

ORIGINAL ARTICLE

Th17-cells in atopic dermatitis stimulate orthodontic root resorption

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OBJECTIVE: The aim of this study was to investigate how atopic dermatitis (AD) contributes to root resorption during orthodontic tooth movement.

MATERIALS AND METHODS: Atopic dermatitis model mice and wild-type mice were subjected to an excessive orthodontic force (OF) to induce movement of the upper first molars. The expression levels of the tartrate-resistant acid phosphatase (TRAP), IL-17, IL-6, and RANKL proteins were determined in the periodontal ligament (PDL) by an immunohistochemical analysis. Furthermore, the effects of the compression force on co-cultures of CD4⁺ cells from AD patients or healthy individuals and human PDL cells were investigated with regard to the levels of secretion and mRNA expression of IL-17, IL-6, RANKL, and osteoprotegerin.

RESULTS: The immunoreactivities for TRAP, IL-17, IL-6, and RANKL in the AD group were found to be significantly increased. The double immunofluorescence analysis for IL-17/CD4 detected immunoreaction. The secretion of IL-17, IL-6, and RANKL, and the mRNA levels of IL-6 and RANKL in the AD patients were increased compared with those in healthy individuals.

CONCLUSION: Th17 cells may therefore be associated with the deterioration of root resorption of AD mice, and may explain why AD patients are more susceptible to root resorption than healthy individuals when an excessive OF is applied.

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Keywords: T-helper 17 cells; IL-17; receptor activator of NF- κ B ligand; root resorption; atopic dermatitis

Introduction

The significance of receptor activator of NF- κ B ligand (RANKL) (Lacey *et al*, 1998; Yasuda *et al*, 1998), an

osteoclast differentiation factor which plays a central role in regulating osteoclast differentiation and bone metabolism by immune cells has recently been demonstrated to at least partially underlie the inflammation leading to bone destruction in rheumatoid arthritis (RA). Arron and Choi (2000) discovered this molecule during their immunological research, and used the term 'osteimmunology' for the first time in the journal *Nature* to define their research.

At the bone destruction sites of RA patients, many multinucleated giant cells are observed. These cells show an osteoclast phenotype (i.e., being positive for tartrate-resistant acid phosphatase (TRAP) staining), suggesting that osteoclasts play an important role in bone destruction due to RA. Additionally, Kotake *et al* (1999) detected IL-17 in the synovial fluids of RA patients, and noted that it served as a cytokine to promote osteoclast differentiation. They also reported IL-17-producing Th17 cells as a new Th-cell subset (Harrington *et al*, 2005; Park *et al*, 2005).

The TRAP-positive odontoclasts observed on the tooth root surface are involved in root resorption that develops during orthodontic treatment. Root resorption is an incidental symptom, in which alveolar bone replaces the root resorption site. According to an epidemiological study by Kaley and Phillips (1991), the roots of all patients who underwent comprehensive orthodontic treatment presented with shortening of the roots, and 3% of all patients with severe root resorption (shortening by more than one-quarter of the root length) presented with root shortening in the maxillary central incisors. This may be caused by the application of an excessive orthodontic force (OF) (Chan and Darendeliler, 2005), long-term treatment (Reitan, 1974), morphological abnormalities of the roots (Tagaya *et al*, 1998), or genetic factors (Al-Qawasmi *et al*, 2006). Nishioka *et al* (2006) reported that many Japanese patients with asthma or allergy presented with severe root resorption, suggesting that the immune system is involved in the development of root resorption during orthodontic treatment. Hayashi *et al* (2012) reported that immunoreactivity for Th17, IL-17, IL-17R, and IL-6 was detected in the resorbed root in periodontal ligament (PDL) tissue of rats subjected to an OF on day seven. Moreover, IL-17 stimulated osteoclastogenesis from human osteoclast precursor

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cells *in vitro*. These results suggested that Th17 cells may aggravate the process of orthodontically induced inflammatory root resorption.

According to a recent report, when peripheral Th17 cells proliferate, they exacerbate the symptoms of patients with atopic dermatitis (AD) (Koga *et al*, 2008). AD is one of the most common skin diseases and is characterized by chronic and recurrent eczema with severe itching. According to a survey of AD in Japan between 2000 and 2002 performed by a study group from the Ministry of Health, Labour and Welfare, the prevalence of AD was 12.8% in 4-month-old infants, 9.8% in 18-month-old children, 13.2% in 3-year-old children, 11.8% in 6-year-old children, 10.6% in 12-year-old children, and 8.2% in 18-year-old adolescents.

Thus, we hypothesized that AD might be involved in the development or exacerbation of severe root resorption. In the present study, TRAP assays and immunohistochemical staining with anti-IL-6, -RANKL, and -IL-17 antibodies were conducted on the pressure sides of the PDL of normal and AD mice using a mouse model of root resorption. Additionally, immunofluorescence staining was conducted to examine the presence of Th17 on the pressure side of the PDL. Furthermore, the IL-17 secretion was examined in a co-culture system employing the PDL and CD4⁺ cells from patients with AD or from healthy individuals to examine the relationship between root resorption and AD. Moreover, the IL-6, RANKL, and osteoprotegerin (OPG) mRNA expression levels and IL-6, RANKL, and OPG secretion were determined in these cultures.

Materials and methods

Animal studies

Animals. Atopic dermatitis (Nc/Nga) and wild-type (BALB/c) mice were purchased from the Sankyo Laboratory (Sankyo Labo Service, Inc., Tokyo, Japan; body weight 20 ± 5 g). Eight-week-old mice were used in the experiments. Fifteen male AD and 15 male wild-type mice were used for the experiments. For each strain, mice were randomly assigned into two groups; control group, where mice received no appliances, and OF group, where mice subjected to the excessive OF. Mice per strain in each group were as follows: AD (control = 7, OF = 8), and wild type (control = 7, OF = 8).

Animals were maintained in the animal center of Nihon University School of Dentistry at Matsudo in separate cages in a 12-h light/dark environment at a constant temperature of 23°C and were provided with food and water *ad libitum*. The health status of each mouse was evaluated by daily body weight monitoring. The animal experimental protocol used for this study was approved by the Ethics Committee for Animal Experiments at Nihon University School of Dentistry at Matsudo (approval No. AP11MD015).

Induction of dermatitis in the AD group. Atopic dermatitis was induced using the method reported by Yamamoto *et al* (2007). On the first induction (day 0), the rostral part of the back skin of the mice was clipped using an electric clipper, and residual hair was depilated using a hair removal cream under ether anesthesia. Antigen challenge was performed by topical application of 100 mg of

dermatophagoides farina body ointment (Dfb ointment; Biostir, Inc., Kobe, Japan) on the dorsal shaved skin. From the second induction on, after the removal of growing hair using an electric clipper and an electric shaver, barrier disruption was achieved by 150 μ l of 4% sodium dodecyl sulfate treatment on the shaved dorsal skin 3 h before Dfb ointment application. These procedures were repeated twice a week. Following the last antigen challenge on day 17, then mice were used for experiments.

Measurement of the serum levels of IgE and IL-17.

Spontaneously induced allergic responses were monitored by measuring the serum levels of IgE and IL-17. Blood was collected from the retroorbital plexus with glass capillary tubes before orthodontic tooth movement, and separated serum samples were stored at -80°C until use. The serum levels of mouse IgE and IL-17 were measured by an IgE ELISA kit (Shibayagi, Gunma, Japan) and an IL-17 ELISA kit (R&D Systems Co., Minneapolis, MN, USA) according to the manufacturer's protocols.

Application of orthodontic devices. The animals were anesthetized with thiamylal sodium (15 mg kg^{-1} body weight) for the application of the orthodontic devices. Experimental tooth movement was induced using the method reported by Yoshimatsu *et al* (2006) with a Nickel-Titanium (Ni-Ti) closed-coil spring (Tomy International, Inc., Fukushima, Japan) inserted between the upper incisors and the upper-left first molar, and the device was fixed with a 0.008-inch stainless steel ligature wire (Tomy International, Inc., Tokyo, Japan) around both teeth by means of a dental adhesive agent (Transbond XT; 3M Unitek, Monrovia, CA, USA). We used the left maxillary molar in each mouse to study the experimental tooth movement. The upper first molar was mesially moved by the closed-coil spring with a force of 25 g, which was based on the report by Al-Qawasmi *et al* (2006) (Figure 1). The force was applied for 9 days.

Tissue preparation. The experimental time point was set at 9 days after the initiation of tooth movement. The animals were deeply anesthetized using thiamylal sodium, and then were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. The maxilla was then immediately dissected and immersed in the same fixative for 18 h at 4°C . The specimens were decalcified in a 10% disodium ethylenediamine tetraacetic acid (EDTA, pH 7.4) solution for 3 weeks, and the decalcified specimens were dehydrated through a graded ethanol series and embedded in paraffin using the usual methods for preparation. Each sample was sliced into $4 \mu\text{m}$ continuous sections in the sagittal direction, and then was prepared for hematoxylin and eosin staining (H&E), and also for immunohistochemical staining. The PDL tissues in the mesial part of the distal buccal root of a first upper molar were observed. The sites of observation were based on the report by Yoshimatsu *et al* (2006). The root length between the bifurcation surface and the apical end of a mouse first molar approximately $600 \mu\text{m}$. There suggests that approximately $300 \mu\text{m}$ of the root from the bifurcation surface at the mesial side is the pressure side

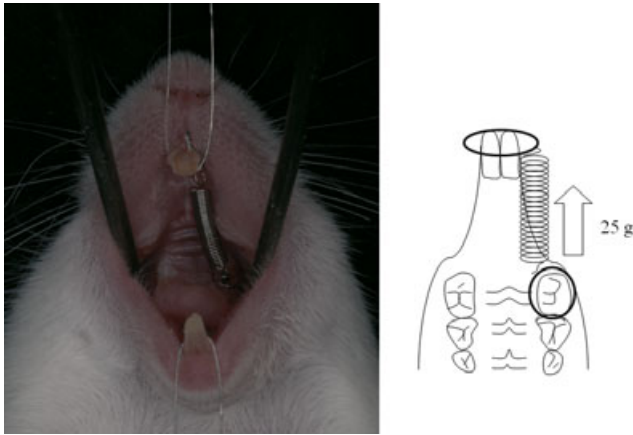


Figure 1 Experimental tooth movement was induced by a Nickel-Titanium closed-coil spring (Tomy International, Inc., Fukushima) inserted between the upper incisor and the upper-right first molar, which was fixed with a 0.008-inch stainless steel ligature wire (Tomy International, Inc., Tokyo) around both teeth by means of a dental adhesive agent (Transbond XT; 3M Unitek). The upper first molar was moved mesially by the closed-coil spring with a force of 25 g. The force was applied for 9 days

during tooth movement. In addition, the alveolar crest is about 80 μm away from the bifurcation surface. Thus, horizontal sections from five levels of the root: 100, 140, 180, 220, and 260 μm away from the bifurcation surface were prepared. These sections were used to count the number of each immunohistochemical staining. The ratio of positive cells/all cells was expressed as the mean from the five sections obtained from the five levels of the root. ($n = 15$ sections per group).

Immunohistochemistry. Immunohistochemical staining was performed as follows: The tissue sections were deparaffinized, and the endogenous peroxidase activities were quenched by incubation in 3% H_2O_2 in methanol for 30 min at room temperature. After washing them in tris-buffered saline (TBS), the sections were incubated with a polyclonal anti-TRAP antibody (Santa Cruz Biotechnology, Inc., CA, USA; working dilution, 1:100), polyclonal anti-IL-17 antibody (Biolegend, San Diego, CA, USA; working dilution, 1:100), polyclonal anti-IL-6 antibody (Santa Cruz Biotechnology; working dilution, 1:200), or polyclonal anti-RANKL antibody (Santa Cruz Biotechnology; working dilution, 1:100) for 18 h at 4°C. The Histofine Simple Stain MAX-Po (G) kit (Nichirei, Co., Tokyo, Japan) was used for the staining of TRAP, IL-6, and RANKL, and the MAX-Po (R) kit (Nichirei, Co.) was used for IL-17, according to the manufacturer's protocols. The sections were rinsed with TBS, and the final color reactions were performed using the 3, 3'-diaminobenzidine tetra-hydrochloride substrate reagent, and the sections were then counterstained with hematoxylin. As immunohistochemical controls, several sections were incubated with 0.01 M phosphate-buffered saline (PBS) instead of the primary antibody.

Double immunofluorescence staining. For double immunofluorescence staining, the sections were deparaffinized and washed in TBS, and then were incubated with a polyclonal anti-rabbit CD4 antibody

(Assay Biotechnology, Inc., Sunnyvale, CA, USA; working dilution, 1:100) for 18 h at 4°C. Subsequently, CD4 staining was performed using Alexa fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA; working dilution, 1:100) for 1 h at RT. The sections were then incubated with a polyclonal anti-rat IL-17 antibody (Biolegend; working dilution, 1:100) for 1 h at RT, and IL-17 staining was performed using Alexa fluor 568-conjugated goat anti-rat IgG (Invitrogen; working dilution, 1:100). After washing the sections with TBS, all were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

In vitro studies

Patients. Human CD4^+ T cells were isolated from the peripheral blood of 30 volunteers. Peripheral blood was obtained from 15 AD patients (eight males and seven females; 20–30 years of age) and 15 healthy individuals (eight males and seven females; 20–30 years of age). The AD patients were diagnosed with AD according to the guidelines for the management of AD reported by Saeki *et al*. The protocol for the human experiments was reviewed and approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo (EC 11-022), and informed consent was obtained from each individual.

Culture of human periodontal ligament (hPDL) cells.

Human periodontal ligament cells were prepared according to a modification of the method reported by Somerman *et al* (1988). Briefly, hPDL tissues were taken from the roots of premolars extracted from six healthy young volunteers (three males and three females; 14–16 years of age). The hPDL tissue specimens were placed in 35-mm tissue culture dishes and covered with a sterilized glass coverslip. The cells were maintained in α -MEM medium (Wako, Osaka, Japan) supplemented with 100 $\mu\text{g ml}^{-1}$ of penicillin-G (Sigma Chemical Co., St. Louis, MO, USA), 50 $\mu\text{g ml}^{-1}$ of gentamicin sulfate (Sigma), 0.3 $\mu\text{g ml}^{-1}$ of amphotericin B (Flow Laboratories; McLean, VA, USA), and 10% fetal calf serum (FCS) (Cell Culture Laboratories, Cleveland, OH, USA). The cultures were kept at 37°C in a humidified incubator (Forma CO₂ incubator MIP-3326; Sanyo Electric Medica System Co., Tokyo, Japan) in the presence of 95% air and 5% CO₂. The medium was changed twice a week. When the cells growing from each explant reached confluence, they were detached with 0.05% trypsin (Gibco, Grand Island, NY, USA) in PBS for 10 min, and then were subcultured in culture flasks. The cells that were still attached to the bottom of the flask were discarded to avoid contamination by epithelial cells.

Isolation of CD4^+ T cells. Isolation of pure CD4^+ T cells from human peripheral blood for the chimerism analyses was performed using Whole Blood MicroBeads (Miltenyi Biotec Miltenyi Biotec Inc., Auburn, CA, USA) and the autoMACS device (Miltenyi Biotec). Labeled leukocyte subpopulations were automatically isolated using the POSSELD2 software program (Miltenyi Biotec). The results were regarded as reliable if the purity exceeded 99%.

Co-culture of human CD4⁺ T cells and hPDL cells. The hPDL cells were seeded at passages 6–9 onto 96-well plates (1.5×10^4 cells per well), and cultured in α -MEM, containing 10% FBS, in the presence of 5% CO₂ at 37°C for 1 day. These cells were co-cultured with freshly prepared human CD4⁺ T cells (1.5×10^5 cells per well) in α -MEM containing 1% FCS.

Application of the compressive force. To reproduce the conditions of pressure during orthodontic tooth movement, we performed the following *in vitro* experiments, as described by Nakajima *et al* (2008). HPDL cells were continuously compressed using a uniform compression method as a model of pressure at the site of orthodontic movement. Static compressive force (CF) is thought to mimic that found *in vivo* during orthodontic treatments. In the present experiments, the cells were stimulated once. Briefly, a cell disk was placed over nearly confluent cell layers in the dishes, on top of which was placed a glass cylinder. The CF was then controlled by placing lead granules in the cylinder. Before the application of the CF, the cells were pre-incubated for 1 h in culture medium containing 1% FCS, after which they were subjected to a CF of 4.0 g cm^{-2} for 12 h. Previous studies have shown that compressive mechanical stress can also be applied by the system utilized in the present experiment (Yamaguchi *et al*, 2006). Control cells were covered with thin glass plates without lead weights, which produced a CF of 0.032 g cm^{-2} . The possibility that removal of the glass plates might cause static electricity, which could affect the cells, was controlled by the use of glass plates in both the CF and control groups.

Real-time polymerase chain reaction (PCR). Total RNA was isolated from cultured hPDL cells using a commercially available kit (RNeasy Plus Micro kit; Qiagen Co., Tokyo, Japan) following the manufacturer's protocol. Aliquots containing equal amounts of mRNA were subjected to real-time PCR. The total RNA was converted to cDNA using a PrimeScript RT reagent kit (TaKaRa Co., Shiga, Japan). Real-time PCR amplification was performed using SYBR Premix Ex Taq (TaKaRa Co.) in a thermal cycler (TP-800 Thermal Cycler Dice; TaKaRa), according to the manufacturer's protocol. The real-time PCR conditions were 95°C for 3 s and 60°C for 30 s for 40 cycles after a hot start, and measurements were taken at the end of the annealing step at 60°C during each cycle. PCR primers for IL-6, IL-8 and GAPDH were purchased from Takara Co. and were designed with reference to the respective cDNA sequences. The primers used were as follows:

IL-6	5'-AAGCCAGAGCTGTGCAGATGAGTA-3' 5'-TGTCCTGCAGCCACTGGTTC-3'
RANKL	5'-ATCGTTGGATCACAGCACATCAG-3' 5'-GGATGTCGGTGGCATTAAATAGTGAG-3'
OPG	5'-AGCTGCAGTACGTCAAGCAGGA-3' 5'-TTTGCAAACGTATTTCGCTCTGG-3'
GAPDH	5'-GCACCGTCAAGGCTGAGAAC-3' 5'-TGTTGAAGACGCCAGTGA-3'

The data were analyzed using the Smart Cycler software program (Multiplate RQ version 1.00; TaKaRa Co.), and the levels of the real-time PCR products corresponding to GAPDH were confirmed to be the same in the various experimental groups. Therefore, it was considered acceptable to assume that the amount of real-time PCR products reflected the level of each mRNA.

Enzyme-linked immunosorbent assay (ELISA). The RANKL, OPG, IL-17, and IL-6 released into the culture supernatants were measured using a Human IL-6 ELISA kit (R&D Systems Co.), a Human IL-17 ELISA kit (Gen-Probe, Besancon, France), a Human soluble RANKL ELISA kit (Biomedica Medizinprodukte GmbH & Co., Wien, Germany), and a Human OPG ELISA kit (RayBiotech, Inc., Norcross, GA, USA) according to the manufacturer's protocols.

Statistical methods

The values in each figure represent the means $\pm$ standard deviation (s.d.) for each group in the *in vivo* study. Inter-group comparisons of average values were performed by a one-way analysis of variance (ANOVA), followed by Tukey's test, with values of $P < 0.05$ considered to indicate a significant difference.

The values shown represent the means $\pm$ standard deviation (s.d.) for each group in the *in vitro* study. A Mann–Whitney *U*-test was used to compare the means of the groups, with values of $P < 0.05$ considered to indicate a significant difference.

Results

In vivo study

Body weights during the experimental period. The body weights of the mice in both groups subjected to the 25 g force decreased transiently on day 1 and then recovered. No significant differences between the two groups were observed during the experimental period (data not shown).

Development of AD-like skin lesions. After the repeated application of Dfb ointment to the dorsal shaved skin of Nc/Nga mice, skin dryness occurred first, followed by mild erythema, and finally, hemorrhage. On day 17, the skin became thick and exhibited severe erythema, hemorrhage, scarring, and excoriation (Figure 2).

Measurement of the serum levels of IgE and IL-17. The serum levels of IgE and IL-17 in the AD and wild-type groups were determined by ELISA. The total serum IgE and IL-17 levels were significantly increased in the AD group compared with the wild-type group before orthodontic tooth movement (Figures 3a,b).

Histological changes in the PDL during tooth movement (H.E. staining). In the control (0 g), wild-type, and AD groups, the mouse PDL specimens were composed of relatively dense connective tissue fibers and fibroblasts that were horizontally aligned from the root cement. The root surface was relatively smooth, with few mononuclear and multinucleated osteoclasts. In the wild-type group

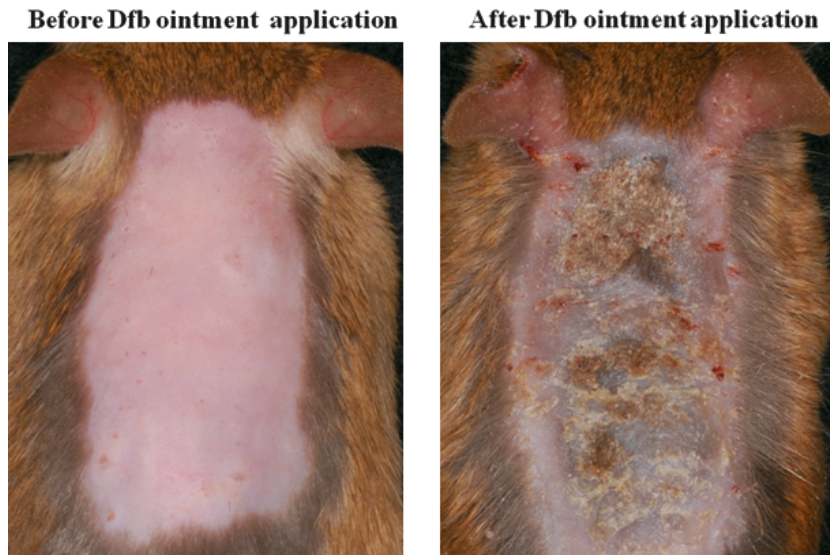


Figure 2 Development of AD-like skin lesions after Dfb ointment application. Dfb ointment was applied on the shaved dorsal skin of Nc/Nga mice. On 17 day, the skin had become thickened, and severe erythema, hemorrhage, scarring, and excoriation were observed

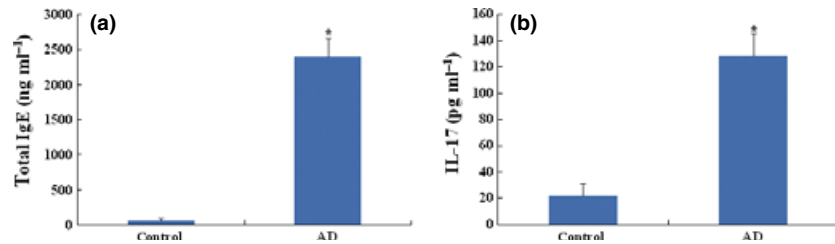


Figure 3 The serum levels of IgE and IL-17 were significantly increased in the AD group compared with the wild-type group before orthodontic tooth movement. (* $P < 0.01$ Mann–Whitney U -test). The data are expressed as the means $\pm$ s.d. of four independent experiments

H.E.

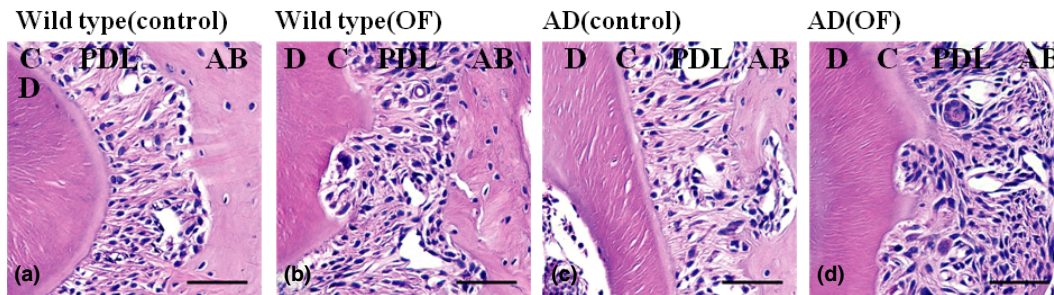


Figure 4 Light macroscopic images of the effects of different orthodontic forces (OFs) on the multinucleated osteoclasts (H&E). The OF was applied for 9 days. Root resorption was observed in the OF group, but not in the control group. Multinucleated cells appeared in the resorbed lacunae in the OF group on day nine (b, d). D, dentine; C, cementum; PDL, periodontal ligament; AB, alveolar bone. Original magnification 400 $\times$, Bar: 50 μ m

subjected to the excessive OF (25 g), there was a coarse arrangement of fibers and compressed blood capillaries. On day nine, several root resorption lacunae with multinucleated odontoclasts were recognized on the surface of the root (Figure 4b). In the AD group subjected to the excessive OF (25 g), the number of root resorption lacunae was higher than that in the wild-type group (Figure 4d).

Immunohistochemical findings of TRAP. In the control (0 g), wild-type, and AD groups, resorption lacunae with TRAP-positive multinucleated osteoclasts were not observed on the surfaces of the root (Figures 5a,c). In the wild-type mice subjected to the excessive OF (25 g), several resorption lacunae with multinucleated TRAP-

positive odontoclasts were observed in the PDL on day 9 (Figure 5b). In the AD group subjected to the excessive OF (25 g), the number of TRAP-positive odontoclasts was higher than that in the wild-type mice (Figure 5d). Intergroup comparisons were performed, with values of $P < 0.05$ considered to indicate a significant difference (Figure 5e).

Protein expression levels of IL-17, IL-6, and RANKL. The immunoreactivity for IL-17, IL-6, and RANKL was examined on day nine after tooth movement. IL-6-, IL-17- and RANKL-positive cells were rarely observed in the control (0 g), wild-type mice, and AD groups (Figures 6a, c, 7a,c and 8a,c). In the wild-type mice subjected to the excessive OF (25 g), several IL-17-, IL-6-, and

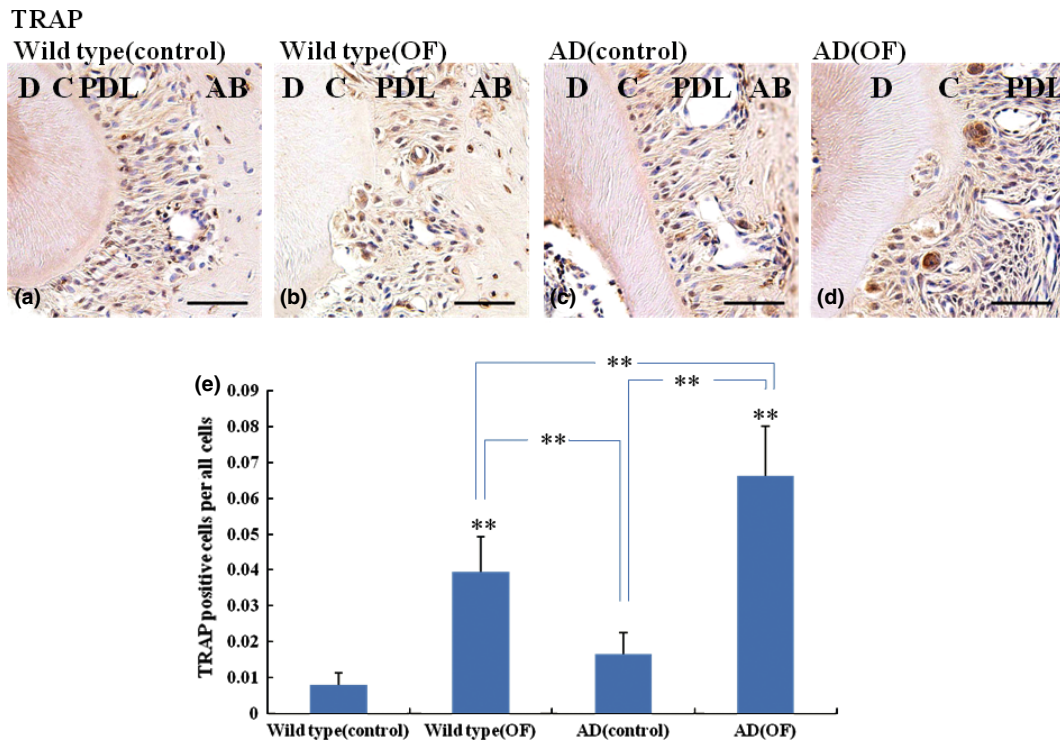


Figure 5 An immunohistochemical comparison of the tartrate-resistant acid phosphatase (TRAP)-positive cells in the AD model and the wild-type mice exposed to excessive orthodontic force (OF). The TRAP-positive cells appeared in the PDL for all groups (a–d). In the AD (OF) group, the number of TRAP-positive cells was increased compared with that in the wild-type mice (b, d). Intergroup comparisons were performed, with values of $P < 0.05$ considered to indicate a significant difference (e) ($*P < 0.05$, $**P < 0.01$ one-way analysis of variance, and Tukey's test). D, dentine; C, cementum; PDL, periodontal ligament; AB, alveolar bone. Original magnification 400 $\times$, Bar: 50 μ m

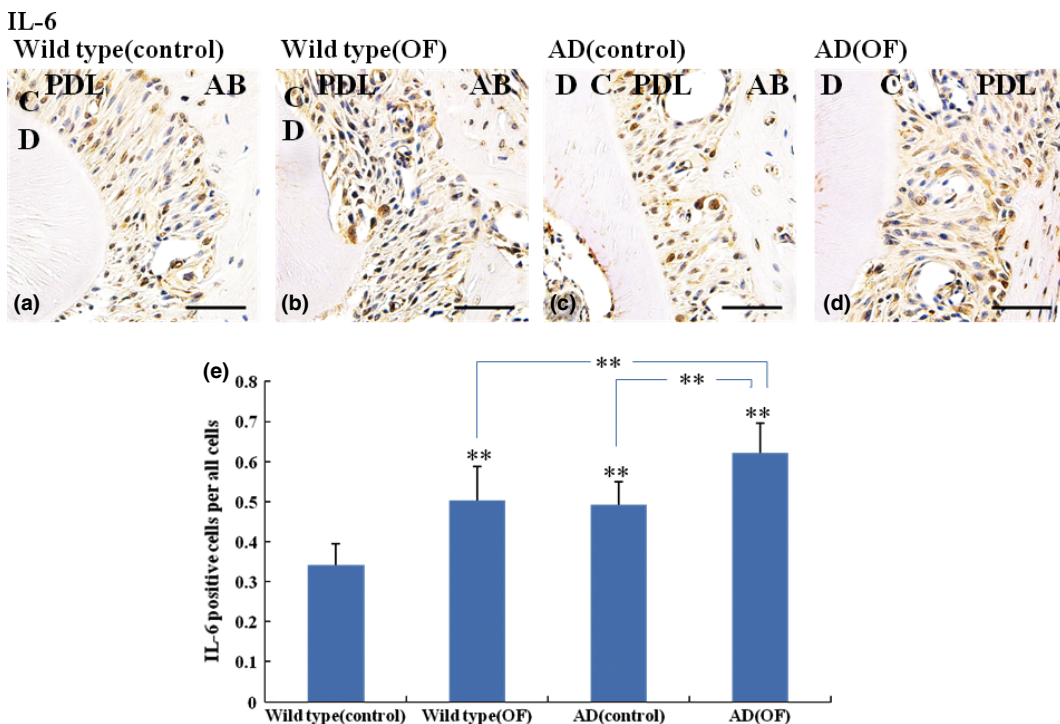


Figure 6 The effects of different orthodontic forces on IL-6-positive cells, as determined by immunohistochemistry. Immunoreactivity for IL-6 was observed on the PDL tissue samples from the compressive force (CF) groups. The immunoreactivity in the AD group was greater than that in the wild-type group (a–d). In addition, The immunoreactivity in the AD (CF) group was greater than that in the wild-type (CF) group (b, d). Intergroup comparisons were performed, with values of $P < 0.05$ considered to indicate a significant difference (e) ($*P < 0.05$, $**P < 0.01$ one-way analysis of variance, and Tukey's test). D, dentine; C, cementum; PDL, periodontal ligament; AB, alveolar bone. Original magnification 400 $\times$, Bar: 50 μ m

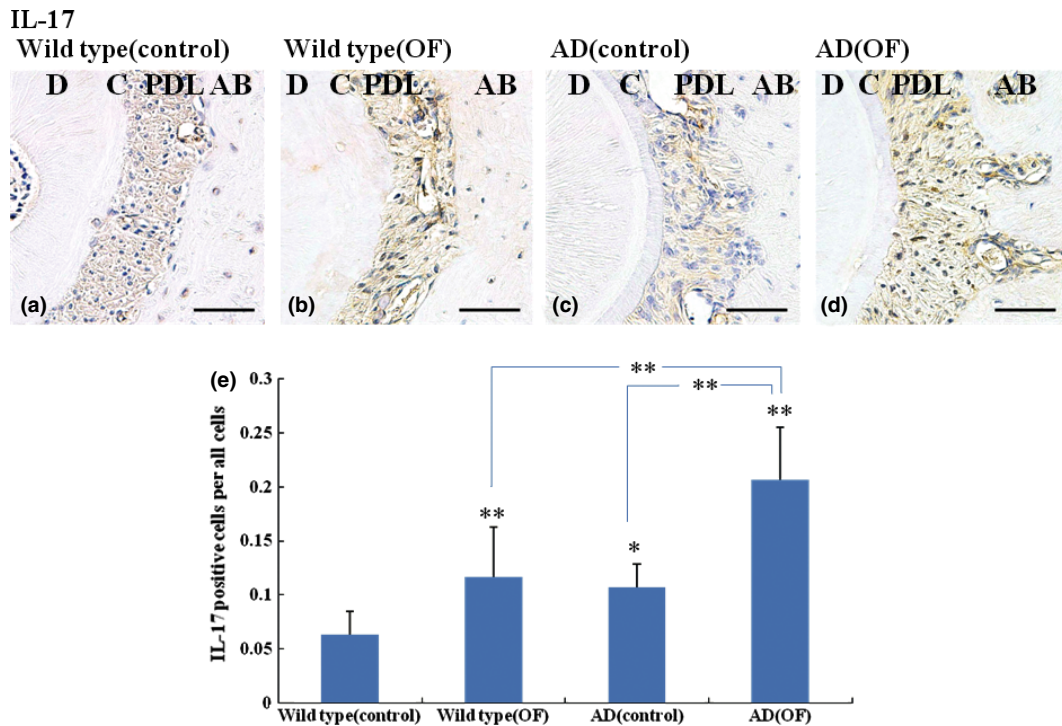


Figure 7 The effects of different orthodontic forces on IL-17-positive cells, as determined by immunohistochemistry. Immunoreactivity for IL-17 was observed on the PDL tissue samples from the compressive force (CF) groups. The immunoreactivity in the AD group was greater than that in the wild-type group (a–d). In addition, The immunoreactivity in the AD (CF) group was greater than that in the wild-type (CF) group (b, d). Intergroup comparisons were performed, with values of $P < 0.05$ considered to indicate a significant difference (e) (* $P < 0.05$, ** $P < 0.01$ one-way analysis of variance, and Tukey's test). D, dentine; C, cementum; PDL, periodontal ligament; AB, alveolar bone. Original magnification 400 $\times$, Bar: 50 μ m

RANKL-positive cells were observed in the PDL tissues (Figures 6b, 7b and 8b). In the AD group subjected to the excessive OF (25 g), the numbers of IL-17-, IL-6-, and RANKL-positive cells were increased in comparison with the wild-type mice exposed to the force (Figures 6d, 7d and 8d). Intergroup comparisons were performed, with values of $P < 0.05$ considered to indicate a significant difference (Figure 6e, 7e and 8e).

Double immunofluorescence findings of Th17 cells. In the control (0 g) mice in both groups, CD4- and IL-17-positive Th17 cells were rarely observed on day nine (Figure 9d,l). In the wild-type mice, several CD4- and IL-17-positive cells appeared in the compressed PDL tissues on day nine (Figure 9h). In the AD group, however, many CD4- and IL-17-positive cells were observed in the PDL tissue slightly removed from the resorbed root surface (Figure 9p).

In vitro studies

The mRNA expression of IL-6 RANKL and OPG in hPDL cells. The mRNA expression levels of IL-6 and RANKL were significantly increased in comparison with those of hPDL cells alone and hPDL cells exposed to the CF after 12 h. Moreover, the mRNA expression levels of IL-6 and RANKL in the cells from AD patients were markedly increased compared with those from the healthy individuals (Figure 10a,b). The mRNA expression of OPG was significantly decreased in comparison with that of hPDL cells alone and hPDL cells exposed to the CF at

12 h. Moreover, the expression of OPG mRNA in the cells from AD patients was markedly decreased compared with that of the healthy individuals (Figure 10c).

The release of IL-17, IL-6, RANKL, and OPG by human CD4⁺ and hPDL cells, as determined by an ELISA. The mRNA expression levels of IL-17, IL-6, and RANKL were significantly increased in comparison with those of hPDL cells alone and hPDL cells exposed to the CF after 12 h. Moreover, the expression of IL-17, IL-6, and RANKL mRNA in the cells from AD patients was markedly increased compared with those from the healthy individuals. The mRNA expression of OPG was significantly decreased in comparison with that of hPDL cells alone and hPDL cells exposed to the CF after 12 h (Figure 11a,b,d). Moreover, the expression of OPG mRNA markedly decreased in the cells from AD patients in comparison with those from the healthy individuals (Figure 11c).

Discussion

Yamamoto *et al* (2007) have reported that the pathophysiological features of dermatitis induced by the repeated application with Dfb ointment in NC/Nga mice were similar to those observed in patients with AD. The percentage of Th17 cells was shown to be increased in the peripheral blood of AD patients and was associated with the severity of AD (Koga *et al*, 2008). Th17 cells constitute a third subset of T-helper cells that are important in the

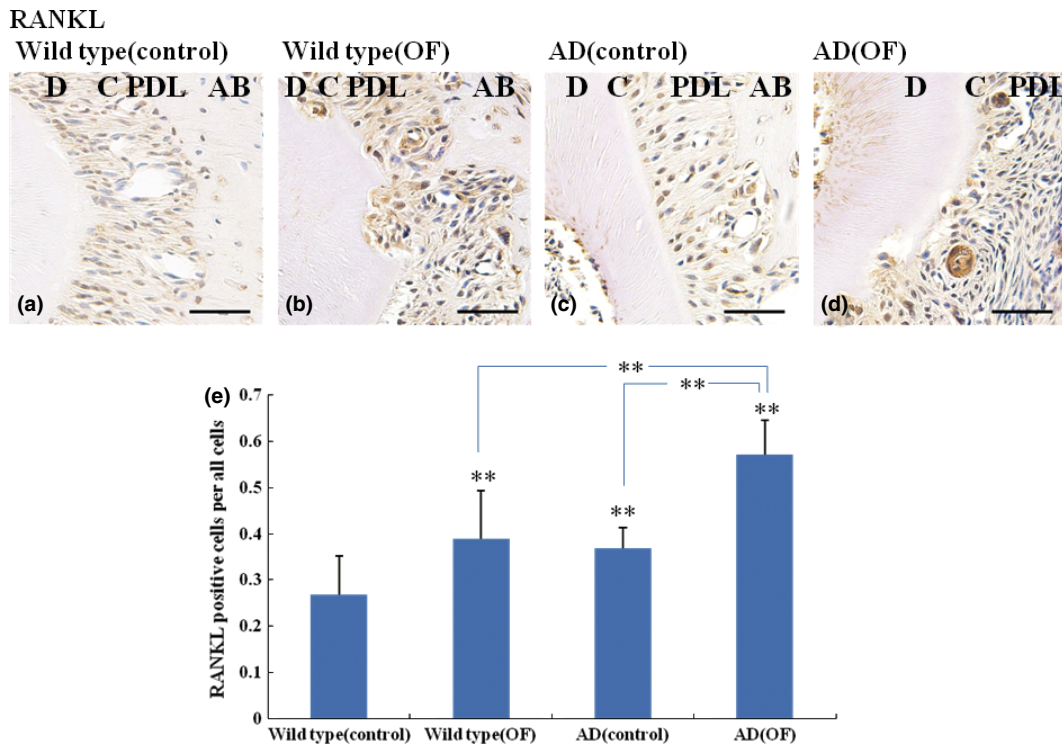


Figure 8 The effects of different orthodontic forces on receptor activator of NF- κ B ligand (RANKL)-positive cells, as determined by immunohistochemistry. Immunoreactivity for RANKL was observed on the PDL tissue samples from the compressive force (CF) groups. The immunoreactivity in the AD group was greater than that in the wild-type group (a–d). In addition, the immunoreactivity in the AD (CF) group was greater than that in the wild-type (CF) group (b, d). Intergroup comparisons were performed, with values of $P < 0.05$ considered to indicate a significant difference (e) (* $P < 0.05$, ** $P < 0.01$ one-way analysis of variance, and Tukey's test). D, dentine; C, cementum; PDL, periodontal ligament; AB, alveolar bone. Original magnification 400 $\times$, Bar: 50 μ m

development of autoimmune diseases and the immune response against infections. IL-17 is produced predominantly by Th17 cells. Therefore, studying experimental root resorption using the Dfb-induced dermatitis NC/Nga model mice will provide important information about the involvement of Th17 cells and IL-17 in external apical root resorption. In the present study, we measured the blood IgE and IL-17 concentrations in NC/Nga mice to which we had applied Dfb, to confirm that they had developed AD (Figure 3a,b).

We found that immunoreactivity for TRAP, which is known as an osteoclast marker, was detected in PDL tissues on day nine after the initial application of the OF, and we noted that the immunoreactivity in the AD group was higher than that in the wild-type mice (Figure 5a–d). Yoshimatsu *et al* (2006) previously reported that TRAP-positive cells were expressed in the PDL and in the resorption lacunae of the mice, which were exposed to an excessive OF. The present study also showed similar results in both groups that were exposed to an excessive OF. In addition, an association of AD with root resorption was assumed based on the presence of a number of TRAP-positive cells in the AD group. Odontoclasts, which are responsible for the resorption of teeth, are thought to be derived from mononuclear precursors of the monocyte/macrophage lineage, and demonstrate characteristics similar to osteoclasts, which are responsible for bone resorption (Sasaki, 2003).

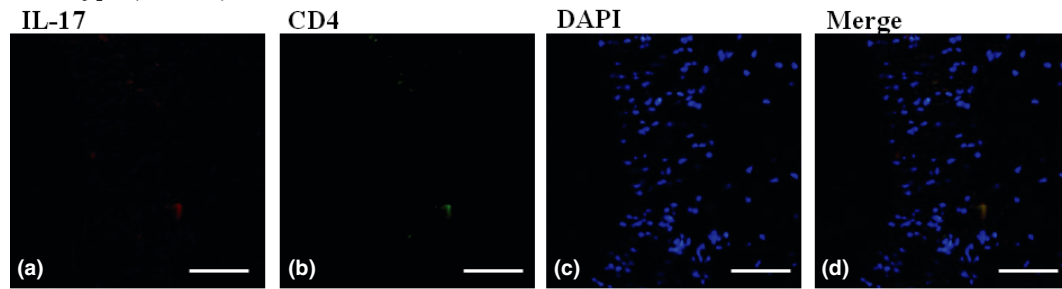
On day nine in the present study, immunoreactivity for IL-17 and RANKL was detected in the PDL tissues exposed to the force, and the immunoreactivity in the AD group for both of these markers was higher than the levels in the wild-type group (Figures 7a–d and 8a–d).

Considering the relationship between IL-17 and RANKL, Okamoto and Takayanagi (2011) concluded from their review that, with regard to the mechanisms underlying the bone destruction found in RA, accumulating evidence lends support to the theory that IL-17-producing Th17 cells induce the expression of RANKL in synovial cells, which in turn stimulates the differentiation and activation of osteoclasts, together with inflammatory cytokines. IL-17 leads to increased sensitivity to RANKL signaling, osteoclast differentiation, and bone loss (Adamopoulos *et al*, 2010). Zhang *et al* (2010) reported that CF induced the expression of IL-17 and its receptor in osteoblast-like cells, and that the IL-17 produced in response to CF might affect osteoclastogenesis.

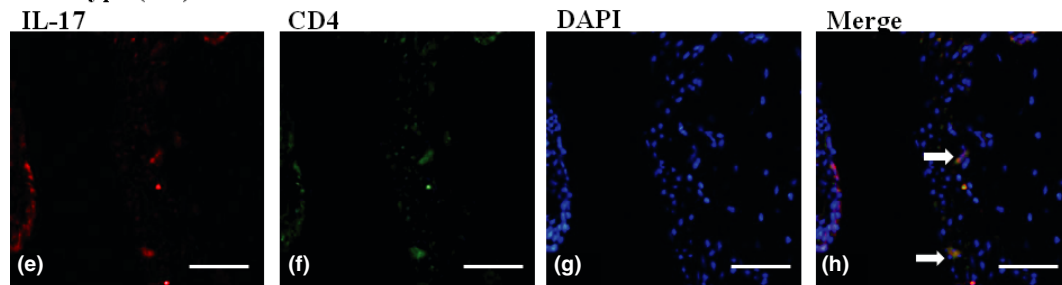
In the present study, immunoreactivity for IL-6 was also detected in mouse PDL tissues and odontoclasts in the AD group, and the level was higher than that in the wild-type mice (Figure 6a–d). IL-6 is a multifunctional cytokine that plays an important role in osteoclastic bone resorption (Ohsaki *et al*, 1992). Kurihara *et al* (1990) demonstrated that IL-6 stimulated the formation of early osteoclast precursors. Adebajo *et al* (1998) reported that IL-6 activated mature osteoclasts. Furthermore, IL-6

Double immunofluorescence

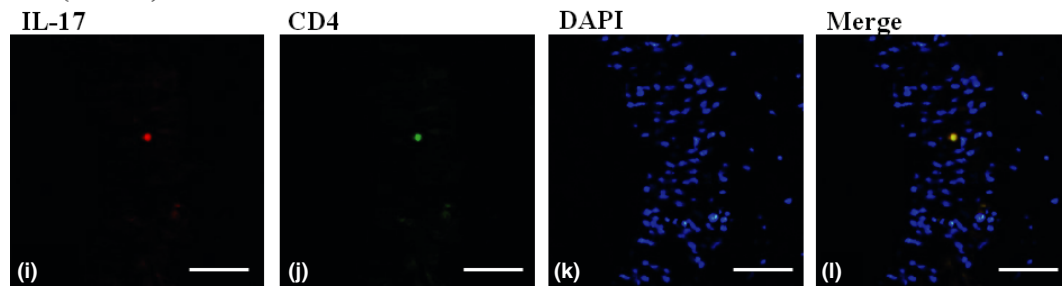
Wild type (control)



Wild type (OF)



AD(control)



AD (OF)

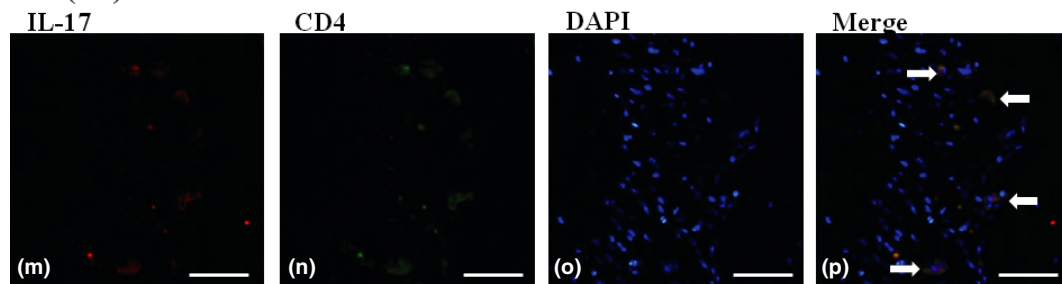


Figure 9 The effects of different orthodontic forces on the presence of Th17 cells as determined by double immunofluorescent staining. The immunolocalization of CD4- (green) and IL-17-(red) positive cells was observed in the PDL tissues from all groups (a–p). More intense immunoreactions for CD4 and IL-17 were noted in the AD [compressive force (CF)] group than in the wild-type (CF) group (d, h, l, p). Original magnification 400×, Bar: 50 μ m

stimulates osteoclastogenesis through stromal/osteoblastic cells (O'Brien *et al*, 1999). Therefore, it has been suggested that IL-6 plays important roles in bone resorption. In addition, Hayashi *et al* (2012) reported that the IL-17 induced by excessive OF stimulates odontoclastogenesis through IL-6 production.

Taken together, these findings and our present results suggest that the increased IL-17 released from Th17 cells,

and the RANKL and IL-6 secretion in PDL tissues induced by excessive OF may activate onto/osteoclastogenesis. Supporting this idea, in the wild-type mice in the present study, several CD4- and IL17-positive cells were detected in the compressed PDL tissues on day nine (Figure 9d,h,l,p). In the AD group, however, many of these cells were observed in the compressed PDL tissue outside of the area of the resorbed root surface (Figure 9p).

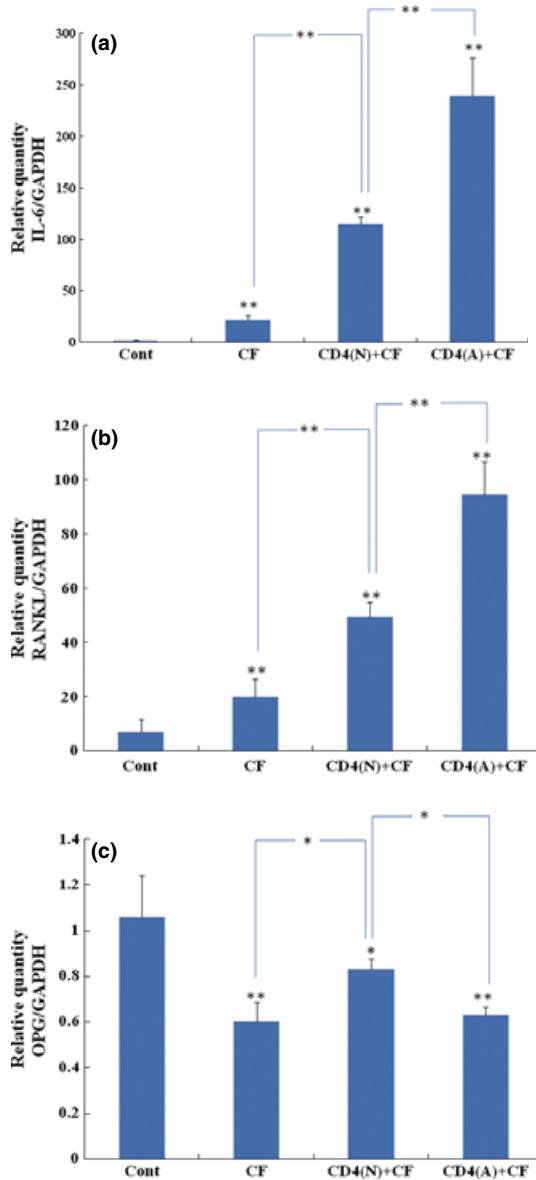
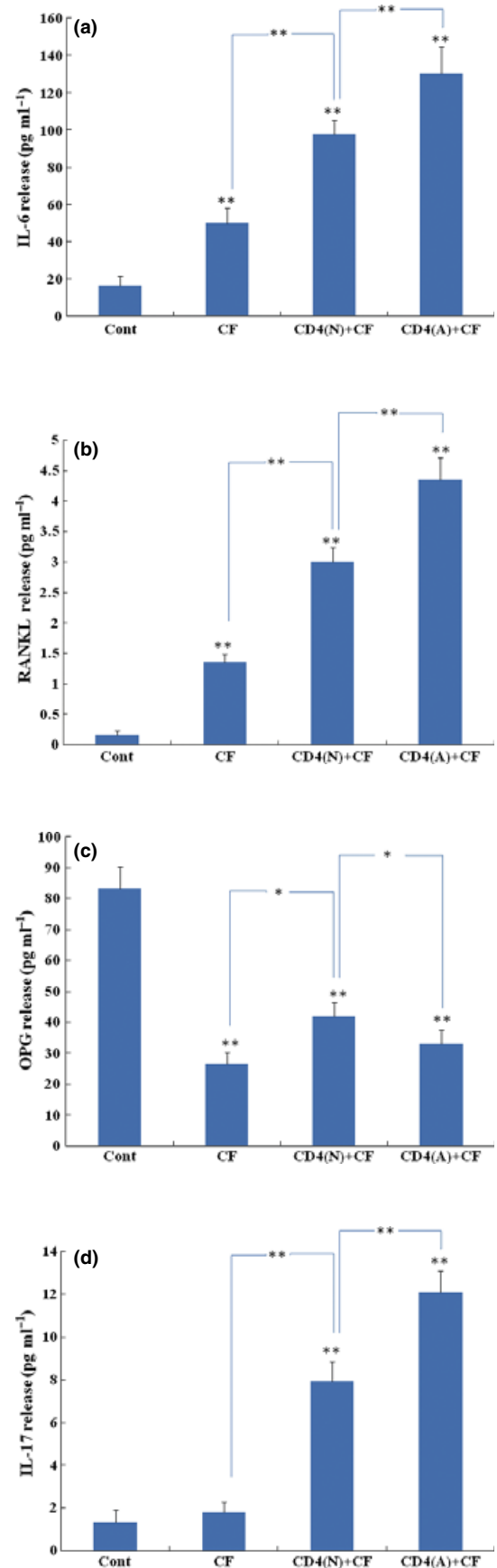


Figure 10 The application of compressive force to the co-cultures of CD4⁺ T cells and human periodontal ligament (hPDL) cells from AD patients and healthy controls led to increased expression levels of IL-6, receptor activator of NF- κ B ligand (RANKL), osteoprotegerin (OPG), and IL-17 mRNA by the PDL cells. The mRNA expression levels of IL-6, RANKL, and IL-17 were all significantly increased in comparison with those of hPDL cells cultured alone and hPDL cells cultured alone and exposed to the compression force for 12 h. Moreover, the cells from AD patients expressed higher levels of IL-6 and RANKL mRNA compared with those from healthy individuals. The mRNA expression of OPG was significantly decreased in comparison with that of hPDL cells cultured alone and hPDL cells cultured alone and exposed to the compression force for 12 h. Moreover, the expression of OPG mRNA was markedly decreased in the cells from AD patients compared with those of the healthy individuals. (* $P < 0.05$, ** $P < 0.01$ Mann-Whitney U -test). The data are expressed as the means $\pm$ s.d. of four independent experiments

In the present study, Th17 cells were found to be expressed in tissue samples from mice that were subjected to the force. Thus, this production of IL-17 has been considered to be the result of immune cells migrating from peripheral vessels. When an OF is applied, local tissue



ischemia and the microenvironment enhance cell damage, and necrotic tissue appears in the compressed root surface adjacent to the PDL (Noxon *et al*, 2001). Subsequently, immune cells accumulate to remove the necrotic tissue (Rygh, 1972). It is thought that these immune cells are the cause of the increased IL-17 production.

In a further experiment, we co-cultivated CD4⁺ T cells and PDL cells from AD patients and healthy individual subjects and applied a CF to elucidate the association between AD and root resorption. The CF was applied in accordance with a report by Nakajima *et al* (2008). We used a CF of 4.0 g cm⁻², which significantly increased the sRANKL expression.

As a result, the mRNA expression levels of RANKL and IL-6 in the PDL cells increased more significantly in the group in which the CD4⁺ T cells and PDL cells were co-cultivated and exposed to the CF than in the group in which the CF was applied to PDL cells alone. In addition, the mRNA expression of RANKL and IL-6 increased more significantly in the group in which the PDL cells and CD4⁺ T cells of AD patients were co-cultivated than in the group in which cells from healthy individuals subjects were co-cultivated (Figure 10a,b). A similar result was obtained with regard to the secretion levels of these molecules and of IL-17 (Figure 11a,b,d), indicating that the IL-17 secretion by CD4⁺ T cells was increased by the CF. In addition, IL-17 increased more significantly in the group in which CD4⁺ T cells from AD patients were used.

Kotake *et al* (1999) reported that IL-17 upregulates the RANKL on human osteoclast precursor cells and induces osteoclast differentiation and bone loss. In addition, Liu *et al* (2005, 2006) reported that the PGE₂ and IL-6 signaling pathways stimulated osteoclastogenesis via their effect on the RANK/RANKL/OPG system. Furthermore, IL-6/sIL-6R directly induced RANKL expression in fibroblast-like synoviocytes (Suzuki *et al*, 2011) and is essential for RANKL induction by TNF- α and IL-17 (Lam *et al*, 2000; Hashizume *et al*, 2008). Therefore, IL-17 may stimulate osteo/odontoclastogenesis via other pathways, such as the RANK/RANKL/OPG system, as well as via the IL-6 pathway. Further studies are necessary to investigate the relationships among IL-17, IL-6, and the RANK/RANKL/OPG system during root resorption.

Thus, IL-17 appears to increase RANKL expression, which accelerates the differentiation of osteoclasts, and also increases IL-6 expression, which directly accelerates the differentiation of osteoclasts. Furthermore, it appears

that IL-6 increases RANKL expression, which accelerates the differentiation of osteoclasts and causes root resorption. In the present study, more IL-17 secretion was observed in CD4⁺ T cells derived from AD patients than CD4⁺ T cells from healthy individuals in the *in vitro* study. In addition, the presence of Th17 cells was also verified in the PDL of the AD mice, which were exposed to an excessive OF in the *in vivo* study.

In conclusion, Th17 cells may therefore be associated with the deterioration of root resorption of AD mice, and may explain why AD patients are more susceptible to root resorption than healthy individuals when an excessive OF is applied.

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Author contributions

Yamada carried out all studies and analysed data. Asano and Kobayashi carried out *in vitro* study. Fujita carried out *in vivo* study. Yamaguchi and Kasai held the design and the support of this study.

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Figure 11 The effects of compressive force exposure on the co-cultures of CD4⁺ T cells and human periodontal ligament (hPDL) cells from AD patients or healthy individuals was assessed by monitoring the release of IL-6, receptor activator of NF- κ B ligand (RANKL), osteoprotegerin (OPG), and IL-17. The mRNA expression levels of IL-6, RANKL, and IL-17 were significantly increased in comparison with those of hPDL cells cultured alone and hPDL cells cultured alone but exposed to the compression force for 12 h. Moreover, the expression of IL-6 and RANKL mRNA in the cells from AD patients were markedly increased compared with those of the healthy individuals. The mRNA expression of OPG was significantly decreased in comparison to that of hPDL cells cultured alone and the hPDL cells cultured alone but exposed to the compression force for 12 h, and compared with the co-cultured cells from healthy individuals. (**P* < 0.05, ***P* < 0.01 Mann–Whitney *U*-test). The data are expressed as the means $\pm$ s.d. of four independent experiments

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