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Lack of Toll-like receptor 4 decreases lipopolysaccharide-induced bone resorption in C3H/HeJ mice *in vivo*

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Nakamura H, Fukusaki Y, Yoshimura A, Shiraishi C, Kishimoto M, Kaneko T, Hara Y. Lack of Toll-like receptor 4 decreases lipopolysaccharide-induced bone resorption in C3H/HeJ mice in vivo*.*
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Introduction: Few *in vivo* studies have demonstrated whether Toll-like receptor 4 (TLR4) is indispensable for lipopolysaccharide (LPS)-induced bone resorption and little is known about the receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) expression induced by LPS under conditions of lack of TLR4.
Methods: We compared bone resorption histomorphometrically in C3H/HeN and C3H/HeJ mice that were repeatedly injected with *Actinobacillus actionmycetemcomitans* LPS into their gingiva every 48 h. RANKL-, interleukin-1β- and OPG-positive cells in the connective tissue were also compared immunohistochemically.
Results: Bone resorption in C3H/HeJ mice in the fourth, seventh, and tenth injection groups was significantly less than that C3H/HeN mice ($P < 0.05$). The number of RANKL-positive cells in C3H/HeJ mice in the 10th injection group was significantly smaller than that in C3H/HeN mice ($P < 0.05$). The numbers of interleukin-1β-positive cells in C3H/HeJ mice in the seventh and tenth injection groups were significantly decreased compared with those in C3H/HeN mice ($P < 0.05$). The numbers of OPG-positive cells in C3H/HeN and C3H/HeJ mice gradually increased, but there was no significant difference between the two strains of mice.
Conclusion: TLR4 is indispensable for LPS-induced bone resorption *in vivo*.

Key words: *Actinobacillus actinomycetemcomitans*; bone resorption; immunohistochemical study; lipopolysaccharide; Toll-like receptor 4

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Bone resorption occurs by formation and activation of osteoclasts. Receptor activator of nuclear factor-κB ligand (RANKL), a member of the superfamily of tumor necrosis factors, induces osteoclastic differentiation from hematopoietic precursors and is essential for the formation, function, and survival of osteoclasts (9, 24, 35, 39). In contrast, osteoprotegerin (OPG) is a soluble decoy receptor for RANKL and inhibits the formation, activation, and survival of osteoclasts (2, 23, 24, 34). Recent studies have shown that RANKL expression in patients with periodontitis was greater than in non-periodontitis subjects and that OPG expression was less in patients with periodontitis (4, 25).

Toll-like receptor 4 (TLR4) is a principal signaling receptor for gram-negative bacterial lipopolysaccharides (LPS) (1). We previously showed that the number of TLR4-positive cells in gingiva with severe inflammation was larger than that in gingiva with mild inflammation, suggesting that TLR4 participates in the innate immune response to stimulation by bacterial products in the periodontal pocket (28).

The C3H/HeJ mouse strain is characterized by hyporesponsiveness to LPS because of a single point mutation of an amino acid in the cytoplasmic portion of TLR4 (14). When bone marrow cells from C3H/HeJ mice were stimulated with *Escherichia coli* and *Actinobacillus actinomycetemcomitans* LPS *in vitro*, the number of tartrate-resistant acid phosphatase

(TRAP)-positive multinucleated cells significantly decreased compared to that in bone marrow cells from control normal mice (C3H/HeN) (15). However, there have been few *in vivo* studies aimed at determining whether TLR4 is indispensable for the LPS-induced bone resorption. Furthermore, little is known about RANKL and OPG expression induced by LPS in the absence of TLR4. Therefore, in the present study, we compared histopathological changes in C3H/HeN mice and C3H/HeJ mice that were injected with *A. actinomycetemcomitans* LPS into their gingiva. Furthermore, RANKL-, interleukin-1β- (IL-1β) and OPG-positive cells in connective tissue were immunohistochemically compared. The results showed that TLR4 is indispensable for LPS-induced bone resorption *in vivo*.

Material and methods

Experimental animals

Twenty-five 7-week-old male C3H/HeJ mice (LPS-non-responsive) and twenty-five 7-week-old male C3H/HeN mice (LPS-responsive) were used in this study.

Purification of LPS

A. actinomycetemcomitans Y4 LPS was prepared as previously described (30). Briefly, *A. actinomycetemcomitans* Y4 in Todd–Hewitt broth supplemented with 1% yeast extract, 5 μg/ml hemin, and 5 μg/ml vitamin K3 was cultured at 37°C under anaerobic conditions (10% H_2, 10% CO_2, 80% N_2) for 3 days. The cells were then sedimented by centrifugation, washed with pyrogen-free water three times, and freeze-dried.

Crude LPS was extracted according to a slight modification of the hot phenol–water procedure (38). Briefly, lyophilized organisms were suspended in 110 ml pyrogen-free water and 88 ml phenol. The mixture was stirred vigorously at 68°C for 20 min, cooled on ice, and then separated by centrifugation at 7000 ***g*** for 20 min. The aqueous phase was removed and insoluble precipitates were re-extracted twice with 110 ml water. The combined aqueous phase was dialyzed against distilled water at 4°C, neutralized with NaOH, and lyophilized.

Crude LPS was ultracentrifuged and digested with nuclease P1 and pronase. The digest was suspended in 100 ml pyrogen-free water and centrifuged at 100,000 ***g*** for 3 h. The pellet was suspended in 10 ml 10 mM Tris buffer (pH 7.4) containing 0.1 mM $ZnCl_2$ and 400 μg nuclease P1. This suspension was incubated at 37°C for 16 h and dialyzed against distilled water at 4°C. The dialyzate was centrifuged twice, and then the pellet was washed with pyrogen-free water and lyophilized. Freeze-dried LPS was suspended in 10 ml 0.1 M borate buffer (pH 7.4) containing 2 mM $CaCl_2$ and 1 mg pronase. The mixture was incubated at 37°C for 24 h, and then heated at 100°C for 5 min, followed by centrifugation at 5000 ***g*** for 10 min. The supernatant was dialyzed against distilled water, neutralized with NaOH, and lyophilized.

The chemical content of purified LPS from *A. actinomycetemcomitans* was expressed as per cent dry weight. The carbohydrate ratio of LPS was 34.4%. LPS was composed of 2.9% heptose, 28.2% hexose, 3.0% hexosamine, and 0.3% 2-keto-3 deoxyoctonate. No protein or nucleic acid was detected and the *Limulus* activity of LPS was 2.51 EU/ng.

Preparation of tissues

Bone resorption was induced by the method used in our previous studies (17, 22, 29, 30). Five micrograms *A. actinomycetemcomitans* LPS in 3 μl phosphate-buffered saline (PBS) was injected using a microsyringe (30 G) every 48 h into the mesial gingival tissue of the first molar of the left mandible of C3H/HeJ or C3H/HeN mice under ether anesthesia. In addition, PBS was injected in the same way as in controls. Groups of five mice were sacrificed 24 h after the first, fourth, seventh, and tenth injections of LPS (Fig. 1). After sacrifice, the left mandible of each mouse was removed and fixed in 4% paraformaldehyde in PBS at 4°C for 6 h, decalcified with 10% ethylenediaminetetraacetic acid for 1 week, and then embedded in paraffin using the AMeX method (33). Specimens were fixed in acetone at –20°C overnight, dehydrated in acetone at 4°C for 15 min and then at room temperature for 15 min, cleared in methyl benzoate for 30 min and then in xylene for 30 min, and then embedded in paraffin for 2 h. Serial sections of 4-μm thickness were prepared to examine the mesio-distal section of the left first molar.

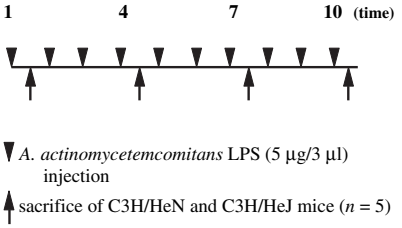

Fig. 1. Experimental schedule. Mice were injected with 5 μg/3 μl LPS every 48 h and sacrificed 24 h after the first, fourth, seventh, and tenth injections of LPS.

Histopathological and histometrical examinations

Ten groups of serial sections, each group containing five subsections, were obtained from each specimen after receipt of a specified number of injections of a given composition. The first subsections from each group of serial sections were stained with hematoxylin & eosin (H&E) to observe the surface of alveolar bone histopathologically.

To examine osteoclasts, the second subsections from each group were stained with TRAP according to the procedure described by Katayama et al. (18). Briefly, a staining solution was made by mixing 0.5 ml pararosanilin solution (1 g pararosanilin in 20 ml distilled water and 5 ml concentrated hydrochloric acid), 0.5 ml 4% sodium nitrite solution, 10 ml 0.1 M acetate buffer pH 5.0, and 10 mg naphthol AS-BI phosphate (Sigma, St. Louis, MO), dissolved in 8 ml distilled water. The mixture was adjusted to pH 5.0 using concentrated NaOH and filtered through No.1 Whatman filter paper. Furthermore, after adding 150 mg L(+)-tartaric acid to a 10-ml aliquot of the solution to a final tartrate concentration of 0.1 M, the solution was adjusted to pH 5.0 with concentrated NaOH. After incubating the second subsections within the stain solution for 30 min at 37°C, they were counterstained with hematoxylin.

Osteoclasts were identified as multinucleated TRAP-positive cells on the bone surface. Quantitative evaluation of the total bone resorption was difficult so the percentage of bone surface (regardless of the presence or absence of resorption lacunae) in intimate contact with osteoclasts (active resorption surface; ARS) was estimated to give a quantitative evaluation of the progression of bone resorption. After counting the number of points of intersection of the bone surface with the line of a micrometer (Olympus, Tokyo, Japan) in 25-μm graduations at 400× magnification, the rate of ARS to total points of intersection was calculated (Fig. 2).

Immunohistological staining and histometric study of RANKL-, IL-1β-, and OPG-positive cells

The third, fourth, and fifth subsections were used for the immunohistological staining of RANKL-, IL-1β-, and OPG-

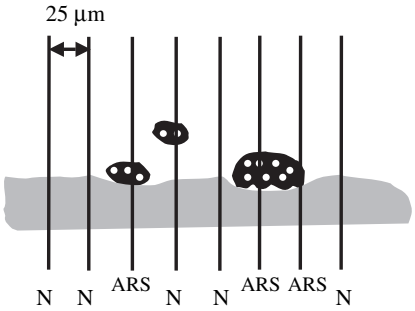

Fig. 2. Histomorphometrical method of bone resorption. Bone surfaces, regardless of the presence or absence of resorption lacunae, in contact with TRAP-positive multinucleated cells were defined as sites of active resorption surface (ARS); others sites (N). Percentage of ARS was derived by dividing the number of sites of ARS by all intersecting points between bone surfaces within a 25-μm scale.

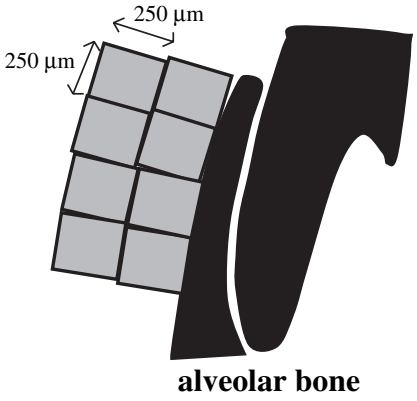

Fig. 3. Immunohistometric analysis of IL-1β-, RANKL-, and OPG-positive cells per unit area. The number of positive cells for each unit area (250 μm^2) was counted. Average of positive cells per unit area was calculated for each section.

bearing cells. Serial subsections were deparaffinized and incubated with 0.1% trypsin for 10 min for unmasking. Endogenous peroxidase activity was blocked by 3% H_2O_2/methanol (for RANKL) and 0.3% H_2O_2/methanol (for IL-1β and OPG) for 20 min. Non-specific immunoglobulin binding was blocked with 500 μg/ml normal rabbit immunoglobulin G (IgG; Sigma, St Louis, MO) for RANKL and IL-1β and with 500 μg/ml normal goat IgG (Sigma) for OPG. The sections were incubated with a primary goat antimouse RANKL polyclonal antibody at a dilution of 1 : 50 (Santa Cruz Biotechnology, Santa Cruz, CA), a goat antimouse IL-1β polyclonal antibody at a dilution of 1 : 100 (R&D Systems, Minneapolis, MN), or a rabbit antimouse OPG polyclonal antibody at a dilution of 1 : 100 (Santa Cruz Biotechnology) overnight at 4°C. On the following day, RANKL and IL-1β were detected by incubating the sections with biotinylated rabbit antigoat IgG (Dako, Carpinteria, CA) for 30 min. Similarly, OPG was detected by incubating the sections with biotinylated antirabbit IgG (Dako) for 30 min. After incubation with streptavidin conjugated to horseradish peroxidase for 10 min, enzyme activity was detected by diaminobenzidine tetrahydrochloride deposition. The sections were counterstained with hematoxylin. Negative controls containing preimmune IgG from the same animal species as the primary antibody ruled out non-specific binding. Using this immunohistological staining, the number of positive cells per 250 μm^2 field (unit area) within 500 μm of the connective tissues from the anterior edge of the periodontal bone was counted in eight fields of five sections per block (Fig. 3). The average number of positive cells was calculated in each unit area.

Statistical analysis

Averaged data of the percentage of ARS as well as immunohistological findings from the injected C3H/HeJ and C3H/HeN mice were obtained from 10 sections of five specimens. Significant differences were analyzed using one-factor analysis of variance and Fisher's protected least significant difference (PLSD) *t*-test. Significance was established at $P < 0.05$.

Results

Increasing the LPS injection times in both mouse strains resulted in strong inflammatory cell infiltration (Fig. 4). On the other hand, PBS injections induced no inflammation.

No TRAP-positive osteoclasts were observed in control mice. They appeared from the fourth injection and gradually increased from the seventh to the tenth injections in C3H/HeN mice. The osteoblast lining on the bone surface was partially interrupted by resorption lacunae with many TRAP-positive osteoclasts in C3H/HeN mice by the 10th injection. In contrast, there were few TRAP-positive osteoclasts in any of the C3H/HeJ mouse groups (Fig. 5). The rate of ARS increased in C3H/HeN mice with increase in LPS injection times, but there was no distinct change in C3H/HeJ mice. The rates of ARS of C3H/HeJ mice in the fourth, seventh and tenth injection groups were significantly lower than those of C3H/HeN mice ($P < 0.05$) (Table 1).

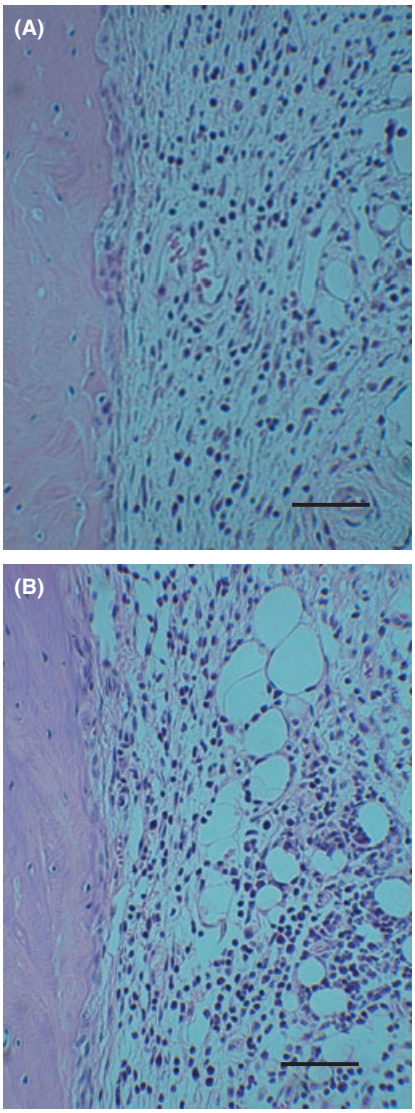

Fig. 4. Histopathological findings after the 10th injection of *Actinobacillus actinomycetemcomitans* LPS in C3H/HeN mice (A) and C3H/HeJ mice (B). There was no difference in inflammatory cell infiltration in the two groups of mice (H&E staining, scale bar = 50 μm).

No RANKL-positive cells were observed in the connective tissue of controls. In LPS-injected groups, they were present in the gingival connective tissue near the anterior edge of the alveolar bone, and they seemed to be fibroblasts, lymphocytes, and endothelial cells based on their histological features. RANKL-positive cells of both mice gradually increased from the fourth injection, peaking at the seventh injection. The number of RANKL-positive cells in the 10th injection group of C3H/HeN mice was significantly higher than that of C3H/HeJ mice ($P < 0.05$) (Table 2).

IL-1β-positive cells were present in the gingival connective tissue away from the

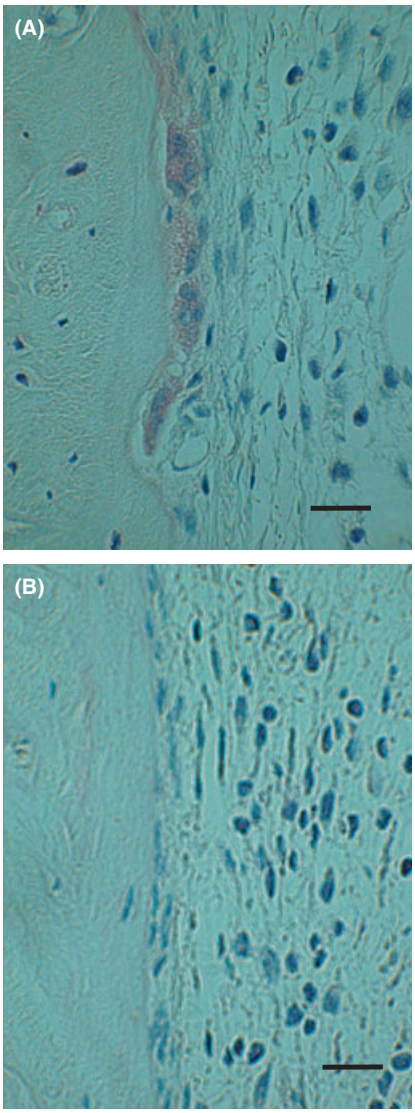

Fig. 5. TRAP-positive cells after the 10th injection of *Actinobacillus actinomycetemcomitans* LPS in C3H/HeN mice (A) and C3H/HeJ mice (B). Osteoclasts were present on the irregular bone surface in C3H/HeN mice, but there were few TRAP-positive osteoclasts in C3H/HeJ mice (TRAP staining, bar = 25 μm).

Table 1. Ratio of active resorption surface (ARS) (%; mean ± SD)

	C3H/HeN	C3H/HeJ
First injection	0.0 ± 0.0	0.14 ± 0.32
Fourth injection	8.85 ± 6.99	0.95 ± 1.42*
Seventh injection	25.89 ± 9.54	3.37 ± 2.33*
Tenth injection	39.12 ± 11.66	2.01 ± 2.08*

*$P < 0.05$.

anterior edge of alveolar bone, and they seemed to be macrophages, neutrophils, fibroblasts, and endothelial cells based on their histological features. The number of IL-1β-positive cells in C3H/HeN mice increased with the increase in LPS injection times, but there was no distinct change in C3H/HeJ mice. The numbers of IL-1β-positive cells in C3H/HeN mice in the seventh and tenth injection groups were significantly elevated compared with those in C3H/HeJ mice ($P < 0.05$) (Table 3).

Table 2. Average number of RANKL positive-cells for each unit area (mean ± SD)

	C3H/HeN	C3H/HeJ
First injection	1.27 ± 0.35	2.18 ± 1.16
Fourth injection	4.28 ± 1.7	4.89 ± 1.39
Seventh injection	14.57 ± 6.16	11.11 ± 6.13
Tenth injection	12.97 ± 5.19	5.65 ± 2.69*

*$P < 0.05$.

The numbers of OPG-positive cells in C3H/HeN and C3H/HeJ mice gradually increased and there was no significant difference between the mouse groups (Table 4). No OPG-positive cells were observed in the connective tissue of control mice.

Discussion

Adult periodontitis is a chronic inflammatory disease initiated by specific subgingival microorganisms, including *Porphyromonas gingivalis* and *A. actinomycetemcomitans* and many other gram-negative anaerobic rods (10). These gram-negative bacteria possess LPS on their cell surface, and LPS regulates various biological activities, such as cytokine production and bone resorption. LPS is recognized by a receptor complex consisting of CD14, TLR4, and MD-2 (8, 32). However, few *in vivo* studies have demonstrated that TLR4 is indispensable for LPS-induced bone resorption. In the present study, bone resorption gradually increased with an increase in the LPS injection times in C3H/HeN mice. On the other hand, bone resorption was not observed in C3H/HeJ mice throughout the experimental period. There was a significant difference between ARS in C3H/HeJ mice and that in C3H/HeN mice. These results clearly demonstrated that TLR4 plays an important role in LPS-induced bone resorption *in vivo*.

Table 3. Average number of IL-1β-positive cells for each unit area (mean ± SD)

	C3H/HeN	C3H/HeJ
First injection	1.46 ± 2.88	0.27 ± 0.49
Fourth injection	2.85 ± 2.96	0.76 ± 0.41
Seventh injection	1.55 ± 0.83	0.0 ± 0.02*
Tenth injection	6.04 ± 2.92	1.4 ± 1.73*

*$P < 0.05$.

Table 4. Average number of OPG-positive cells for each unit area (mean ± SD)

	C3H/HeN	C3H/HeJ
First injection	1.47 ± 1.31	3.59 ± 3.85
Fourth injection	11.39 ± 9.77	2.79 ± 1.37
Seventh injection	25.95 ± 15.52	14.35 ± 5.81
Tenth injection	16.47 ± 6.91	20.83 ± 19.85

RANKL induces the differentiation of osteoclast precursors into osteoclasts through RANKL–RANK interaction (5, 21). In contrast, OPG inhibits osteoclastogenesis and bone resorption as a decoy receptor for RANKL (7, 26). In the present study, the number of RANKL-positive cells in C3H/HeJ mice in the 10th injection group was significantly decreased compared to that in C3H/HeN mice ($P < 0.05$). The number of IL-1β-positive cells in C3H/HeJ mice in the seventh and the tenth injection groups were also significantly decreased compared to those in C3H/HeN mice ($P < 0.05$). These results suggested that the lack of an LPS–TLR4 signal pathway in C3H/HeJ mice led to decreases in RANKL expression and in production of cytokines such as IL-1β. However, there was a significant difference in ARS between the two strains of mice in the fourth injection group, although no significant difference in numbers of RANKL- and IL-1β-positive cells was found. These findings suggest that osteoclast-activating factors other than RANKL act in the early period of LPS-induced bone resorption. For example, tumor necrosis factor-α (TNF-α) can stimulate osteoclast differentiation through a mechanism independent of the RANKL–RANK system (20), while immunohistological detection of TNF-α expression was methodologically difficult in this study.

Osteoclasts spontaneously die as a result of apoptosis. A previous study showed that the number of purified osteoclasts decreased in a time-dependent manner and that LPS, as well as RANKL, significantly reduced the spontaneous apoptosis of osteoclasts (16). Apoptosis was not observed in either of the groups of mice in the present study despite the fact that the TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) method was used to detect osteoclast apoptosis (data not shown).

TLR4 is expressed in bone marrow macrophages and osteoclasts (16). Several studies have shown that LPS activated macrophages via TLR4 to produce TNF-α, IL-1, and prostaglandin E_2, which induced RANKL expression on osteoblasts (13, 31). Furthermore, macrophages activate T cells and B cells to produce RANKL (12, 37). In addition, it has been demonstrated that the expression of OPG messenger

RNA in osteoblasts was enhanced by LPS stimulation (36) and that IL-1β and TNF-α elevated OPG expression in human microvascular endothelial cells (3). In the present study, the numbers of RANKL- and OPG-positive cells in the first, fourth, and seventh LPS-injection groups showed no significant difference between the two strains of mice, whereas the numbers of those cells gradually increased with the increase in injection times and only a few positive cells were observed in the control group. These results seem to conflict with the lack of TLR4. Although TLR4 is probably the main LPS signaling receptor, other receptors such as macrophage scavenger receptor and CD11b/CD18 are able to recognize LPS in the absence of TLR4 and CD14, the major LPS-binding molecule (6, 11, 19, 27). It is likely that such receptors are also involved in mediating TLR4-independent signal transduction and RANKL and OPG expression.

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