

Interleukin-17/T-helper 17 cells in an atopic dermatitis mouse model aggravate orthodontic root resorption in dental pulp

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Interleukin (IL)-17 is an important mediator of orthodontically induced inflammatory root resorption (OIIRR). However, its role in the dental pulp (DP) has not been studied. The aim of this study was to investigate, using an atopic dermatitis (AD) model, how IL-17 contributes to OIIRR in DP. Atopic dermatitis is the most common IL-17-associated allergic disease. Atopic dermatitis model mice (AD group) and wild-type mice (control group) were subjected to an excessive orthodontic force. The localization of T-helper (Th)17 cells, IL-17, IL-6, and keratinocyte chemoattractant (KC; an IL-8-related protein in rodents) were determined in DP. In addition, CD4⁺ T cells, including IL-17 production cells, were obtained from patients with AD and from healthy donors, and the effects of IL-17 on the production of IL-6 and IL-8 were investigated using a co-culture of CD4⁺ T cells with human dental pulp (hDP) cells stimulated with substance P (SP). Immunoreactivity for Th17 cells, IL-17, IL-6, and KC was increased in DP tissue subjected to orthodontic force in the AD group compared with DP tissue subjected to orthodontic force in the control group. The cells obtained from the AD patients displayed increased IL-6 and IL-8 production. These results suggest that IL-17 may aggravate OIIRR in DP.

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Interleukin (IL)-17 is an inflammatory cytokine that is produced exclusively by the activated T-helper (Th)17 cell subset of CD4⁺ T lymphocytes (1). Interleukin-17 has been shown to be an important mediator of autoimmune diseases, including rheumatoid arthritis (RA) (2), multiple sclerosis (3), and psoriasis (4). Recently, IL-17 was implicated in the pathogenesis of atopic dermatitis (AD) (5). Atopic dermatitis is the most common skin disease and is characterized by chronic and recurrent eczema with severe itching (6). KOGA *et al.* (5) reported that the percentage of Th17 cells is increased in the peripheral blood of patients with AD and that these cells are involved in the process of skin inflammation in AD.

Furthermore, IL-17 is involved in osteoclastic bone destruction in some diseases, including RA. With regard to the mechanism underlying the bone destruction observed in RA, evidence lends support to the theory that IL-17-producing Th cells induce the expression of receptor activator of nuclear factor kappa B ligand (RANKL) in synovial cells, which stimulates the differentiation and activation of bone-resorbing osteoclasts, together with inflammatory cytokines (7). Recently, IL-17, when used alone, has been reported to be able to

induce osteoclastogenesis directly from monocytes (8). Moreover, IL-17 has been shown to stimulate fibroblastic cells to produce pro-inflammatory cytokines, such as IL-6 and IL-8 (9). Interleukin-6 and IL-8 play important roles in osteoclastic bone resorption (10, 11). The concentrations of IL-6 and IL-8 are increased during orthodontic tooth movement (12, 13), and ZEHNDER *et al.* (14) reported that IL-6 and IL-8 are expressed at significantly higher levels in inflammatory pulp than in normal pulp.

Orthodontic forces are known to produce inflammatory reactions in dental pulp (DP) (15). KUCUKKELES & OKAR (16) reported that the inner root surface of the pulp in teeth subjected to intrusive forces exhibits resorption cavities. Previous studies have found that endodontically treated teeth are significantly less resorbed than are contralateral non-rootfilled controls, suggesting that DP might be involved in orthodontically induced inflammatory root resorption (OIIRR) (17, 18). Therefore, in the present study, we focused on the relationship between DP and OIIRR.

Substance P (SP) is known to act as a potent immunostimulator that can sustain the inflammatory process in DP. Various neuropeptides, including SP, are

increased in DP in response to orthodontic tooth movement in vivo (19, 20). Therefore, we used SP in place of orthodontic forces in DP in an in vitro study.

Orthodontically induced inflammatory root resorption is an unavoidable complication associated with orthodontic tooth movement. The causes are reported to vary and include the use of heavy force (21), the length of treatment, the type of root (22), and genetic predisposition (23). Recently, NISHIOKA *et al.* (24) found that allergy patients, including those with higher levels of IL-17, demonstrate increased orthodontic root resorption. Moreover, NIGUL & JAQOMAQI (25) also reported that allergic patients exhibit more resorption than do non-allergic patients. These data suggest that having allergies is a risk factor for the development of OIIR.

Therefore, we hypothesized that IL-17 might be involved in the development of OIIR in DP. In the present study, we investigated AD as a common IL-17-associated disease because it is considered to be of great clinical importance owing to the likelihood for patients with AD to receive orthodontic treatment. *Dermatophagoides farinae* body (Dfb) ointment-treated Nc/Nga mice have been reported to be useful as an AD model (26). Therefore, we used these mice as a model of AD and investigated the immunolocalization of Th17 cells, IL-17, IL-17 receptor (IL-17R), IL-6 and keratinocyte chemoattractant (KC; an IL-8-related protein in rodents) with regard to root resorption during tooth movement. In addition, CD4⁺ T cells, including IL-17 production cells, were obtained from AD patients and healthy donors using the automated MACS device. The levels of IL-6 and IL-8 proteins and of *IL6* and *IL8* mRNAs were examined in a co-culture of CD4⁺ T cells, obtained from AD patients, with human dental pulp (hDP) cells stimulated with SP in vitro.

Material and methods

Animals

Twelve male AD model mice (AD group; Nc/Nga) and 12 male wild-type mice (control group; BALB/c) were purchased from the Sankyo Laboratory (Sankyo Labo Service, Tokyo, Japan). Eight-week-old mice were used in all experiments. The animals were maintained at the Animal Center of Nihon University School of Dentistry at Matsudo in separate cages in a 12-h light/12-h 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 using daily body-weight monitoring. The animal experimental protocol in this study was approved by the Ethics Committee for Animal Experiments at Nihon University School of Dentistry at Matsudo (approval no. AP11MD015).

Development of AD-like skin lesions

Dermatitis was induced using the method reported by YAMAMOTO *et al.* (26). On the first induction (day 0), the mice were anesthetized with ether, the rostral part of the back skin of each mouse was clipped using an electric clipper, and residual hair was depilated using a hair-removal cream. An antigen challenge was performed using topical

application of 100 mg of Dfb ointment (Biostir, Kobe, Japan) on the dorsal shaved skin. From the second induction onward (day 3), after the removal of growing hair using an electric clipper and an electric shaver, barrier disruption was achieved by application of 150 μ l of 4% SDS onto the shaved dorsal skin. The Dfb ointment was applied onto the dorsal skin 3 h after the skin was shaved. These procedures were repeated twice a week. The mice were used for experiments following the last antigen challenge on day 17 (Fig. 1A).

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 retro-orbital plexus, into glass capillary tubes, before orthodontic tooth movement, and separated serum samples were stored at -80°C until use. The levels of IgE and IL-17 in murine serum were measured using an IgE ELISA kit (Shibayagi, Gunma, Japan) and an IL-17 ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the protocol supplied by each manufacturer.

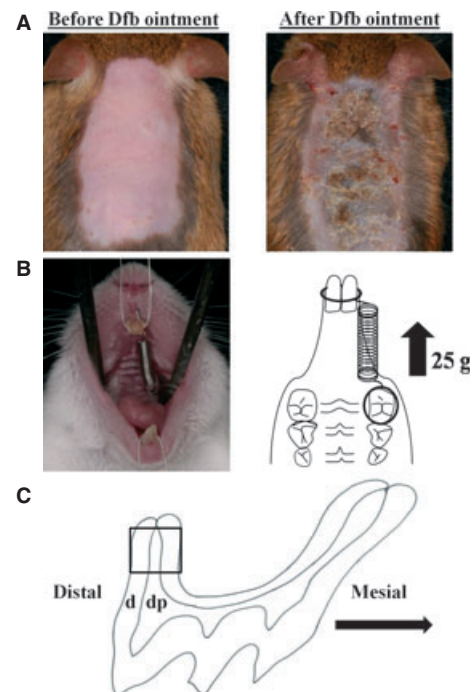


Fig. 1. (A) Development of atopic dermatitis-like skin lesions after the application of *Dermatophagoides farinae* body (Dfb) ointment. The Dfb ointment was applied onto the dorsal shaved skin of Nc/Nga mice. On day 17, the skin had become thick and severe erythema, hemorrhage, scarring, and excoriation were observed. (B) Experimental tooth movement was performed using a nickel–titanium (Ni–Ti) closed-coil spring that was inserted between the upper incisors and the upper-left first molar and fixed with a 0.2-mm stainless-steel ligature wire around both teeth by means of a dental adhesive agent. The upper first molar was mesially moved by the closed-coil spring with a force of 25 g. The force was applied for 9 d. (C) Schematic illustration showing the area of investigation (box) in the radicular pulp of the distal root of the first molar. The large arrow indicates the direction of the force. d, dentin; dp, dental pulp.

Orthodontic devices

The animals were anesthetized with thiamylal sodium (15 mg/kg of body weight) before application of the orthodontic devices. Experimental tooth movement was induced according to the method reported by YOSHIMATSU *et al.* (27), using a nickel-titanium (Ni-Ti) closed-coil spring (Tomy International, Fukushima, Japan) that was inserted between the upper incisors and the upper-left first molar and fixed with a 0.2-mm stainless-steel ligature wire (Tomy International) around both teeth by means of a dental adhesive agent (Transbond XT; 3M Unitek, Monrovia, CA, USA). The left maxillary molar in each mouse was used 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 selected based on the report of AL-QAWASMI *et al.* (28) (Fig. 1B). The force was applied for 9 d.

Tissue preparation

The experimental period was set at 9 d after tooth movement was initiated. The animals were deeply anesthetized with thiamylal sodium and then transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, after which the maxilla was 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 wk, and the decalcified specimens were dehydrated through a graded ethanol series and embedded in paraffin using the usual methods of preparation. Each sample was sliced into 4- μ m serial sections in the sagittal direction and then prepared for hematoxylin and eosin (29, 30) and immunohistochemical staining. The DP in the distal buccal root of the first upper molar was analyzed using a previously described method (27) (Fig. 1C). The side that was not moved was evaluated as a control. Sagittal serial sections were generated to include 15 consecutive slides from in front of to behind the apical end of the DP. The number of positive cells on each slide was counted after immunohistochemical staining. The mean values from six mice were used to calculate the ratio of positive cells/all cells.

Immunohistochemistry

The tissue sections were deparaffinized and the endogenous peroxidase activities were quenched by incubation in 3% H₂O₂ in methanol for 30 min at room temperature. After washing in Tris-buffered saline (TBS), the sections were incubated with polyclonal anti-[goat tartrate-resistant acid phosphatase (TRAP)] IgG (working dilution, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal anti-(rat IL-17) IgG (working dilution, 1:100; Biolegend, San Diego, CA, USA), polyclonal anti-(rabbit IL-17R) IgG (working dilution, 1:200; Santa Cruz Biotechnology), polyclonal anti-(goat IL-6) IgG (working dilution, 1:200; Santa Cruz Biotechnology), or polyclonal anti-(goat KC) IgG (working dilution, 1:100; Santa Cruz Biotechnology) for 18 h at 4°C. Tartrate-resistant acid phosphatase, IL-17, IL-17R, IL-6, and KC were stained using the Histofine Simple Stain MAX-Po kit (Nichirei, Tokyo, Japan), according to the manufacturer's protocol. The sections were rinsed with TBS, and the final color reactions were performed using the 3,3'-diaminobenzidine tetrahydrochloride substrate reagent. The sections were then counterstained with hematoxylin. As immunohistochemical

controls, several sections were incubated with 0.01 M PBS instead of with the primary antibodies.

Double immunofluorescence

For double-immunofluorescence staining, the sections were deparaffinized and washed in TBS, then incubated with a polyclonal anti-(rabbit CD4) IgG (working dilution, 1:100; Assay Biotechnology, Sunnyvale, CA, USA) for 18 h at 4°C. Subsequently, CD4 staining was performed using Alexa Fluor 488-goat anti-rabbit IgG conjugate (working dilution, 1:100; Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. The sections were then incubated with polyclonal anti-(rat IL-17) IgG (working dilution, 1:100; Biolegend) for 1 h at room temperature, and IL-17 staining was performed using Alexa Fluor 568-goat anti-rat IgG conjugate (working dilution, 1:100; Invitrogen). After washing with TBS, all sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) and analyzed using a fluorescence microscope (Olympus, Tokyo, Japan).

Cell sampling

Human CD4⁺ T cells were isolated from the peripheral blood of 30 volunteers. Peripheral blood was obtained from 15 AD patients (eight men and seven women, 20–30 yr of age) and from 15 healthy controls (eight men and seven women, 20–30 yr of age). The patients were diagnosed with AD according to the guidelines for the management of AD reported by SAEKI *et al.* (31). The protocol for the human experiments was reviewed and approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo (approval no. EC 11-022), and informed consent was obtained from each individual.

hDP cell culture

Human dental pulp cells were prepared using a modification of the method reported by HOSOYA *et al.* (32). Briefly, hDP tissues were taken from the roots of premolars extracted from six healthy young volunteers (three male subjects and three female subjects, 14–16 yr of age). The hDP tissue specimens were placed in 35-mm dishes and covered with a sterilized glass coverslip. The cells were maintained in α -minimum essential medium (α -MEM) (Wako, Osaka, Japan) supplemented with 100 μ g/ml of penicillin-G (Sigma Chemical, St Louis, MO, USA), 50 μ g/ml of gentamicin sulfate (Sigma), 0.3 μ g/ml of amphotericin B (Flow Laboratories, McLean, VA, USA), and 10% fetal calf serum (FCS) (Cell Culture Laboratories, Cleveland, OH, USA). The cultures were maintained at 37°C in a humidified incubator (Forma CO₂ incubator MIP-3326; Sanyo Electric Medica System, 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 subcultured in culture flasks.

Isolation of CD4⁺ T cells and co-culture with SP-stimulated hDP cells

Isolation of pure CD4⁺ T cells from human peripheral blood for the chimerism analyses was performed using Whole Blood Micro Beads (Miltenyi Biotec, Auburn, CA,

USA) and the auto MACS device (Miltenyi Biotec). Labeled leukocyte subpopulations were isolated automatically using the POSSELD2 program (Miltenyi Biotec). The results were regarded as reliable if the purity exceeded 99%.

The hDP cells were seeded at passages 6–9 onto 96-well plates (1.5×10^4 cells/well) and cultured in α -MEM containing 10% FCS in the presence of 5% CO₂ at 37°C for 1 d. These cells were co-cultured with freshly prepared human CD4⁺ T cells (1.5×10^5 cells/well) in α -MEM containing 1% FCS and 10^{-6} M SP (Ana Spec, Fremont, CA, USA) for 24 h. In order to reproduce the conditions of pressure during orthodontic tooth movement, we treated the cells with SP, in accordance with the method reported by KOJIMA *et al.* (33).

Enzyme-linked immunosorbent assay

The levels of IL-17, IL-6, and IL-8 released into the culture supernatants were measured using a human IL-17 ELISA kit (Gen-Probe, Besancon, France), a human IL-6 ELISA kit (R&D Systems), and a human IL-8/CXCL8 ELISA kit (R&D Systems), according to each manufacturer's protocol.

Real-time PCR

Total RNA was isolated from cultured hDP cells and from CD4⁺ T cells using a commercially available kit (RNeasy Plus Micro kit; Qiagen, Tokyo, Japan) according to 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 Prime Script RT reagent kit (TaKaRa, Shiga, Japan). Real-time PCR amplification was performed using SYBR Premix Ex Taq (TaKaRa) in a thermal cycler (TP-800 Thermal Cycler Dice; TaKaRa), according to the manufacturer's protocol. The real-time PCR conditions were as follows: 95°C for 3 s and 60°C for 30 s, for 40 cycles, after a hot start. Measurements were obtained at the end of the 60°C annealing step for each cycle. PCR primers for IL-6, IL-8, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from TaKaRa and designed with reference to the respective cDNA sequences (Table S1). The data were analyzed using the SMART CYCLER software program (Multiplate RQ version 1.00; TaKaRa) and the levels of the real-time PCR products corresponding to those of GAPDH were confirmed to be the same in the experimental groups. Therefore, it was considered acceptable to assume that the amount of real-time PCR products reflected the level of each mRNA.

Statistical methods

The values in each table represent the mean $\pm$ SD of each group. The Mann–Whitney *U*-test was used to compare the means of the groups. Values of $P < 0.05$ were considered to indicate significant differences.

Results

Serum levels of IgE and IL-17

The serum levels of IgE and IL-17 in the AD and control groups were determined using ELISA. The total

Table 1
Serum levels of IgE and interleukin-17 (IL-17)

Group	IgE (ng/ml)	IL-17 (pg/ml)
Control	55.33 $\pm$ 17.56	19.17 $\pm$ 6.85
AD	2231.67 $\pm$ 271.03*	130.75 $\pm$ 24.97*

The serum levels of IgE and IL-17 in the atopic dermatitis (AD) and control groups were determined by ELISA. The serum levels of IgE and IL-17 were significantly increased in the AD group compared with the control group (* $P < 0.01$ Mann–Whitney *U*-test). The data are expressed as the mean $\pm$ SD of 12 mice.

levels of IgE and IL-17 in serum were significantly higher in the AD group compared with the control group before orthodontic tooth movement (Table 1).

Histological changes in DP during tooth movement

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 (Fig. S1). In both of the 0-g groups, the murine DP specimens were composed of relatively dense connective-tissue fibers and fibroblasts that were sagittally aligned from the root dentin. The root surface was relatively smooth (Fig. 2A,C). In both of the 25-g groups, there was a coarse arrangement of fibers. Furthermore, several root-resorption lacunae with multinucleated odontoclasts were recognized on the inner surface of the root on day 9 (Fig. 2B,D).

Tartrate-resistant alkaline phosphatase

In both of the 0-g groups, resorption lacunae with few TRAP-positive multinucleated odontoclasts were observed on the inner surface of the root (Fig. 2E,G). In both of the 25-g groups, several resorption lacunae with multinucleated TRAP-positive odontoclasts were observed in the DP tissues on day 9 (Fig. 2F,H). The number of TRAP-positive odontoclasts was significantly higher in the 25-g AD group compared with the 25-g control group (Table 2).

Expression of IL-17, IL-17R, IL-6, and KC proteins

The immunoreactivity for IL-17, IL-17R, IL-6, and KC was examined on day 9 after tooth movement. Interleukin-17-, IL-17R-, IL-6-, and KC-positive cells were rarely observed in either of the 0-g groups (Fig. 3A,C,E,G,I,K,M,O). In both of the 25-g groups, IL-17-, IL-17R-, IL-6-, and KC-positive cells were observed in the DP tissues (Fig. 3B,D,F,H,J,L,N,P). The numbers of IL-17-, IL-17R-, IL-6-, and KC-positive cells were significantly higher in the 25-g AD group compared with the 25-g control group (Table 2, Fig. S2 and Table S2).

Immunolocalization of Th17 cells

In the 0-g control group, no CD4-positive or IL-17-positive Th17 cells were observed (Fig. 4M). In the 0-g

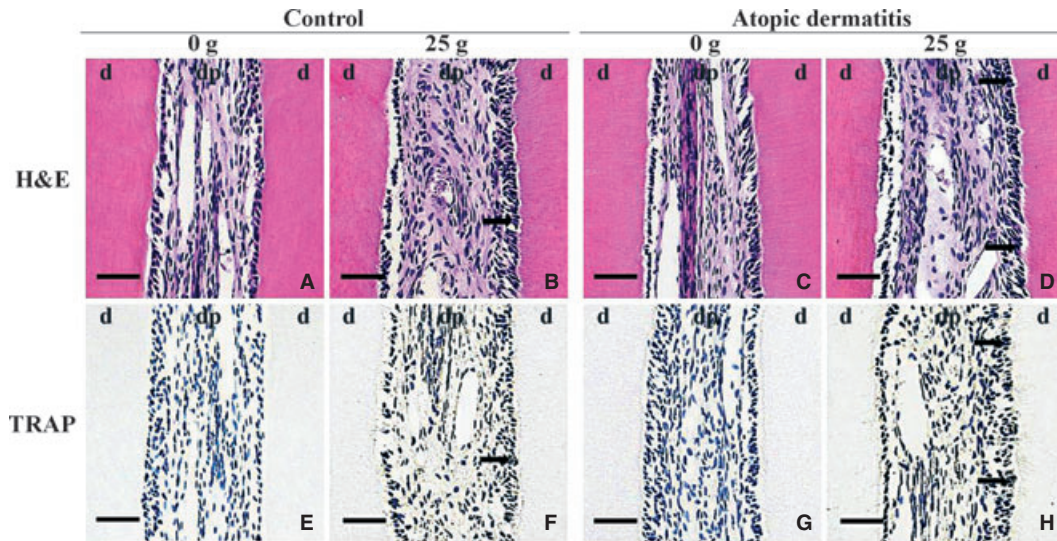


Fig. 2. Light-microscopy images of the effects of different orthodontic forces on multinucleated odontoclasts [indicated by hematoxylin and eosin (H&E) staining] and the result of immunohistochemical staining for tartrate-resistant alkaline phosphatase (TRAP). The orthodontic force was applied for 9 d. Root resorption was observed in both control and atopic dermatitis groups when 25 g of orthodontic force was applied but in neither of these groups when treated with 0 g of orthodontic force. Multinucleated cells had appeared by day 9 in the resorbed lacunae (arrows) in both groups treated with 25 g of orthodontic force (B, D). TRAP immunoreactivity was observed (arrows) in the multinucleated cells (F, H). d, dentin; dp, dental pulp. Original magnification 200 $\times$, Bar: 50 μ m.

Table 2
Ratio of positive cells/all cells (%)

Group	TRAP	IL-17	IL-17R	IL-6	KC
Control 0 g	0.28 $\pm$ 0.04	2.52 $\pm$ 0.35	12.00 $\pm$ 2.74	6.88 $\pm$ 1.07	7.88 $\pm$ 1.14
Control 25 g	3.44 $\pm$ 0.57	8.32 $\pm$ 0.84	21.28 $\pm$ 4.03	22.50 $\pm$ 1.76	25.10 $\pm$ 3.28
AD 0 g	0.33 $\pm$ 0.08 *	4.34 $\pm$ 0.81 *	14.08 $\pm$ 1.62 *	8.80 $\pm$ 0.85 *	9.60 $\pm$ 2.47 *
AD 25 g	5.67 $\pm$ 0.71	13.64 $\pm$ 2.70	34.12 $\pm$ 4.49	38.48 $\pm$ 2.31	42.16 $\pm$ 3.90

The ratio of positive cells/all cells was determined by immunohistochemical analysis. The numbers of tartrate-resistant alkaline phosphatase (TRAP)-, interleukin (IL)-17-, IL-17 receptor (IL-17R)-, IL-6-, and keratinocyte chemoattractant (KC)-positive cells were found to be significantly higher in the 25-g atopic dermatitis (AD) group compared with the 25-g control group (* P < 0.01 Mann-Whitney U -test). The data are expressed as the mean $\pm$ SD of six mice.

AD group and the 25-g control group, a few CD4-positive and IL-17-positive Th17 cells appeared in the DP tissues on day 9 (Fig. 4N,O). In the 25-g AD group, however, many CD4-positive and IL-17-positive Th17 cells were observed in the DP tissues at that time (Fig. 4P).

Release of IL-17, IL-6, and IL-8 by human CD4⁺ T cells and hDP cells in vitro

Following stimulation of the co-culture of CD4⁺ T cells (obtained from AD patients and healthy donors) and hDP cells with SP for up to 24 h, the release of IL-6 and IL-8 was increased compared with that observed in the monoculture. Moreover, the co-culture of the cells obtained from AD patients led to the release of higher amounts of IL-17, IL-6, and IL-8 compared with that observed in the cells obtained from the healthy donors (Table 3).

Expression of IL6 and IL8 mRNAs by human CD4⁺ T cells and hDP cells in vitro

Stimulation of the co-culture of CD4⁺ T cells (obtained from AD patients and healthy donors) and hDP cells with SP led to an increase in the expression of IL6 and IL8 mRNAs compared with that observed in a monoculture in the presence of SP at 24 h. Moreover, the levels of IL6 and IL8 mRNAs expressed in the co-cultures with cells obtained from AD patients were higher compared with the levels expressed in the cells obtained from healthy donors (Table 3).

Discussion

In this study, we used an AD model to investigate whether Th17 cells and IL-17 are involved in OIIR in vivo and in vitro. Atopic dermatitis is considered to be

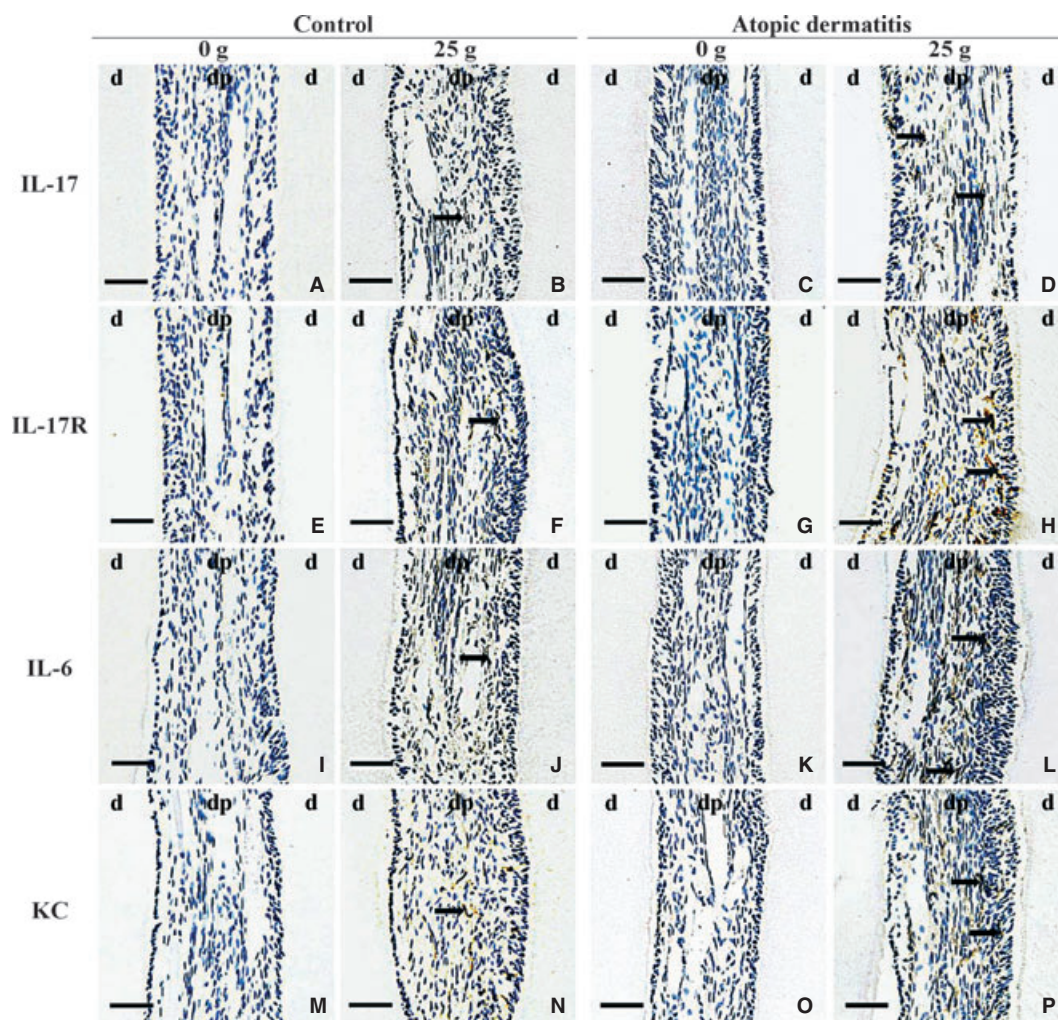


Fig. 3. Effects of orthodontic force on the expression of interleukin (IL)-17, IL-17 receptor (IL-17R), IL-6, and keratinocyte chemoattractant (KC), as determined using immunohistochemistry. Immunoreactivity for IL-17, IL-17R, IL-6, and KC was observed in the dental pulp tissues (arrows) of control and atopic dermatitis groups when 25 g of orthodontic force was applied (B, D, F, H, J, L, N, P) but was rarely observed in either of these groups when treated with 0 g of orthodontic force (A, C, E, G, I, K, M, O). d, dentin; dp, dental pulp. Original magnification 200 $\times$, Bar: 50 μ m.

a Th2-cell-mediated disease characterized by the induction of Th2 lymphocytes expressing IL-4, IL-10, and IL-13. T-helper 2 cells secrete prototypical cytokine IL-4, which suppresses the polarization of Th1 cells and Th17 cells (34, 35). In patients with RA, Th1 cells and Th17 cells are known to produce cytokines related to progressive resorption (7). Therefore, AD appears to suppress the resorption induced by IL-4. Indeed, it has been demonstrated that airway allergies result in the attenuation of bone resorption (36), although a previous study reported that allergies and asthma increase root resorption (24, 25). An RA mouse model appeared to experience more root resorption in that study than in this case. However, RA patients rarely receive orthodontic treatment. Therefore, we decided to use AD in this study.

YAMAMOTO *et al.* (26) reported that the clinical and histological symptoms observed in Dfb ointment-treated Nc/Nga mice are similar to those observed in

human cases of AD. The percentage of IL-17-secreting Th17 cells is increased in the peripheral blood of AD patients (5). Therefore, to confirm the development of AD, we measured the serum levels of IgE and IL-17 in Nc/Nga mice to which we had applied Dfb.

Many investigators have reported that root resorption is aggravated by increasing force magnitudes (12, 13, 21). For example, AL-QAWASMI *et al.* (28) evaluated root resorption after application of excessive orthodontic force (25 g) for 9 d. Therefore, 25 g was used as the excessive orthodontic force in our model. In the present study, several resorption lacunae with odontoclasts appeared on the inner surface of the root after 9 d, in accordance with the findings of a previous report (28).

Moreover, intense immunoreactions of Th17 cells were observed in the DP tissues subjected to excessive orthodontic force. The production of IL-17 is considered to be a result of the migration of immune cells from peripheral vessels (37). Furthermore, in this study,

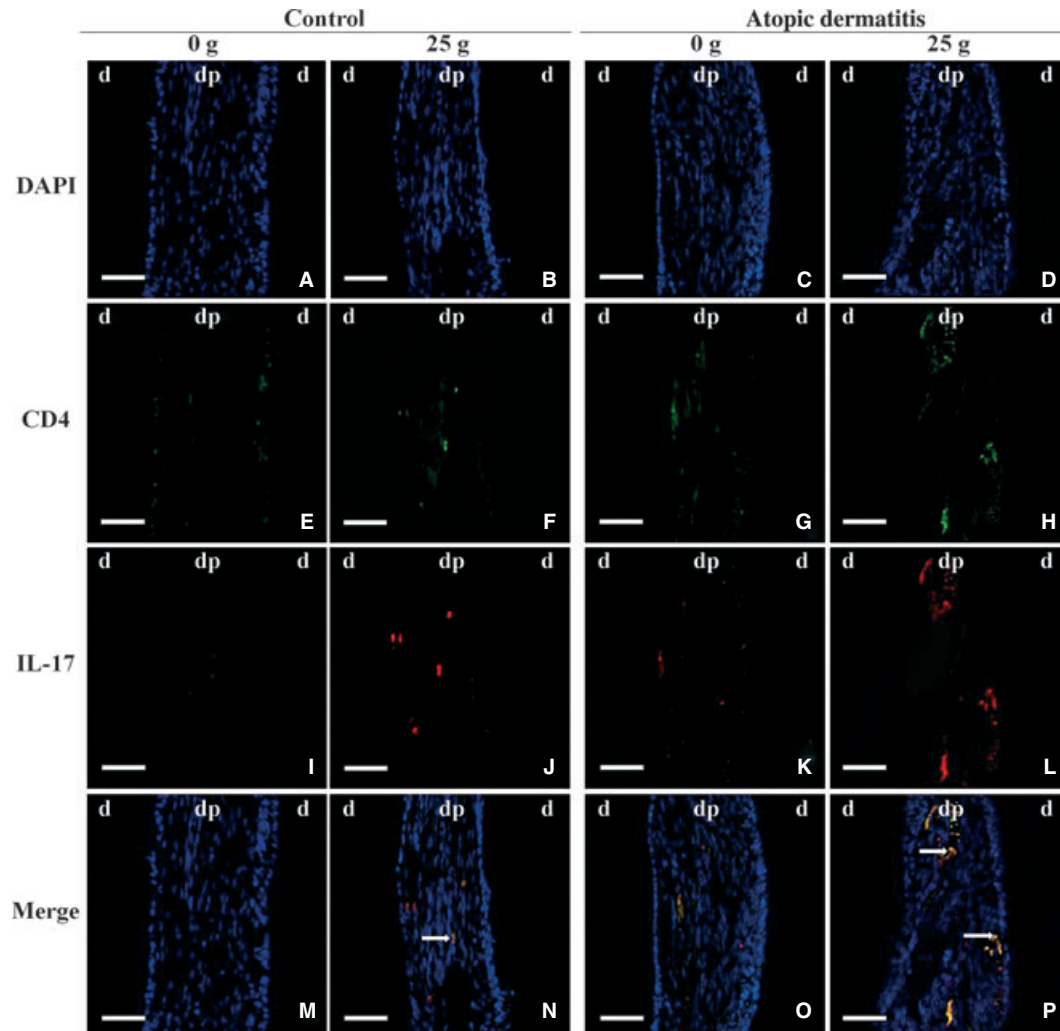


Fig. 4. Effects of different orthodontic forces on the presence of T-helper 17 cells, as determined by double-immunofluorescence staining. The immunolocalization of CD4-positive (green) and IL-17-positive (red) cells was observed in the dental pulp tissues of both control and atopic dermatitis groups when 25 g of orthodontic force was applied (F, H, J, L) but not in the control group treated with 0 g of orthodontic force (E, I). CD4-positive and IL-17-positive cells were also rarely observed in the atopic dermatitis group treated with 0 g of orthodontic force (G, K); however, many CD4-positive and IL-17-positive cells were noted (white arrows) in the atopic dermatitis group treated with 25 g of orthodontic force (P). DAPI, 4',6-diamidino-2-phenylindole; d, dentin; dp, dental pulp. Original magnification 200 $\times$, Bar: 50 μ m.

the total serum IL-17 levels were significantly increased in the AD group compared with the control group without orthodontic force. Before the application of orthodontic force, the number of IL-17-positive cells was not significantly different between the two groups; however, after exposure to force, the number of IL-17-positive cells was markedly higher in the AD group than in the control group. These results indicated a higher capacity for immune-cell migration in the DP tissues of AD mice compared with control mice.

Interleukin-17 has been shown to stimulate epithelial and fibroblastic cells to secrete pro-inflammatory cytokines, such as IL-6 and IL-8, during cutaneous remodeling in AD (5). Furthermore, many studies have also reported that IL-17 up-regulates the production of IL-6 and IL-8 by fibroblasts (9, 38, 39). Therefore, we investigated IL-6 and KC in the DP tissue and found that

the numbers of IL-6-positive and KC-positive cells were significantly increased in the 25-g AD group in comparison with the numbers of IL-6-positive and KC-positive cells observed in the 25-g control group. It was therefore concluded that the increased level of IL-17 is probably responsible for the higher levels observed of IL-6 and KC. In addition, IL-6 and IL-8 play important roles in osteoclastic bone resorption. Interleukin-6 stimulates the formation of early osteoclast precursors and activates mature osteoclasts (10, 40). Interleukin-8 is one of the most potent chemokines and is required for the trafficking of osteoblast and osteoclast precursors and also for the development, activity, and survival of bone cells (11). Osteoclasts, which are responsible for the resorption of bone, are thought to be derived from mononuclear precursors of the monocyte/macrophage lineage and demonstrate characteristics similar to

Table 3

Effects of stimulation with substance P (SP) on the co-culture of CD4⁺ T cells, obtained from atopic dermatitis (AD) patients or healthy donors, with human dental pulp (hDP) cells in vitro

Group	Protein release level (pg/ml)			mRNA expression level	
	IL-17	IL-6	IL-8	IL6	IL8
hDP	1.83 ± 0.57	8.75 ± 2.98	14.30 ± 3.50	8.11 ± 2.26	9.53 ± 2.03
CD4(H)	1.84 ± 0.44	18.40 ± 3.96	21.23 ± 4.00	18.78 ± 5.14	18.55 ± 5.50
CD4(AD)	2.29 ± 0.39	20.20 ± 4.45	25.55 ± 4.45	25.73 ± 3.34	33.93 ± 6.12
hDP+SP	2.13 ± 0.73	40.65 ± 4.72	47.33 ± 8.86	39.93 ± 11.43	66.70 ± 12.22
CD4(H)+SP	7.61 ± 0.91	28.20 ± 3.29	42.30 ± 5.65	44.15 ± 8.06	58.43 ± 9.66
CD4(AD)+SP	11.50 ± 1.41 *	40.53 ± 3.20 *	51.53 ± 6.52 *	53.98 ± 7.38 *	79.10 ± 12.09 *
hDP+CD4(H)+SP	9.20 ± 0.98 *	81.13 ± 3.47 *	100.23 ± 6.76 *	83.08 ± 9.90 *	119.90 ± 9.81 *
hDP+CD4(AD)+SP	13.75 ± 1.73 *	119.03 ± 13.59 *	131.83 ± 8.52 *	118.22 ± 17.01 *	179.68 ± 18.85 *

CD4(AD), CD4⁺ T cells obtained from atopic dermatitis patients; CD4(H), CD4⁺ T cells obtained from healthy donors; hDP, human dental pulp cells; SP, substance P.

The levels of interleukin (IL)-17, IL-6, and IL-8 protein were determined by ELISA and the levels of IL6 and IL8 mRNA were determined using real-time PCR. The levels of IL-17, IL-6 and IL-8 protein and of IL6 and IL8 mRNA were increased in the AD patients compared with the healthy donors (**P* < 0.01; Mann-Whitney *U*-test). The data given represent the mean ± SD of four independent experiments.

odontoclasts, which are responsible for tooth resorption (41). Therefore, IL-17 and related cytokines may participate in the activation of odontoclasts.

Taken together, these findings and our present results suggest that increased release of IL-17 from Th17 cells and increased production of IL-6 and KC in DP tissues is induced by excessive orthodontic force. In a previous study, it was reported that IL-17 is involved in both the inflammatory response and bone resorption in periodontal diseases (42, 43) and periapical lesions (44, 45). However, experimental root resorption is caused by excessive orthodontic force. In periodontal diseases and periapical lesions, bone resorption is caused by inflammatory cytokines, including IL-17 induced by infection. We found that root resorption is also induced by inflammatory cytokines. Therefore, the triggers are different between these processes; however, it is thought that the mechanisms of resorption are similar.

To investigate the pathways involved in the IL-17-mediated regulation of IL-6 and IL-8 in OIHR during orthodontic tooth movement, we measured the levels of IL-17, IL-6, and IL-8 induced by co-culture of CD4⁺ T cells (obtained from AD patients or healthy controls) with hDP cells stimulated with SP in vitro. Treatment with SP was used to reproduce the conditions of pressure during orthodontic tooth movement, in accordance with the method reported by KOJIMA *et al.* (33). Substance P is involved in the progression of inflammation in pulp tissue and the incidence of severe root resorption during orthodontic treatment (46).

Previously, patients with AD have been reported to exhibit an increased percentage of Th17 cells in the peripheral blood (5). It is thought that many Th17 cells are likely to be included in the CD4⁺ T cells of patients with AD. Therefore, we used CD4⁺ T cells from patients with AD. BARROS *et al.* (47) demonstrated that SP elevates the Th17-cell response. Another previous study reported that IL-17 stimulates fibroblas-

tic cells to secrete IL-6 and IL-8 (9). These findings show that the IL-17 induced by SP stimulation in co-culture cells might increase the production of IL-6 and IL-8. In our study, IL-17 markedly increased the levels of these cytokines in co-culture cells compared with the levels in the monoculture. HAYASHI *et al.* (12) showed that the IL-17 induced by excessive orthodontic force stimulates odontoclastogenesis through the production of IL-6. Furthermore, ASANO *et al.* (13) reported that IL-8 (CINC-1) and monocyte chemotactic protein 1 (MCP-1) in periodontal ligament (PDL) tissues may facilitate the process of root resorption caused by excessive orthodontic force. In addition, DP cells are involved in regulating the differentiation and function of osteoclasts as well as of PDL cells (48). In these studies, IL-17 appeared to stimulate osteoclastogenesis/odontoclastogenesis via the IL-8 and IL-6 pathways. Furthermore, the AD patients were found to have significantly increased levels of these cytokines compared with the healthy donors. Therefore, the incidence of root resorption is thought to involve DP tissue.

In conclusion, IL-17-secreting Th17 cells in patients with AD might aggravate root resorption via cytokine production in the presence of excessive orthodontic force in DP.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primer sequences for real-time PCR amplification.

Table S2. Ratio of SP-positive cells to all cells.

Fig. S1. Body weights of the mice in the groups.

Fig. S2. Immunoreactivity for SP examined on day 9 after tooth movement.

Supporting Information

IL-17/Th17 cells in an atopic dermatitis mouse model aggravate orthodontic root resorption in dental pulp

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Table S1

Primer sequences for real-time PCR amplification

Gene	Forward primer	Reverse primer
IL-6	AAGCCAGAGCTGTGCAGATGAGTA	TGTCCTGCAGCCACTGGTTC
IL-8	ACACTGCGCCAACACAGAAATTA	TTTGCTTGAAGTTTCACTGGCATC
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table S2

The ratio of positive cells/all cells (%)

Group	SP
Control 0 g	2.98±0.82
Control 25 g	21.68±4.84
AD 0 g	4.71±0.56
AD 25 g	40.0±4.29

The ratio of positive cells/ all cells were determined by an immunohistochemical analysis. The number of SP-positive cells was found to be significantly increased in both of the 25 g groups in comparison with the 0 g groups (* $p < 0.01$ Mann-Whitney U -test). The data are expressed as the means $\pm$ SD of 6 mice.

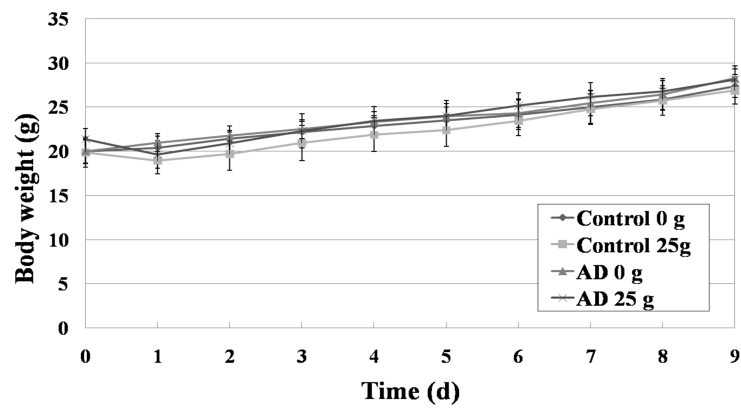


Fig. S1: The health status of each mouse was evaluated by daily body weight monitoring. The body weights of the mice in both groups subjected to the 25 g force decreased transiently on day 1 and then recovered. No significant differences between the two groups were observed. The data are expressed as the means $\pm$ SD of six mice. $\blacklozenge$: Control 0 g $\blacksquare$: Control 25 g $\blacktriangle$: AD 0 g $\times$: AD 25 g

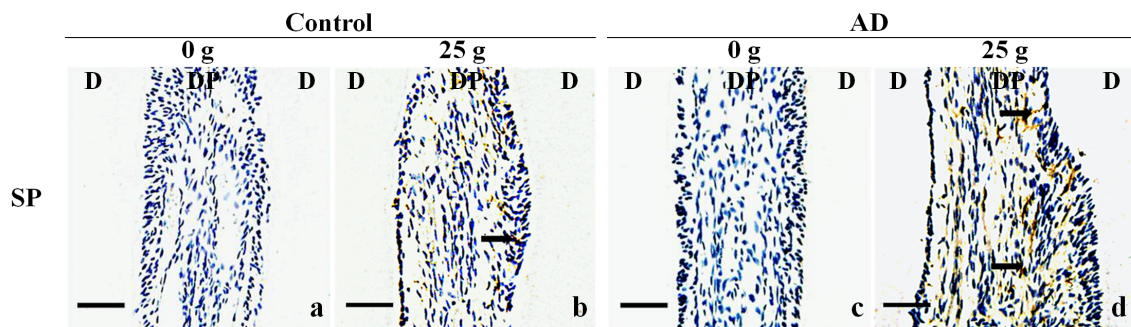


Fig. S2: Immunohistochemical staining was performed according to protocol of in this study. SP staining was performed using a monoclonal anti-mouse substance P antibodies (R&D systems, MN, USA; working dilution, 1:100) and the Histofine Simple Stain MAX-Po kit (Nichirei, Tokyo, Japan). The immunoreactivity for SP was examined on day 9 after tooth movement. SP-positive cells were rarely observed in either of the 0 g groups (a, c). In both of the 25 g groups, SP- positive cells were observed in the DP tissues on day 9 (b, d).