Inflammatory Bone Loss in Experimental Periodontitis Induced by *Aggregatibacter actinomycetemcomitans* in Interleukin-1 Receptor Antagonist Knockout Mice

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The interleukin-1 receptor antagonist (IL-1Ra) binds to IL-1 receptors and inhibits IL-1 activity. However, it is not clear whether IL-1Ra plays a protective role in periodontal disease. This study was undertaken to compare experimental periodontitis induced by *Aggregatibacter actinomycetemcomitans* in IL-1Ra knockout (KO) mice and wild-type (WT) mice. Computed tomography (CT) analysis and hematoxylin-and-eosin (H&E) and tartrate-resistant acid phosphatase (TRAP) staining were performed. In addition, osteoblasts were isolated; the mRNA expression of relevant genes was assessed by real-time quantitative PCR (qPCR); and calcification was detected by Alizarin Red staining. Infected IL-1Ra KO mice exhibited elevated ($P, <0.05$) levels of antibody against *A. actinomycetemcomitans*, bone loss in furcation areas, and alveolar fenestrations. Moreover, protein for tumor necrosis factor alpha (TNF- α) and IL-6, mRNA for macrophage colony-stimulating factor (M-CSF), and receptor activator of NF- κ B ligand (RANKL) in IL-1Ra KO mouse osteoblasts stimulated with *A. actinomycetemcomitans* were increased ($P, <0.05$) compared to in WT mice. Alkaline phosphatase (ALP), bone sialoprotein (BSP), osteocalcin (OCN)/bone gla protein (BGP), and runt-related gene 2 (Runx2) mRNA levels were decreased ($P, <0.05$). IL-1 α mRNA expression was increased, and calcification was not observed, in IL-1 Ra KO mouse osteoblasts. In brief, IL-1Ra deficiency promoted the expression of inflammatory cytokines beyond IL-1 and altered the expression of genes involved in bone resorption in *A. actinomycetemcomitans*-infected osteoblasts. Alterations consistent with rapid bone loss in infected IL-Ra KO mice were also observed for genes expressed in bone formation and calcification. In short, these data suggest that IL-1Ra may serve as a potential therapeutic drug for periodontal disease.

Subgingival biofilm plays a major role in the pathogenesis of periodontal disease by stimulating immune responses that can lead to periodontal tissue damage (1, 2). In addition to genetic and acquired risk factors that can modify host responses, susceptibility to periodontal disease, as well as its severity and progression, is influenced by environmental factors (3–5). *Aggregatibacter actinomycetemcomitans* expresses components that may mediate adherence to mucosal surfaces, enable penetration into epithelial cells, inhibit host defense mechanisms, and elicit gingival tissue destruction and alveolar bone resorption (6, 7). *A. actinomycetemcomitans* is clearly associated with the pathogenesis of aggressive periodontitis (AgP). AgP is typically an aggressive, rapidly developing disease that often manifests itself in a severe form in juveniles. *A. actinomycetemcomitans* induces massive titers of antibody against the serotype b-specific capsular polysaccharide antigen (CPA) of *A. actinomycetemcomitans* (8), coupled with elevated proinflammatory cytokine production (9) and rapid periodontal bone loss (10). Furthermore, there is evidence that people susceptible to AgP have a genetic predisposition to develop this disease (11, 12). Accordingly, we reasoned that *A. actinomycetemcomitans* would be an excellent pathogen to analyze in a model where a defined genetic lesion results in a lack of ability to regulate cytokine production properly. Furthermore, our group has reported that *A. actinomycetemcomitans* lipopolysaccharide (LPS) induced bone resorption in BALB/c mouse calvaria organ cultures (13). *A. actinomycetemcomitans* CPA and LPS promote the formation of osteoclast-like cells; LPS also supports the survival of osteoclasts induced by interleukin-1 α (IL-1 α) in mouse bone marrow cul-

tures (14, 15), and CPA suppresses the production of IL-6 and IL-8 by human fibroblasts (16). During these processes, a range of proinflammatory cytokines (including IL-1 α and IL-1 β) that may initiate connective tissue inflammation and alveolar bone resorption are induced (17). Three gene products, IL-1 α , IL-1 β , and IL-1 receptor antagonist (IL-1Ra), bind to IL-1 receptors. Of these, IL-1 α and IL-1 β exert similar biologic activities via IL-1 receptor type I, whereas IL-1Ra is a naturally occurring inhibitor of IL-1 that acts by binding competitively to the receptor. IL-1Ra levels in gingival crevicular fluid (GCF) are negatively correlated with disease severity in chronic periodontitis patients (18), while IL-1Ra levels in moderate and deep pockets from patients with aggressive periodontitis were lower than those at control sites (19). In contrast, IL-1Ra mRNA expression was found to be upregulated with IL-1 β or the IL-6/soluble IL-6 receptor (sIL-6R) complex in inflamed human gingival tissue (20). Peripheral blood mononuclear cells from patients with generalized aggressive periodontitis exhibited higher levels of IL-1Ra and IL-10 than those from healthy

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controls with or without LPS stimulation (21). Nevertheless, whether IL-1Ra plays a role in the defense against the development of periodontal disease remains unknown.

Previous studies of proinflammatory cytokine production, osteoclast formation, and bone resorption in IL-1Ra knockout (KO) and wild-type (WT) mice in response to *A. actinomycetemcomitans* LPS have demonstrated that IL-1Ra regulates IL-1 activity, and it appears to reduce the levels of other proinflammatory cytokines, including tumor necrosis factor alpha (TNF- α) and IL-6, while reducing the expression of the EP4 receptor, which is related to prostanoid sensitivity and osteoclast formation *in vitro* (22). IL-1Ra inhibited osteoclast formation (23), and it decreased bone resorption in ovariectomized rats (24). However, the effects of IL-1Ra on the expression of bone resorption-related genes, such as those encoding receptor activator of NF- κ B ligand (RANKL), osteoprotegerin (OPG), and macrophage colony-stimulating factor (M-CSF), in osteoblasts have not been investigated. In the present study, experimental periodontitis was induced by *A. actinomycetemcomitans*, and the expression of selected genes and osteoblast activity were determined for IL-1Ra KO mice and were compared to those for WT mice.

MATERIALS AND METHODS

IL-1Ra KO and WT mice. IL-1Ra knockout (KO) mice with a BALB/cA background (25) were kindly provided by Y. Iwakura (Tokyo University of Science, Chiba, Japan), while control wild-type (WT) BALB/cA mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). 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.

Aggregatibacter actinomycetemcomitans culture. *A. actinomycetemcomitans* (ATCC 29524) cells were grown in brain heart infusion medium (Difco Laboratories, Detroit, MI, USA) with 1% yeast extract (Difco Laboratories, Detroit, MI, USA) at 37°C under a CO₂-rich atmosphere with an AnaeroPack system (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) (26).

Experimental periodontitis stimulated by *A. actinomycetemcomitans*. IL-1Ra KO and WT mice were infected with 300 μ l of a solution consisting of 1×10^{10} CFU/ml of live *A. actinomycetemcomitans* (each mouse was orally infected with 3×10^9 CFU) suspended in sterile phosphate-buffered saline (PBS) containing 2.5% carboxymethyl cellulose. Mice were infected a total of 5 times at 1-day intervals. Forty-two days after the last infection, blood was collected from the inferior vena cava, and sera were obtained; mandibles were removed and immediately fixed in 4% paraformaldehyde overnight at 4°C, and then micro-computed tomography (micro-CT) analysis was performed (27, 28).

Quantification of anti-*A. actinomycetemcomitans* IgG antibodies. Titers of anti-*A. actinomycetemcomitans* IgG in serum were measured using an enzyme-linked immunosorbent assay (ELISA), as described previously (29). Ninety-six-well ELISA plates (Sumilon; Sumitomo Bakelite Company, Tokyo, Japan) were coated with *A. actinomycetemcomitans* Y4 CPA (1 μ g/ml) (30), followed by overnight incubation at 4°C. The wells were then blocked for 30 min at room temperature (RT) with 2% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in PBS-Tween (PBST), and serum samples were incubated for 2 h at RT. A horseradish peroxidase-conjugated mouse IgG antibody (lot FIM2412051; R&D Systems, Minneapolis, MN) was added, and the mixture was incubated for 1 h at RT; a tetramethylbenzidine (TMB) microwell peroxidase substrate solution (KPL, Gaithersburg, MD) was used as a substrate. The reaction was stopped using a stop solution, and absorbance at 450 nm was measured using a SpectraFluor Plus microplate reader (Tecan, Durham, NC).

The titer was defined as the reciprocal of the last dilution resulting in an absorbance greater than 0.1 (31, 32).

Quantification of alveolar bone resorption. Micro-CT analysis was performed to measure bone resorption. 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, and the data obtained were analyzed using LaTheta software (Aloka, Tokyo, Japan). Morphological evaluation of the alveolar crest was carried out using linear measurements from the cemento-enamel junction (CEJ) to the root apex (RA) and from the alveolar bone crest (ABC) to the RA in the intradental region between the lower first and second molars (M1-M2). All images were reoriented such that the CEJ and RA appeared in the micro-CT slice to be analyzed. Measurements of root length (RL) from the CEJ to the RA were also performed in order to assess the percentage of vertical bone remaining. The ratio of the remaining alveolar bone crest to the root length was calculated as $(ABC - RA)/RL$, where $(ABC - RA)$ is the distance from the ABC to the RA and RL is the distance from the CEJ to the RA (33, 34).

Histological analysis of mandibles. Following micro-CT analysis, mandible specimens were decalcified with a 10% EDTA solution (pH 7.6) for 1 day at 4°C. Each mandible was then processed for paraffin embedding, and 4- μ m-thick sections were obtained for each. The first section was stained with hematoxylin and eosin (H&E) for histological observations, while the next serial section was stained with tartrate-resistant acid phosphatase (TRAP) to reveal osteoclasts, which are considered to be TRAP-positive multinucleated cells (35–37). The number of osteoclasts on the interradicular septum (bone surface) (Oc.N/BS) was determined, and the osteoclast surface on the interradicular septum (bone surface) (Oc.S/BS) was evaluated, by scoring the TRAP-positive multinucleated cells attached to the bone surface as defined by Parfitt et al. (38, 39). These cells were counted using a light microscope (Nikon, Tokyo, Japan).

Protocol for isolation and culture of osteoblasts. Osteoblasts were isolated from the calvarias of 5-day-old IL-1Ra KO and WT mice. Each calvaria was digested in 10 ml of α -minimum essential medium (α -MEM) (Gibco-BRL, Grand Island, NY) containing 0.1% collagenase (Wako Pure Chemical Industries, Osaka, Japan) and 0.2% dispase (Eidia, Tokyo, Japan) for 10 min at 37°C with vigorous shaking and was then centrifuged at $300 \times g$ for 5 min. The first supernatant fluid was discarded, after which another 10 ml of collagenase/dispase enzyme solution was added, and the mixture was incubated for 10 min. The digestion procedure was repeated 4 times, and cells isolated by the last 3 digestions were combined as osteoblasts (40). They were cultured for 3 to 4 days in α -MEM containing 10% fetal bovine serum (FBS) (MP Biomedicals, Solon, OH) and antibiotic solution (100 U/ml of penicillin, 100 μ g/ml of streptomycin) under a humidified atmosphere of 5% CO₂ at 37°C, with a change of medium every 3 days. Osteoblasts at passages 4 to 6 were subjected to mRNA expression assays.

Stimulation of osteoblast cell cultures by *A. actinomycetemcomitans* infection. Osteoblasts were seeded at a concentration of 3.3×10^5 cells/well in 24-well plates. The cells were infected with *A. actinomycetemcomitans* in serum-free α -MEM containing no antibiotics. The cells were washed 3 times, cultured for 24 h in serum-free α -MEM, infected with viable *A. actinomycetemcomitans* at a multiplicity of infection (MOI) of 100, and cultured for a further 12 h in the presence of the bacteria in an incubator at 37°C under a 5% CO₂ atmosphere (35, 41). After the culture periods, attached osteoblasts were collected for examination of the mRNA expression of bone resorption-related genes encoding RANKL, OPG, and M-CSF, and supernatant fluids were collected for the measurement of cytokines IL-1Ra, TNF- α , and IL-6 produced in the supernatant.

Assay for the formation of a mineralized osteoblast matrix. Osteoblasts were seeded at a concentration of 4×10^5 cells/well in 24-well plates. After 24 h of culture, α -MEM containing 10% FBS was replaced with an osteogenic medium containing L-ascorbic acid-2-phosphate (50 μ g/ml), dexamethasone (10^{-7} M), and β -glycerophosphate (10^{-3} M), and the medium was changed every 3 days. After 7, 14, and 21 days, cell culture samples were fixed for 10 min at room temperature in 4% paraformaldehyde.

FIG 1 Comparison of *A. actinomycetemcomitans*-specific antibody responses in IL-1Ra KO and WT mice. Antibody titers in IL-1Ra KO mice ($n = 5$) infected with *A. actinomycetemcomitans* were higher than those in infected WT mice ($n = 6$), uninfected WT mice ($n = 5$), and uninfected IL-1Ra KO mice ($n = 5$). Differences among groups were analyzed using one-factor ANOVA and Tukey's test. Data are expressed as means $\pm$ SD. Asterisks indicate significant differences ($P < 0.05$) from IL-1Ra KO mice infected with *A. actinomycetemcomitans*.

hyde, washed with PBS, and stained with 40 mM Alizarin Red S (Kanto Chemical, Tokyo, Japan), pH 6.4, for 10 min at room temperature. Next, the cell preparations were washed 3 times with deionized water (DW) and were incubated in DW for 12 h to eliminate nonspecific staining. At the same time, other cell culture samples were collected and were used for examination of the mRNA expression of bone formation-related genes and IL-1.

Determination of mRNA expression from infected and mineralized osteoblasts using real-time PCR. Total RNA was extracted by using the NucleoSpin RNA II system (Macherey-Nagel Inc., Bethlehem, PA, USA) according to the manufacturer's instructions. The purity and level of RNA were assessed by calculating A_{230}/A_{260} and A_{260}/A_{280} ratios using a NanoDrop ND-1000 fluorospectrometer (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized from total RNA by the extension of random primers with 200 U of SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). To quantify RANKL, OPG, M-CSF, IL-1 α , IL-1 β , alkaline phosphatase (ALP), osteocalcin (OCN)/bone gla protein (BGP), bone sialoprotein (BSP), and runt-related gene 2 (Runx2) mRNA levels, quantitative PCR (qPCR) analyses were performed using a TaqMan gene expression assay for mouse RANKL (Mm00441906_m1), OPG (Mm01205928_m1), M-CSF (Mm00432686_m1), IL-1 α (Mm99999060_m1), IL-1 β (Mm01336189_m1), ALP (Mm00475834_m1), OCN/BGP (Mm03413826_m1), BSP (Mm00436767_m1), and Runx2

(Mm00501580_m1) with the TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA, USA), and the mRNA levels obtained were normalized against the level of eukaryotic 18S rRNA (Hs99999901_s1). Analysis was performed using the ABI Prism 7000 sequence detection system (with the associated software, version 1.0; Applied Biosystems, Foster City, CA, USA), programmed with the following cycle parameters: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The relative amounts of mRNA for the target cytokines were determined by subtracting the cycle threshold (C_T) value for the gene from that for rRNA (ΔC_T). Next, the ΔC_T value for the control group was subtracted from that for the treated group ($\Delta\Delta C_T$). The results are expressed as the fold difference ($2^{-\Delta\Delta C_T}$) between the mRNA expression level for the control group and that for the treated group, where $\Delta\Delta C_T$ is calculated as follows: $[(C_T \text{ for the target mRNA} - C_T \text{ for 18S rRNA}) \text{ for the treated group}] - [(C_T \text{ for the target mRNA} - C_T \text{ for 18S rRNA}) \text{ for the control group}]$ [35].

Measurement of cytokine levels in supernatant fluids of *A. actinomycetemcomitans*-infected osteoblasts. We used mouse TNF- α , IL-6, and IL-1Ra enzyme ELISA kits (the TNF- α and IL-6 kits were from Diaclone [Besançon, France], and the IL-1Ra kit was from R&D Systems [Minneapolis, MN, USA]) to determine the levels of TNF- α , IL-6, and IL-1Ra, respectively, according to the manufacturer's instructions. The minimum detectable doses were found to be 12.9, 10.0, and 7.0 pg/ml, respectively. Cytokine determinations were performed in triplicate for each sample.

Statistical analysis. Data were analyzed using PASW Statistics software (version 18.0; SPSS Japan, Tokyo, Japan). Differences among groups were examined using one-factor analysis of variance (ANOVA), Tukey's test, and Bonferroni's multiple comparison test. Differences in ALP, OCN/BGP, BSP, Runx2, and IL-1 α values were determined using Student's t test. Data are expressed as means $\pm$ standard deviations (SD).

RESULTS

Antibody responses. Forty-two days after infection, titers of antibody against *A. actinomycetemcomitans* capsular polysaccharide antigen (CPA) in serum were significantly higher in infected IL-1Ra KO mice (527.88 ± 174.97) than in infected WT mice (61.81 ± 53.97) ($P < 0.05$), uninfected WT mice (23.61 ± 16.37), or uninfected IL-1Ra KO mice (21.53 ± 18.39) (Fig. 1). The titers of antibody against *A. actinomycetemcomitans* in 2 out of 5 infected WT mice were modestly elevated (153.25 and 116.81, respectively). However, the average titers of antibody against *A. actinomycetemcomitans* CPA did not differ significantly between the uninfected and infected groups of WT mice (Fig. 1).

CT analysis. No bone resorption was seen around the second mandibular molars in *A. actinomycetemcomitans*-infected or uninfected WT mice or in uninfected IL-1Ra KO mice (Fig. 2A, B, and C), whereas infected IL-1Ra KO mice exhibited alveolar fenestration and bone resorption in the furcation area of the second

FIG 2 Comparison of mandibles from *A. actinomycetemcomitans*-infected IL-1Ra KO and WT mice using micro-CT. Micro-CT images of mandibles are shown. (A) Uninfected WT mice; (B) uninfected IL-1Ra KO mice; (C) WT mice infected with *A. actinomycetemcomitans*; (D) IL-1Ra KO mice infected with *A. actinomycetemcomitans*. Infected IL-1Ra KO mice exhibited alveolar fenestrations in the mesial root and bone loss in furcation areas of the second mandibular molar, in contrast to WT infected mice and uninfected IL-1Ra KO mice.

Ratio of remaining alveolar crest bone
(ABC – RA)/RL

A. actinomycetemcomitans
infection

FIG 3 Ratio of the remaining alveolar crest bone to the root length in *A. actinomycetemcomitans*-infected IL-1Ra KO and WT mice. The ratio of the remaining alveolar crest bone to the root length was calculated using the formula (ABC – RA)/RL, where (ABC – RA) is the distance from the ABC to the RA and RL is the distance from the CEJ to the RA. That ratio in the mesial roots of the second mandibular molars in IL-1Ra KO mice was significantly lower than the ratios for infected WT mice ($n = 16$), uninfected WT mice ($n = 12$), and uninfected IL-1Ra KO mice ($n = 7$). Differences among groups were analyzed using one-factor ANOVA and Tukey's test. Data are expressed as means $\pm$ SD. Asterisks indicate significant differences ($P < 0.05$) from infected IL-1Ra KO mice. RA, root apex; CEJ, cemento-enamel junction; ABC, alveolar bone crest.

mandibular molars (Fig. 2D). The ratio of the remaining alveolar crest bone to the root length in infected IL-1Ra KO mice (0.63 ± 0.04) was significantly lower than the ratios in uninfected WT mice (0.81 ± 0.04), uninfected IL-1Ra KO mice (0.82 ± 0.05), and infected WT mice (0.76 ± 0.03) ($P < 0.05$) (Fig. 3).

Histological analysis of mandibles from IL-1Ra KO and WT mice. Histological images of periodontal tissues showed epithelial and connective tissue elements characteristic of periodontal disease, such as apical migration of junctional epithelium, the presence of inflammatory cells, and epithelial attachment loss between the first and second molars in IL-1Ra KO mice infected with *A. actinomycetemcomitans*, in contrast to infected WT mice. Inflammatory infiltration in infected IL-1Ra KO mice was characterized mainly by the presence of macrophages and neutrophils, while lymphocytes were also observed (Fig. 4). Furthermore, histological analysis of periodontal tissues showed a greater number of

TRAP-positive multinucleated cells in the alveolar bone crest in infected IL-1Ra KO mice than in infected WT mice (Fig. 5). Oc.N/BS was significantly (3.5-fold) higher in the interradicular septa of IL-1Ra KO mice infected with *A. actinomycetemcomitans* than in those of infected WT mice ($P < 0.05$). Oc.S/BS was significantly (3.7-fold) higher in the interradicular septa of IL-1Ra KO mice infected with *A. actinomycetemcomitans* than in those of infected WT mice ($P < 0.05$) (Fig. 5).

Expression of mRNAs for RANKL, OPG, and M-CSFs in osteoblasts following stimulation with viable *A. actinomycetemcomitans*. Unstimulated WT mice served as the background controls. IL-1Ra KO and WT mice were stimulated with *A. actinomycetemcomitans* (MOI, 100). RANKL mRNA expression in IL-1Ra KO mice was increased by 48.41 ± 13.45 -fold, compared with 29.04 ± 8.06 -fold for WT mice ($P < 0.05$) (Fig. 6). OPG mRNA expression did not differ between control osteoblasts and

FIG 4 Histologic views of first and second mandibular molars from *A. actinomycetemcomitans*-infected IL-1Ra KO and WT mice (H&E staining; magnification, $\times 200$). Histological findings in periodontal tissues included epithelial attachment loss (arrow) and a considerable inflammatory reaction in IL-1Ra KO mice infected with *A. actinomycetemcomitans*, in contrast to infected WT mice. These histological findings were obtained for 3 out of 5 IL-1Ra KO mice.

FIG 5 TRAP enzymology views of second mandibular molars from *A. actinomycetemcomitans*-infected IL-1Ra KO and WT mice (magnification, $\times 100$). Histological examination of periodontal tissues showed TRAP-positive multinucleated cells (arrowheads) in IL-1Ra KO mice infected with *A. actinomycetemcomitans*, in contrast to infected WT mice. The number of osteoclasts on the interradicular septum (bone surface) (Oc.N/BS) was determined, and the osteoclast surface on the interradicular septum (bone surface) (Oc.S/BS) was evaluated, by scoring the TRAP-positive multinucleated cells attached to the bone surface. These histological findings were obtained for 5 out of 5 IL-1Ra KO mice.

infected osteoblasts from both groups of mice (data not shown). M-CSF mRNA expression in IL-1Ra KO mouse osteoblasts was increased by 8.60 ± 1.93 -fold, as opposed to 4.27 ± 0.34 -fold for WT mice ($P, < 0.05$) (Fig. 6). These results suggest that IL-1Ra strongly downregulated the expression of RANKL and M-CSF mRNAs in osteoblasts stimulated with viable *A. actinomycetemcomitans*.

TNF- α , IL-6, and IL-1Ra production in osteoblasts following stimulation with viable *A. actinomycetemcomitans*. ELISA results for TNF- α , IL-6, and IL-1Ra revealed significant differences between WT and IL-1Ra KO mouse osteoblasts stimulated with viable *A. actinomycetemcomitans* (Fig. 7). Viable *A. actinomycetemcomitans* induced significantly higher levels of TNF- α and IL-6 in IL-1Ra KO mouse osteoblasts than in WT mouse osteoblasts (32.38 ± 17.30 pg/ml TNF- α for WT mice versus 261.47 ± 15.56 pg/ml for KO mice [$P, < 0.05$]; 3.70 ± 0.53 ng/ml IL-6 for WT

mice versus 8.19 ± 0.82 ng/ml for KO mice [$P, < 0.05$]) (Fig. 7). Interestingly, a significantly higher level of IL-1Ra was found in WT mice infected with viable *A. actinomycetemcomitans* for 24 h than in uninfected WT mice (87.08 ± 8.59 pg/ml IL-1Ra for uninfected WT mice versus 516.01 ± 64.53 pg/ml for infected WT mice) (Fig. 7). IL-1Ra was not found in IL-1Ra KO mice under any culture condition.

ALP, OCN/BGP, BSP, Runx2, IL-1 α , and IL-1 β mRNA expression in osteoblasts from IL-1Ra KO and WT mice. Comparisons of gene expression for ALP, OCN/BGP, BSP, Runx2, IL-1 α , and IL-1 β are shown in Fig. 8. Significant differences were apparent for all genes except IL-1 β .

ALP mRNA expression at 1, 2, and 3 weeks was significantly lower than the baseline, at 0.20 ± 0.06 -fold, 0.19 ± 0.01 -fold, and 0.15 ± 0.05 -fold, respectively, in IL-1Ra KO mouse osteoblasts, in contrast to 1.04 ± 0.28 -fold, 0.88 ± 0.23 -fold, and

FIG 6 RANKL and M-CSF mRNA expression in IL-1Ra KO and WT mouse osteoblasts stimulated with *A. actinomycetemcomitans*. IL-1Ra KO ($n = 4$) and WT ($n = 4$) mouse osteoblasts were stimulated with *A. actinomycetemcomitans* (MOI, 100). The mRNA levels of RANKL and M-CSF were determined using real-time PCR and are expressed as fold differences from levels in unstimulated WT mouse osteoblasts. RANKL and M-CSF mRNA expression was significantly increased in IL-1Ra KO mouse osteoblasts. Differences among groups were analyzed using one-factor ANOVA and Tukey's test. Data are expressed as means $\pm$ SD. Asterisks indicate significant differences ($P, < 0.05$).

FIG 7 TNF- α , IL-6, and IL-1Ra levels in osteoblast culture supernatant fluids from IL-1Ra KO ($n = 4$) and WT ($n = 4$) mice either left unstimulated or stimulated with *A. actinomycetemcomitans*. Supernatant fluids were studied after 12 h of culture for TNF- α , IL-6, and IL-1Ra and after 12 and 24 h of culture for IL-1Ra. The levels of TNF- α , IL-6, and IL-1Ra were measured by ELISA. Differences among groups were analyzed using one-factor ANOVA and Bonferroni's multiple comparison test. Data are expressed as means $\pm$ SD. Asterisks indicate significant differences ($P, < 0.05$) between KO and WT osteoblasts.

0.66- $\pm$ 0.22-fold ALP mRNA expression in WT mouse osteoblasts ($P, < 0.05$) (Fig. 8). BSP mRNA expression in IL-1Ra KO mouse osteoblasts at 1 and 2 weeks was decreased, at 0.38- $\pm$ 0.01-fold and 0.31- $\pm$ 0.026-fold, in contrast to 1.01- $\pm$ 0.14-fold and 0.81- $\pm$ 0.13-fold BSP mRNA expression in WT mouse osteoblasts ($P, < 0.05$) (Fig. 8). OCN/BGP mRNA levels in IL-1Ra KO mouse osteoblasts at 1, 2, and 3 weeks were decreased, at 0.46- $\pm$ 0.04-fold, 0.32- $\pm$ 0.04-fold, and 0.13- $\pm$ 0.01-fold, in contrast to 1.01- $\pm$ 0.15-fold, 0.59- $\pm$ 0.04-fold, and 0.32- $\pm$ 0.02-fold OCN/BGP mRNA expression in WT mouse osteoblasts ($P, < 0.05$). The levels of Runx2 mRNA expression were 0.66- $\pm$ 0.08-fold and 0.46- $\pm$ 0.06-fold in IL-1Ra KO mice, as opposed to 1.57- $\pm$ 0.18-fold and 1.91- $\pm$ 0.23-fold in WT mice, at 2 and 3 weeks, respectively ($P, < 0.05$) (Fig. 8). The IL-1 α mRNA level in IL-1Ra KO mouse osteoblasts was increased at 3 weeks, at 6.27- $\pm$ 0.67-fold, in contrast to 3.19- $\pm$ 0.41-fold IL-1 α mRNA expression in WT mice at that time ($P, < 0.05$) (Fig. 8). IL-1 β mRNA expression in osteoblasts cultured in an osteogenic medium did not differ between the groups at any time point. These results suggest that IL-1Ra strongly upregulates the expression of ALP, OCN/BGP, BSP, and Runx2 and downregulates IL-1 α mRNA expression in osteoblasts cultured in an osteogenic medium.

Mineralized matrix formation. In studies of mineralized matrix formation, the osteoblasts were not infected with *A. actinomycetemcomitans* but were just cultured for 1 to 3 weeks (Fig. 9). Osteoblasts from IL-1Ra KO and WT mice were cultured in an

osteogenic medium, which was red at 1, 2, and 3 weeks. No evidence of staining was seen in osteoblasts from IL-1Ra KO or WT mice after 1 and 2 weeks. However, after 3 weeks, osteoblasts from WT mice were clearly stained, whereas osteoblasts from IL-1Ra KO mice were not (Fig. 9).

DISCUSSION

Alveolar bone resorption may (42, 43) or may not (44, 45) be recognized in inbred strains of mice challenged by oral *Porphyromonas gingivalis* infection. Moreover, normal Wistar rats exhibited no alveolar bone loss induced by oral *A. actinomycetemcomitans* infection (46). In short, bone loss does not occur or develops slowly in wild-type rodents infected with human periodontal pathogens. In the present study, alveolar bone resorption was not observed in infected BALB/cA WT mice (Fig. 2C). However, more-dramatic bone loss has been reported in certain genetically engineered mutant mice that lack cell adhesion molecules (47–49). Genetically modified mice have become essential to our understanding of how a particular gene functions *in vivo*. For example, IL-10 is an anti-inflammatory cytokine, and comparisons of IL-10 KO mice and WT mice with experimental periodontitis (44) suggest that IL-10 inhibits the progression of periodontal disease. It may also be relevant that IL-10 is also known to enhance the production of IL-1Ra (50, 51). IL-1Ra KO mice with a BALB/cA background spontaneously develop a chronic inflammatory polyarthropathy that closely resembles arthritis in humans. Histo-

FIG 8 ALP, OCN/BGP, BSP, Runx2, IL-1 α , and IL-1 β mRNA expression in osteoblasts from IL-1Ra KO ($n = 3$) and WT ($n = 3$) mice. Osteoblasts were cultured for 1, 2, and 3 weeks in an osteogenic medium; then mRNA levels were determined using real-time PCR and are expressed as fold differences from levels in WT mouse osteoblasts cultured for 1 week. ALP, BSP, OCN/BGP, and Runx2 mRNA levels were significantly decreased, while the IL-1 α mRNA level was significantly increased, in IL-1Ra KO mouse osteoblasts. Differences among groups were analyzed using Student's t test. Data are expressed as means $\pm$ SD. Asterisks indicate significant differences ($P < 0.05$) from WT mouse osteoblasts.

pathological analysis has revealed marked synovial and periarticular inflammation, accompanied by articular erosion, caused by invasion of granulation tissue in those mice (25, 52). Mizutani et al. reported that stimulation of supernatant fluids from IL-1Ra KO mouse peritoneal macrophages with *A. actinomycetemcomitans* LPS induced severe calvarial bone resorption, in contrast to the findings for WT mice (22). Accordingly, we reasoned that IL-1Ra KO mice might be a suitable model animal for studies of experimental periodontitis.

In the present study, we induced experimental periodontitis with *A. actinomycetemcomitans* in IL-1Ra KO and WT mice. Using this murine model, we have replicated features typically associated

with *A. actinomycetemcomitans* in AgP. These features include rapid periodontal bone loss (Fig. 2 and 3), a potent immune response to the serotype-specific CPA (Fig. 1), and elevated proinflammatory cytokine production (Fig. 7), which is related to the inherited lack of ability to produce IL-1Ra. Thus, this murine model illustrates how a genetic defect in cytokine regulation can lead to the phenotype seen in human AgP. Moreover, these relationships suggest that AgP patients may be an interesting population to treat with IL-1Ra. Our group has purified and determined the structure of the CPA from *A. actinomycetemcomitans* (30), and this antigen is serotype specific, so in the present study, this antigen was used for the measurement of titers of antibody against *A.*

FIG 9 Assay of mineralized matrix formation in osteoclasts from IL-1Ra KO and WT mice. IL-1Ra KO and WT mouse osteoblasts were cultured for 1, 2, and 3 weeks in an osteogenic medium and were stained with Alizarin Red. After 3 weeks, there was evidence of staining in WT mouse osteoblasts, whereas osteoblasts from IL-1Ra KO mice remained unstained. The experiments were repeated twice with similar results.

actinomycetemcomitans in IL-1Ra KO and WT mice. Titers of antibody against *A. actinomycetemcomitans* in IL-1Ra KO mice were markedly higher than those in WT mice. It has been reported that IL-1Ra KO mice have enhanced T cell-dependent antibody production through induction of CD40 ligand and OX40 on T cells (53), and IL-1Ra inhibited the production of IgG2, which dominates the responses to carbohydrates from periodontal pathogenic bacteria, by peripheral blood mononuclear cells from healthy subjects stimulated with pokeweed mitogen (54). This may be related to the enhanced *A. actinomycetemcomitans*-specific antibody titers in IL-1Ra KO mice (Fig. 1). Although titers of antibody against *A. actinomycetemcomitans*-infected WT mice did tend to be slightly increased, they were not significantly different from those for the control group of uninfected WT mice. Further analysis also revealed gingival junctional epithelium attachment loss, inflammatory cell infiltration in connective tissue, and a significantly increased number of multinucleated TRAP-positive cells on alveolar bone surfaces of the second molar interradicular septum in IL-1Ra KO mice (Fig. 4 and 5). These data provide substantial support for the concept that IL-1Ra minimizes periodontal disease progression and inflammatory bone resorption in experimental periodontitis induced by a periodontal pathogen. We infected IL-1Ra KO mice with *A. actinomycetemcomitans* for the reasons stated above; however, *P. gingivalis* infection is commonly used for studies of bone loss in experimental periodontitis (31, 44, 45). Accordingly, we plan to study *P. gingivalis* in the IL-1Ra KO mouse experimental periodontitis model for comparative purposes.

IL-1 induces osteoclast formation and bone-resorbing activity, and these activities are inhibited by IL-1Ra (23). The expression of IL-1Ra-related genes, such as those encoding RANKL, OPG, and M-CSF, in osteoblasts stimulated with *A. actinomycetemcomitans* has not been investigated previously. RANK is expressed at very high levels on osteoclast precursors and is required for osteoclast differentiation and activation (55). RANK mRNA expression is elevated in IL-1Ra KO mouse bone marrow cells (22). RANKL binds to RANK, to induce bone resorption (56, 57). M-CSF is the most effective colony-stimulating factor in osteoclast formation in normal murine bone marrow (58) and promotes bone resorption in an organ culture system designed to examine osteoclast formation (59). RANKL, M-CSF, TNF- α , and IL-6 mRNA expression in IL-1Ra KO mouse osteoblasts was significantly higher than that in WT mice (Fig. 6). Interestingly, IL-1Ra production was

increased in osteoblasts from WT mice stimulated with *A. actinomycetemcomitans* (Fig. 7). This result suggests that bacterial infection can induce IL-1Ra in an autocrine fashion in WT mice. Serum IL-1 and IL-6 levels were elevated in TNF- α transgenic mice (60), and Hughes et al. demonstrated that IL-1 can stimulate osteoblasts to produce other proinflammatory cytokines, such as IL-6 and TNF- α , and inflammatory mediators, such as prostaglandin E₂ and nitric oxide (61). 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 NF- κ B p65 protein levels were higher in untreated hepatocytes from IL-1Ra KO mice (BALB/c background) than in those from WT mice, although cytosolic NF- κ B p65 protein levels did not differ (62). Hence, an IL-1Ra gene deficiency increases the expression of IL-1 and other inflammatory cytokines through NF- κ B activation, and upregulated inflammatory cytokines enhance RANKL and M-CSF mRNA expression in our experiment as well.

On the other hand, osteoblast differentiation and bone formation are well controlled by bone-associated proteins: the osteoblast-specific transcription factor Runx2 and the osteoblast differentiation markers ALP and OCN/BGP. IL-1 α mRNA expression was elevated in IL-1Ra KO osteoblasts cultured in an osteogenic medium. This is consistent with the findings of Tanabe et al., who reported that ALP activity and calcification in rat osteoblasts decreased, and that BSP mRNA expression decreased slightly, with IL-1 α stimulation (63). It is thought that high levels of IL-1 α in IL-1Ra KO mouse osteoblasts downregulate ALP, OCN/BGP, BSP, and Runx2 mRNA expression, and our results suggest that the aggravation of experimental periodontitis in IL-1Ra KO mice may be due to high levels of the inflammatory cytokines IL-1 α , TNF, and IL-6 permitted by the IL-1Ra gene deficiency, promoting the upregulation of osteoclasts and the downregulation of osteoblast-related gene expression. This dysregulation promotes an imbalance of bone formation and bone resorption. Thus, it appears that IL-1Ra is a very important molecule, in that it helps to control periodontal tissue breakdown. We are eager to investigate the therapeutic effects of IL-1Ra in experimental periodontitis in IL-1Ra KO and other genetically modified mice.

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