fig. 4B and 4C).

However, it remains unclear whether this phenomenon is dependent on the elevated concentration of inflammatory cytokines in PM-SF from IL-1Ra KO mice or the result of an *Il1Ra* gene deficiency in the bone marrow cells. IL-1Ra produced by macrophages was found to be contained in WT L-PM-SF, and thus we added IL-1Ra KO L-PM-SF to WT and IL-1Ra KO bone marrow cells. Our results showed that PGE_2 production and *Ep4* mRNA expression, as well as the osteoclast fusion index of IL-1Ra KO bone marrow cells, were significantly higher compared with those from WT mice (Table 1). Moreover, Lee *et al.* reported that IL-1 produced by bone marrow cells more effectively induced osteoclast formation compared with IL-1 produced by osteoblasts in co-culture experiments (37). Together, these results suggest that increased osteoclast formation and bone resorption

in IL-1Ra KO mice caused by *Il1Ra* gene deficiency up-regulate proinflammatory cytokine production by Mϕs stimulated with *A. actinomycetemcomitans* LPS. Consequently, the expressions of IL-1, COX-2, PGE_2 and EP4 are up-regulated in bone marrow cells of *Il1Ra* gene-deficient mice.

Studies of anti-inflammatory mediators have shown that adenosine suppresses proinflammatory cytokine production in fibroblasts stimulated with periodontal pathogenic bacteria, while the inhibitory effect on inflammatory bone resorption has not been determined (38). IL-1Ra has been used for approximately 10 years as a treatment for RA, and has been shown to be a strong regulator of severe inflammation and bone resorption (39,40).

Thus, IL-1Ra is a very important molecule because it helps to control periodontal tissue inflammation and tissue breakdown, similarly to that which occurs in RA. Baker *et al.* created an experimental mouse model of periodontitis that exhibits alveolar bone destruction following oral infection with periodontal pathogenic bacteria (41). Based on the present results, we plan to investigate periodontitis susceptibility *in vivo* using IL-1Ra KO mice in the near future.

Acknowledgements

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