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Human Periodontal Ligament Fibroblasts Stimulate Osteoclastogenesis in Response to Compression Force Through TNF-α-Mediated Activation of CD4+ T Cells

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ABSTRACT

Periodontal ligament fibroblasts (PLF) sense and respond to mechanical stimuli and participate in alveolar bone resorption during orthodontic treatments. This study examined how PLF influence osteoclastogenesis from bone marrow-derived macrophages (BMM) after application of tension or compression force. We also investigated whether lymphocytes could be a primary stimulator of osteoclastic activation during alveolar bone remodeling. We found that mechanical forces inhibited osteoclastic differentiation from BMM in co-cultures with PLF, with PLF producing predominantly osteoprotegerin (OPG) rather than receptor activator of nuclear factor-kappaB (NF-κB) ligand (RANKL). In particular, PLF increased the expression of tumor necrosis factor (TNF)-α in response to compression. Additional experiments showed the presence of CD4- and B220-positive cells with a subsequent increase in tartrate-resistant acid phosphatase (TRAP)-positive cells and RANKL expression only at the compression side of the force-subjected periodontal tissues. Exogenous TNF-α increased the number of TRAP-positive cells and pit formation in the co-cultures of BMM with Jurkat, but not with BJAB cells and this effect was almost completely inhibited by antibodies to TNF-α or TNF receptor. Collectively, the current findings suggest that PLF secrete relatively higher levels of TNF-α at the compression side than at the tension side and this imbalance leads to RANKL expression by activating CD4+ T cells, thereby facilitating bone resorption during orthodontic tooth movement. J. Cell. Biochem. 112: 2891–2901, 2011.

KEY WORDS: PERIODONTAL LIGAMENT FIBROBLASTS; MECHANICAL FORCES; ALVEOLAR BONE REMODELING; TUMOR NECROSIS FACTOR-α; OSTEOCLASTOGENESIS; LYMPHOCYTES

Orthodontic tooth movement is the result of complicated and exquisite changes within the periodontium consisting of cementum, alveolar bone, periodontal ligament (PDL), and gingival [Bartold and Narayanan, 2006]. There are several phases in the process of tooth movement, in which the PDL plays a most important role [Pilon et al., 1996; Vas Leeuwen et al., 1999]. The initial phase is the recruitment of osteoclast and osteoblast progenitor cells, and the chemoattraction of inflammatory cells around and/or in the PDL. Development and removal of the hyalinized zones required for alveolar bone remodeling is made possible by the recruitment of phagocytic cells in the compression sides, while in tension regions collagenous and noncollagenous matrix substances are produced as a consequence of homeostasis to maintain the width of the PDL.

The PDL has extremely rapid turnover and high remodeling rates, properties that are important for its renewal and reparation of itself [Beertsen et al., 1997]. The regenerative potential of the PDL is believed to be related to the functions of the fibroblasts present in the PDL. PDL fibroblasts (PLF) are the most abundant cells in the PDL [Sodek and Limeback, 1979] and are known to play critical roles in maintaining PDL integrity and in mediating cellular responses to

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physiological and mechanical stresses [Lekic and McCulloch, 1996].

As bone metabolism is controlled by the balanced activation of osteoblasts and osteoclasts, numerous studies have suggested that PLF modulate the processes of alveolar bone remodeling through the production of various active cytokines which are essential for osteoclastic and osteoblastic differentiation [Kanzaki et al., 2002; Nishijima et al., 2006; Yamaguchi et al., 2006]. In fact, PLF induced osteoclast formation in co-culture with peripheral blood mononuclear cells or bone marrow cells through a receptor activator of nuclear factor-kappaB (NF-κB) ligand (RANKL)-dependent mechanism [Wada et al., 2001; Hasegawa et al., 2002; de Vries et al., 2006]. PLF also produce a decoy receptor for RANKL, osteoprotegerin (OPG), which is a soluble member of the tumor necrosis factor (TNF) receptor superfamily [Simonet et al., 1997; Kanzaki et al., 2006]. PLF produce RANKL and OPG in response to mechanical stimuli and this production is affected differently according to the mode of force applied. Overall, it is believed that PLF are a cell population with a central role in regulating osteoclastogenesis through RANKL/OPG-dependent pathways during alveolar bone remodeling by orthodontic force. However, the precise mechanisms by which PLF affect osteoclastogenesis in the process of alveolar bone resorption are still unclear.

Periodontitis is an inflammatory injury caused by a convergence of many bacteria adhering to and growing up on the tooth surface. Persistent and prolonged periodontitis induces periodontal tissue destruction and bone resorption, eventually leading to loss of the tooth. Bone resorption in periodontal disease is thought to be related to the activation of RANKL-expressing lymphocytes such as T and B cells [Baker et al., 2001; Harada et al., 2006; Kawai et al., 2006]. It is also apparent that both antigen-induced arthritis and inflammation-mediated osteoclastogenesis are driven by a sustained local production of pro-inflammatory cytokines derived mainly from lymphocytes [Udagawa, 2003; Wong et al., 2006]. It is important to consider that orthodontic tooth movement is a result accompanied by inflammatory responses in the periodontal tissue. Orthodontic force can stimulate PLF to secrete various neuropeptides which induce the migration of leucocytes into the capillaries present in and around PDL [Krishnan and Davidovitch, 2006]. Based on these findings, T and B lymphocytes are believed to participate in alveolar bone remodeling by orthodontic force. However, the possible roles of lymphocytes in bone resorption have not yet been defined.

Therefore, this study examined the exact roles of PLF in osteoclastic differentiation from bone marrow-derived macrophages (BMM) after the application of tension or compression force. This study also investigated whether lymphocytes are significant stimulators of osteoclastic activation in the compression region during orthodontic tooth movement using an experimental animal model.

MATERIALS AND METHODS

ANIMALS, CHEMICALS, AND LABORATORY WARES

Male wistar rats and BALB/c mice (6 weeks old) were purchased from Orient Co. (Seoul, South Korea). Flexible-bottomed six-well plates coated with type I collagen (COL I) were obtained from BioFlex® plates (Flexcell International Corporation, Hillsborough, NC). Recombinant human TNF-α was purchased from R&D systems, Inc. (Minneapolis, MN) and primary antibodies against OPG, RANKL, CD45R/B220, and CD4 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-TNF-α, anti-TNFR1, and anti-TNFR-2 antibodies were obtained from Pharmingen (San Diego, CA). Unless specified otherwise, other chemicals and laboratory wares were obtained from Sigma Chemical Co. (St. Louis, MO) and SPL Life Sciences (Pochun, South Korea), individually.

CELL CULTURES

PLF were obtained from healthy male individuals aged 20–30 years and cultured according to methods described elsewhere with slight modifications [Howard et al., 1998]. Written informed consent for tissue use was obtained from all donors. This study was approved by the Ethical Committee of Chonbuk National University Hospital (A084283). Briefly, single cell suspensions of PLF were incubated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and antibiotics (100 IU/ml penicillin G and 100 μg/ml streptomycin) in 100-mm culture dishes. After the cells reached confluence, they were subcultured for several passages before the application of mechanical force. All the experiments were performed at passages 4 through 7.

Several fibroblastic cells including human gingival fibroblasts (GF), normal skin fibroblast cell line (SFN), and skin fibrosarcoma cell line (SFC), as well as human lymphoma BJAB and Jurkat cells were cultured in DMEM supplemented with 10% FBS and antibiotics before co-culturing with BMM. Unless specified otherwise, the fibroblastic cells were divided into culture plates at density of 1×10^6 cells/ml and applied to experiments when they had reached 80% confluence.

CHARACTERIZATION OF PLF

Before the application of mechanical forces, PLF were characterized by staining for a specific marker, fibromodulin, followed by counterstaining with 4′-6-diamidino-2-phenylindole (DAPI) and FITC-tubulin [Lallier et al., 2005].

APPLICATION OF MECHANICAL FORCES IN VITRO

PLF suspensions (1×10^6 cells/ml) were spread onto flexible-bottomed six-well plates coated with COL I and further cultured until the cells had reached 80% confluence. For application of tension force, the flexible plates were subjected to static waves of 1.5% elongation for 1 h using a computer-controlled vacuum stretch apparatus (FX-4000 Tension/Compression System). Other PLF cultured on the plates were embedded in the COL I gel matrix and received a compression force with the magnitude of 0.5% elongation for 1 h. At various times after the force application, cells were processed for analyses of cytokine expression and osteoclast formation.

PREPARATION OF HUMAN GINGIVAL CREVICULAR FLUID (GCF)

Five healthy male volunteers, aged 27.0 ± 1.0 year, were used for the collection of GCF, and informed written consent for use of the GCF was obtained from all patients before this experiment. This study was also approved by the Ethical Committee of Chonbuk National University Hospital (A084283). In brief, deposits of plaque around

the premolar teeth were removed with a periodontal probe before collection of the GCF samples. Filter paper strips were inserted into the mesiobuccal and distobuccal gingival crevice around the teeth to a depth of 1 mm for 4 min. The volunteers were given oral hygiene motivation after GCF collection. GCF volume for each sample was measured by weighing the strips which were then stored at −80°C until the samples were analyzed by enzyme-linked immunosorbent assay (ELISA).

BMM PREPARATION

Bone marrow cells were obtained from 6- to 8-week-old BALB/c mice and cultured according to methods described elsewhere [Cho et al., 2010]. Animal care and use was approved by the Chonbuk National University Committee on Ethics in the Care and Use of Laboratory Animals (CBU 2010-0007). In brief, the tibiae and femora were removed from mice, and bone marrow samples were harvested by flushing the bones with modified essential medium (MEM) supplemented with 10% fetal calf serum (FCS; Hyclone) and antibiotics. Five milliliters of bone marrow suspension (2×10^6 cells/ml) were divided into 100 mm culture plates in the presence of 50 ng/ml monocyte-colony stimulating factor (M-CSF). After culturing for 3 days, adherent cells were collected using a scraper and examined by flow cytometric analysis for the presence of CD3, B220, and F4/80 (data not shown). This analysis revealed that approximately 95% of the cells were considered to be BMM (CD3- and B220-negative and F4/80-positive).

OSTEOCLASTIC DIFFERENTIATION AND TRAP STAINING

BMM were divided on the top of PLF cultured in the flexible plates at a density of 10^5 cells per well and incubated with or without 10 nM 1,25-dihydroxy vitamin D_3 (1,25-$(OH)_2D_3$) and 100 nM dexamethasone (Dex) in the presence or absence of 1 μg/ml anti-OPG antibody. After 1 day of co-incubation, these cells were subjected to mechanical forces as described above and further incubated for various times. In parallel, GF, SFN, or SFC were co-cultured with BMM with or without 1,25-$(OH)_2D_3$, Dex, and/or anti-OPG antibody. BJAB and/or Jurkat cells were also co-cultured with BMM in the presence or absence of 10 ng/ml TNF-α for 4 days. As positive controls, BMM were incubated in medium supplemented with 50 ng/ml M-CSF, 50 ng/ml RANKL, and 10 ng/ml TNF-α.

The control and force-applied cultures were fixed in 4% PBS-buffered para-formaldehyde and stained for tartrate-resistant acid phosphatase (TRAP) using a Sigma Aldrich kit according to the manufacturer's instructions. TRAP-positive mononuclear cells were counted using optic microscopy, and cells containing three or more nuclei were considered to be multinucleated osteoclasts (MNCs).

MEASUREMENT OF CYTOKINES

Conditioned media were prepared from PLF cultures or the co-cultures with BMM for the determination of OPG, RANKL, and TNF-α levels using ELISA kits. In addition, samples of GCF collected on filter paper strips were resuspended in 200 μl of phosphate buffered saline (PBS) buffer before centrifugation at 12,000*g* for 10 min. The supernatants were processed for the analyses of cytokines. ELISA kits for human OPG (BI-20402, Biomedica, Vienna, Austria), RANKL (BI-20422H, Biomedica), and TNF-α (EA-0203, Signosis, Inc., Sunnyvale, CA) were used to detect the cytokines, and each ELISA was performed according to the manufacturer's instructions. The experiment was performed in triplicate and the data were compared to a standard curve.

WESTERN BLOT ANALYSIS

Whole cell lysates were prepared from PLF at various times of incubation without force application using a NP-40 lysis buffer (30 mM Tris-Cl, pH 7.5, 1 mM EDTA, 150 mM NaCl, and 1% NP-40). Equal amounts (30 μg/sample) of protein extracts were separated by 12% SDS-PAGE and blotted onto poly vinyl difluoride membranes. The blots were probed with primary antibodies specific for RANKL and OPG overnight at 4°C prior to incubation with secondary antibody in a blocking buffer for 1 h. The blots were developed with enhanced chemiluminescence (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK) and exposed on X-ray film (Eastman-Kodak, Rochester, NY).

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted from the control or force-exposed PLF according to the manufacturer's instructions (TRI reagent, Molecular Research Center, Cincinnati, OH). Reverse transcription and PCR amplification were performed using an Access RT-PCR System (Promega, Madison, WI) according to the manufacturer's protocol. The primer sequences used were as follows: OPG (−253 bp), 5′-GAG TGT CTA TAC TGC AGC CC-3′ (forward) and 5′-TGT TTT CTA CAG GGT GCT TT-3′ (reverse); RANKL (−233 bp), 5′-TGG TTC CCA TAA AGT GAG TC-3′ (forward) and 5′-AGG GTA TGA GAA CTT GGG AT-3′ (reverse); TNF-α (−128 bp) 5′-CTG GTA TGA GCC CAT CTA TC-3′ (forward) and 5′-GCA ATG ATC CCA AAG TAG AC-3′ (reverse). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified with the primers 5′-AAC CTG CCA AAT ATG ATG AC-3′ (forward) and 5′-ATA CCA GGA AAT GAG CTT GA-3′ (reverse). PCR was performed for 30–35 cycles at 94°C for 30 s, at 50–55°C for 30 s, and at 72°C for 1 min in a DNA thermal cycler (PTC-100, Waltham, MA). PCR products were analyzed on a 1.5–2% agarose gel and visualized by ethidium bromide staining. PCRs were repeated in at least three independent RNA preparations, and band intensities were calculated using a gel imaging system (model F1-F2 Fuses type T2A, BIO-RAD, Segrate, Italy).

APPLICATION OF MECHANICAL FORCE IN VIVO AND IMMUNOHISTOCHEMISTRY

Mechanical force was applied to wistar male rats (n = 10, each weighing 180-200 g) by placing elastic bands (diameter 4.8 mm, TP Orthodontics Inc., AZ, USA) between the maxillary first and second molars after anesthetizing the rats with a mixture of ketamine (90 mg/kg) (Yuhan Co., Seoul, Korea) and xylazine (5 mg/kg) (Sigma Chemical Co.). Placing the elastic bands between the molars leads to a wedging effect and simultaneously creates both compression and tension areas around the roots of the first and second molars.

For immunohistochemistry, the maxillae of the rats were dissected after 3 days of banding and then processed for

decalcification in 0.5 M EDTA (pH 7.2) for 3 weeks. The decalcified specimens were dehydrated in ascending concentrations (from 40 to 100%) of ethanol before being immersed in xylene substitute for 1 h. Specimens were embedded in paraffin, horizontally cut to 5 μm thickness, and mounted on microscope slides. After removal of paraffin with xylene and washing with distilled water, the specimens were exposed to hydrogen peroxide and then 5% goat serum. The sections were incubated with primary antibodies to OPG (polyclonal goat antibody, 1:50), RANKL (polyclonal goat antibody, 1:50), CD45R/B220 (monoclonal mouse antibody, 1:50), and CD4 (monoclonal rat antibody, 1:50) in a humid chamber for 2 h. After washing with PBS three times, the sections were treated with secondary anti-mouse IgG-FITC, anti-rabbit IgG-FITC, or anti-goat IgG-TRICT antibody for 1 h, or with horseradish peroxidase-conjugated secondary antibody (Vector Laboratories, CA) for 10 min, followed by conjugation with DAB substrate kit (Vector Laboratories, CA). After counterstaining with DAPI and Mayer's hematoxylin, the sections were observed under fluorescence microscopy (Axiovision 3.1, Carl Zeiss, Germany) or confocal laser scanning microscopy (LSM 510 META, Carl Zeiss).

BONE RESORPTION ASSAY

BMM (1×10^5 cells/ml) were divided onto the top of PLF or Jurkat cells cultured in a 24-well plate coated with calcium phosphate nano crystal (OAAS-24; Osteoclast Activity Assay Substrate, Oscotec Inc., Choongnam, South Korea) at a density of 2×10^4 cells/cm^2. For the positive control, BMM only were divided into the calcium-coated plates in the presence of 50 ng/ml M-CSF, 50 ng/ml RANKL, and 10 ng/ml TNF-α. Seven days after incubation, the cells were removed from the plates by treatment with 5% sodium hypochlorite, and pit formation was observed under an optic microscope. The resorbed area was also measured by image analyzer and expressed as a percentage (%) of the control value.

STATISTICAL ANALYSIS

Unless specified otherwise, all data were expressed as the mean ± standard deviation (SD). One-way ANOVA was used for multiple comparisons using SPSS version 18.0 software. A P value <0.05 was considered statistically significant.

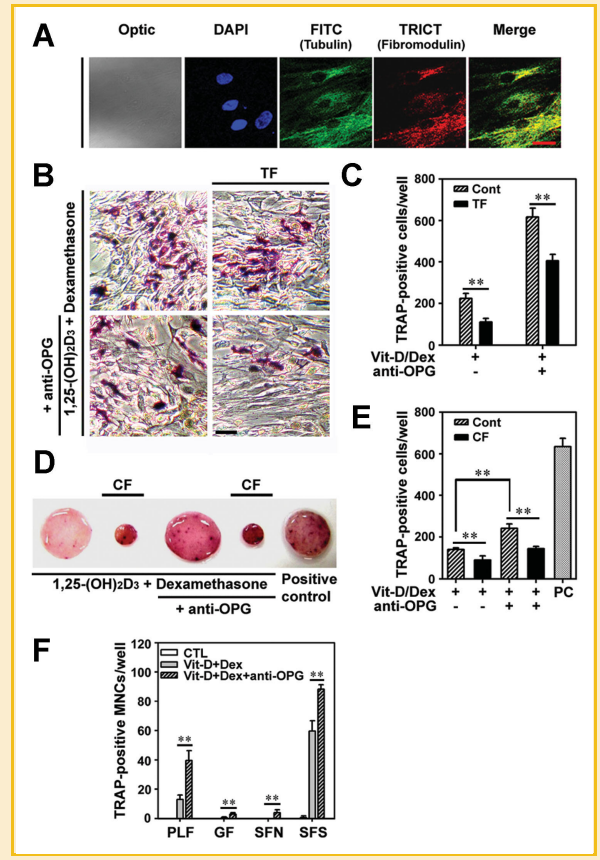

Fig. 1. Mechanical force inhibits osteoclastic differentiation of BMM in co-cultures with PLF through an OPG-dependent mechanism. A: Characterization of human PLF. Bar = 25 μm. PLF were co-cultured with BMM in the presence of 10 nM 1,25-$(OH)_2D_3$ and 100 nM Dex with and without 1 μg/ml anti-OPG antibody and then subjected either to tension (B) or compression force (D) for 1 h. Bar = 50 μm. These cells were subjected to TRAP staining 4 days after exposure to force and the numbers of TRAP-positive cells were counted from triplicate experiments (C and E). F: Various types of fibroblast cells including PLF, GF, SFN, and SFS were incubated in combination with BMM in the presence and absence of 10 nM 1,25-$(OH)_2D_3$, 100 nM Dex, and/or 1 μg/ml anti-OPG antibody for 4 days. TRAP-positive MNCs were calculated from triplicate experiments. $^{**}P < 0.01$ versus the experiments. TF, tension force; CF, compression force; PC, positive control. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

RESULTS

MECHANICAL FORCE INHIBITS OSTEOCLASTIC DIFFERENTIATION FROM BMM IN CO-CULTURES WITH PLF

We initially evaluated if PLF were contaminated with other cells by immunostaining the cells. As shown in Figure 1A, the cells were mostly stained with a specific marker of PLF, fibromodulin (red), where the cells were counterstained with DAPI (blue) for nucleus and tubulin (green) for cytoplasm. In the absence of 1,25-$(OH)_2D_3$ and Dex, PLF did not stimulate osteoclastic differentiation from BMM in the co-cultures and applying tension or compression force to the cultures did not affect this outcome (Supplement Fig. 1A and 1B). The combined treatment of the co-cultures with 1,25-$(OH)_2D_3$ and Dex induced the formation of TRAP-positive cells and this formation was further augmented by adding anti-OPG antibody to the co-cultures (Fig. 1B–E). However, the application of tension or compression force to the co-cultures significantly diminished the number of TRAP-positive cells that were induced by treating with 1,25-$(OH)_2D_3$ and Dex (Fig. 1C and E). This reduction was related to the production of OPG, in that anti-OPG antibody attenuated the force-induced decrease in TRAP-positive cells in the co-cultures. In addition to the PLF, anti-OPG antibody accelerated the osteoclastic differentiation of BMM in co-cultures with GF, SFN, or SFS in the presence of 1,25-$(OH)_2D_3$ and Dex (Supplement Fig. 1C), although the number of TRAP-positive MNCs was different according to the origins of fibroblastic cells used in the co-culture (Fig. 1F). Further, the presence of Dex in the induction of osteoclastic differentiation did not cause any side effects on the results, although it was also known as osteogenic inducer [Guzmán-Morales et al., 2009].

PLF PRODUCE MORE OPG THAN RANKL IN VITRO SYSTEM

We determined the levels of OPG and RANKL expressed by PLF grown in vitro and in vivo with neither mechanical stimulation nor addition of 1,25-$(OH)_2D_3$ and Dex. The results revealed that OPG was naturally more expressed than RANKL, as shown by the relatively high ratio of OPG to RANKL in the conditioned media of PLF cultures (Fig. 2A) and the mRNA levels (Fig. 2B). The level of OPG in the media was approximately sevenfold higher than RANKL at 3 days of the incubation. Figure 2C shows the protein profiles of RANKL and OPG in PLF at various times (0–120 h) after incubation. Similarly, PLF that were embedded only within the collagen gel matrix showed more prominent FITC intensity for OPG than RANKL (Fig. 2D). These results suggest that the nature of PLF is more inclined towards the production of OPG than RANKL. However, immunostaining of periodontal tissue sections did not show a significant difference between the protein levels of OPG and RANKL in the PDL (Fig. 2E). Further, the level of RANKL in human GCF was approximately sixfold higher than that of OPG (Fig. 2F). In the presence of 1,25-$(OH)_2D_3$ and Dex, the level of RANKL in the culture supernatants of PLF also gradually increased according to the times incubated, whereas the level of OPG was not changed (Fig. 2G).

COMPRESSION FORCE INCREASES THE PRODUCTION OF TNF-α, WHICH STIMULATES OSTEOCLASTOGENESIS OF BMM IN CO-CULTURES WITH PLF

We next investigated the properties of PLF regarding production of OPG, RANKL, and TNF-α in response to mechanical stimuli. Application of either tension or compression force to PLF increased mRNA expression of OPG, while it decreased the level of RANKL (Fig. 3A–D). TNF-α mRNA levels were differently changed according to the condition of forces administered; tension force reduced TNF-α expression in PLF, but compression force dramatically increased its mRNA levels. The results from ELISA showed that, unlike RANKL, OPG levels in the conditioned medium of PLF cultures were significantly increased after the application of tension or compression force (Fig. 3E). The tension-mediated reduction and the compression-mediated increase in TNF-α levels were also seen in the PLF cultures. The combined treatment of the co-cultures of

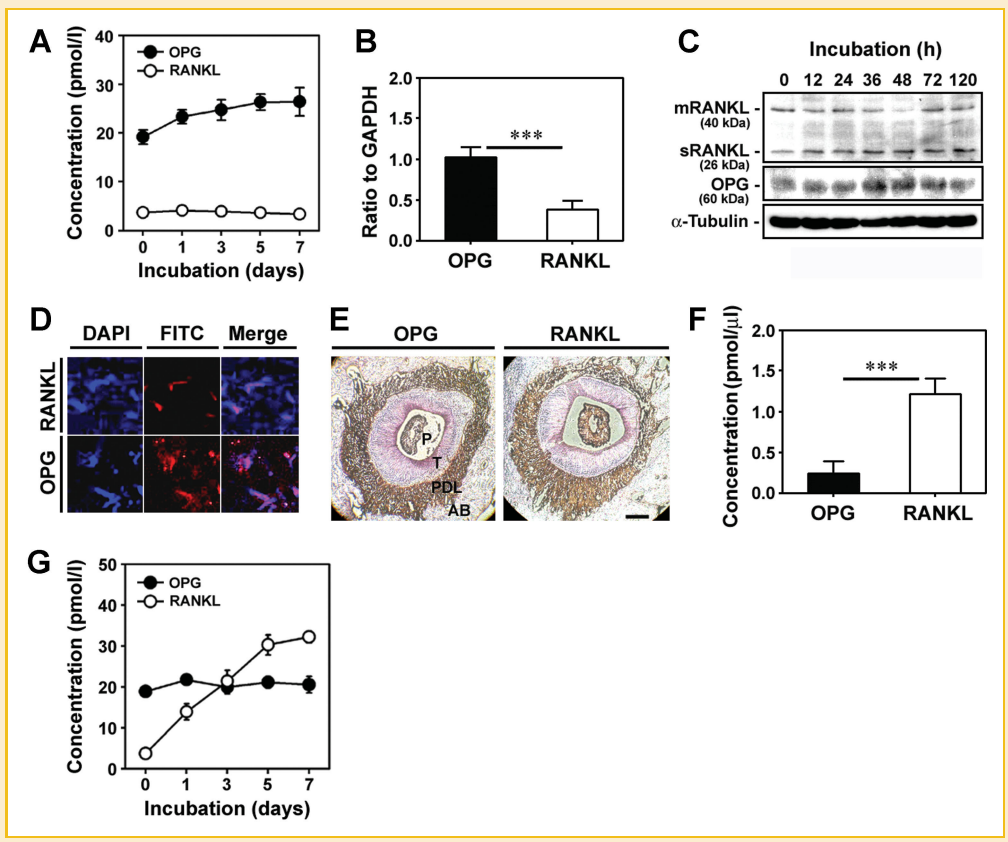

Fig. 2. PLF themselves express predominantly more OPG than RANKL in vitro. A: The levels of OPG and RANKL secreted in the culture supernatants of PLF were determined by ELISA at the indicated times (0–7 days). B: After 3 days of incubation, the mRNA levels specific for OPG and RANKL were analyzed by RT-PCR. C: The levels of cellular RANKL and OPG proteins were evaluated by Western blot analysis at the indicated times (0–120 h). D: Paraffin sections of PLF embedded in COLI gel matrix were stained with FITC-bound OPG and RANKL antibodies. E: Paraffin sections of the unloaded periodontal tissues were also stained with antibodies against OPG and RANKL. Bar = 500 μm. F: OPG and RANKL levels in GCF samples were determined using ELISA kits. G: At the indicated times, OPG and RANKL levels in the conditioned media of PLF cultures were determined by ELISA. $^{***}P < 0.001$ versus the experiments. P, pulp; T, tooth; AB, alveolar bone. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

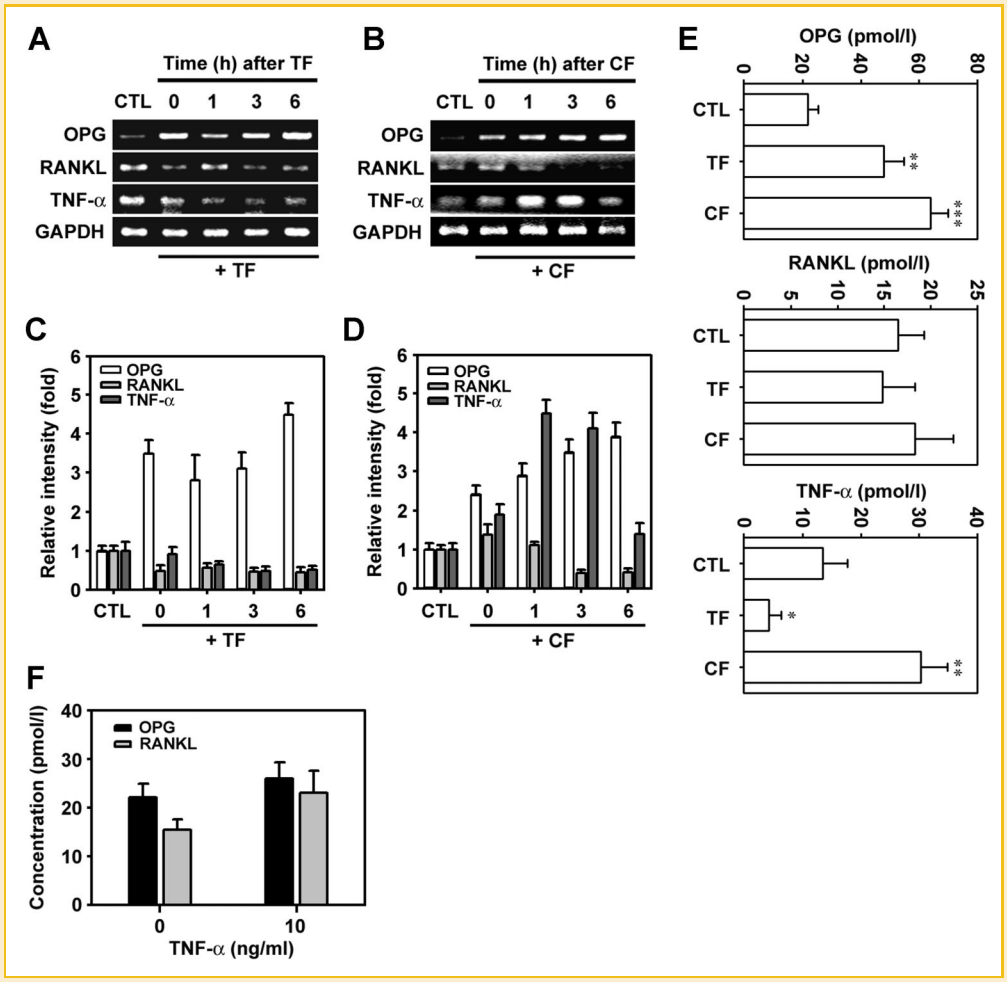

Fig. 3. Compression force increases the production of TNF-α which stimulates osteoclastogenesis of BMM in co-cultures with PLF. PLF were subjected to tension (A) or compression force (B) for 1 h and at the indicated times, the levels of OPG, RANKL, and TNF-α mRNA expression were determined by RT-PCR. C–D: The ratios of mRNA expression of OPG, RANKL, and TNF-α were determined from triplicate experiments quantified by densitometry after normalizing the bands to GAPDH. E: Culture supernatants of PLF cultures were collected after 24 h of applied force and then processed for the determination of these cytokines by ELISA. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the unloaded controls. F: PLF were exposed to 10 ng/ml TNF-α for 24 h and the levels of OPG and RANKL proteins were determined by ELISA.

PLF and BMM with 1,25-$(OH)_2D_3$, Dex, and TNF-α did not facilitate the formation of TRAP-positive cells, but significantly augmented the induction of TRAP-positive MNCs as well as the activity of bone resorption (data not shown). Exogenous TNF-α did not affect the levels of OPG and RANKL produced by PLF itself in the presence of 1,25-$(OH)_2D_3$ and Dex (Fig. 3F).

INCREASED NUMBERS OF B AND T LYMPHOCYTES ARE FOUND AT THE COMPRESSION SIDE WITH A CORRESPONDING INDUCTION OF RANKL AFTER MECHANICAL FORCE APPLICATION

The upper molars of periodontal tissues were prepared from rats subjected to mechanical force just before immunostaining (Supplement Fig. 2). Dramatic increases in both CD4 and B220 expression appeared around the compression sides with a subsequent increase in TRAP-positive cells, as compared to the untreated control tissues (Fig. 4A and B). These increases were not found on the tension side (data not shown). The data from confocal analysis also showed that the apparent stimulation of RANKL expression was accompanied by an increase in lymphocytes, thereby suggesting the involvement of B and CD4+ T cells in alveolar bone resorption during orthodontic tooth movement (Fig. 4C and D).

TNF-α STIMULATES OSTEOCLASTOGENESIS FROM BMM IN CO-CULTURES WITH JURKAT CELLS

In order to determine if lymphocytes were actually related to the induction of osteoclastic differentiation, BJAB or Jurkat cells alone or co-cultures of the two cell types were exposed to TNF-α (Fig. 5). The basal levels of cytokines produced in the co-cultures were apparently higher in RANKL than in OPG, where the level of RANKL produced by BJAB or Jurkat cells was approximately 3.4- or 4-fold higher than that of OPG. Stimulation of these cells with TNF-α significantly augmented RANKL levels in Jurkat alone or in combination with BJAB cells, although the level of OPG was also increased after treatment with TNF-α only in the co-cultures of these

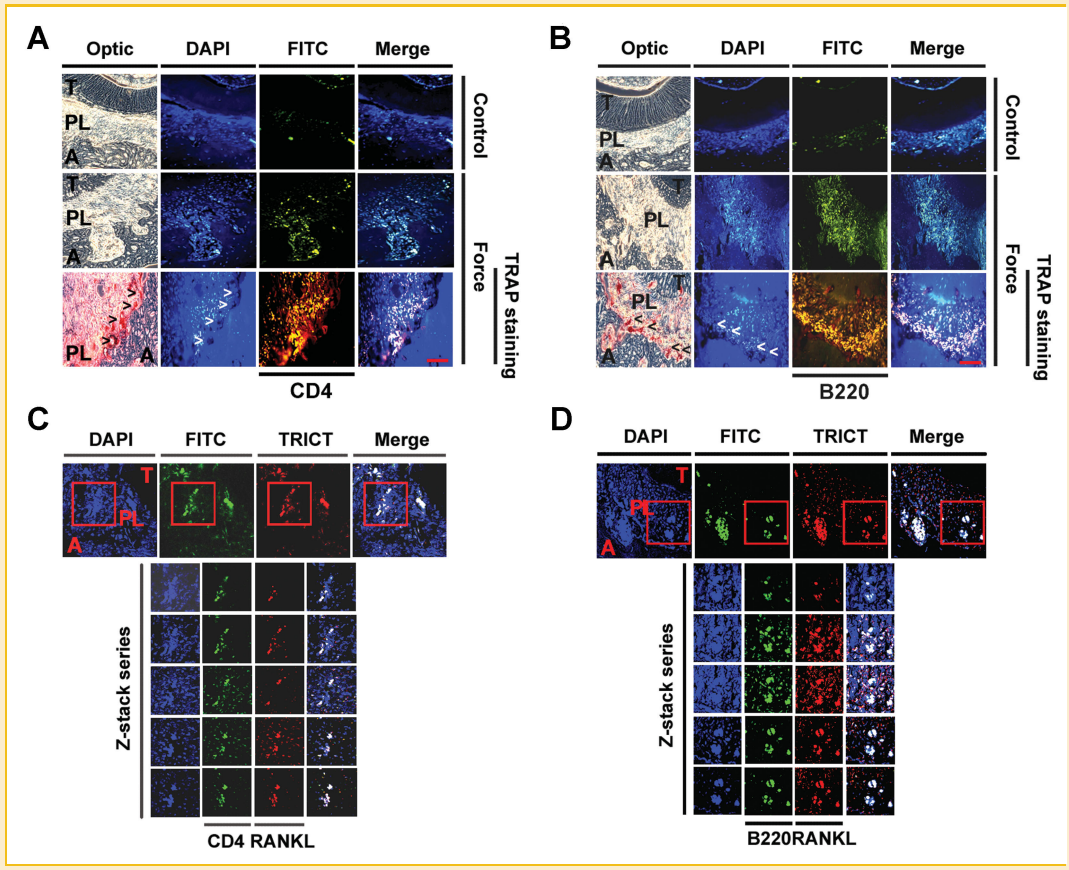

Fig. 4. Numerous B and T lymphocytes are found at the compression side with an attendant increase in RANKL after mechanical force application. Paraffin sections of the first and second molars were stained with CD4- (A) or B220-specific markers (B), followed by double-staining with TRAP. Black and white arrows represent TRAP-positive cells. Bar = 250 μm. C and D: These paraffin sections were immunohistochemically stained with antibodies against CD4, B220, and RANKL, and the results were visualized using a confocal laser scanning microscope. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

cells. Especially, the exposure of Jurkat cells to TNF-α augmented the RANKL levels up to 6.1 pmol/L, compared to the untreated controls (1.2 pmol/L).

Induction of TRAP-positive cells was found in the co-cultures of BMM with Jurkat in the presence of TNF-α (Fig. 6A). The number of TRAP-positive cells was approximately fourfold higher when BMM cells were co-cultured with Jurkat cells than when they were grown in combination with BJAB cells (Fig. 6B). When BMM were incubated with TNF-α in the presence of M-CSF, TRAP-positive cells were induced and this was not affected by adding OPG into the cultures (Fig. 6C). The combined treatment of BMM with RANKL dramatically accelerated the formation of TRAP-positive cells, whereas this additive effect was almost completely inhibited by the addition of OPG. Therefore, we suggested that exogenous TNF-α could stimulate T cells to induce osteoclastogenesis through the production of RANKL. This possibility was supported by the finding that, in the presence of TNF-α, the co-cultures of BMM and Jurkat cells showed pit formation, which was slightly reduced in the presence of BJAB cells (Fig. 6D and E). Furthermore, TNF-α-mediated increases in TRAP-positive MNCs and pit formation in the co-cultures with Jurkat cells were almost completely inhibited by pre-treating them with anti-TNF-α antibody or a mixture of anti-TNFR1 and anti-TNFR2 antibodies (Fig. 6F). Although the facilitative effects of TNF-α on osteoclast formation were not as dramatic as those observed in the positive controls where BMM were incubated in combination with RANKL, these data indicate that TNF-α is, at least in part, involved in osteoclastogenesis through an interaction with T lymphocytes.

DISCUSSION

Osteoclastogenic processes are tightly regulated by the ratio of RANKL and OPG [Jimi et al., 1999]. This study shows that PLF themselves tend to prevent osteoclastic differentiation through the predominant production of OPG rather than RANKL. Evidence of this property was also supplied by the finding that exogenous anti-OPG antibody augmented the formation of TRAP-positive cells in the co-cultures of BMM with PLF. These findings were in agreement with a report showing that the addition of conditioned media from PLF culture into BMM inhibited osteoclastic activation and pit formation [Wada et al., 2001]. It was also reported that in co-cultures of PLF with BMM, TRAP-positive MNCs were not visible unless 1,25-$(OH)_2D_3$ and Dex were co-administered [Hasegawa et al., 2002]. It

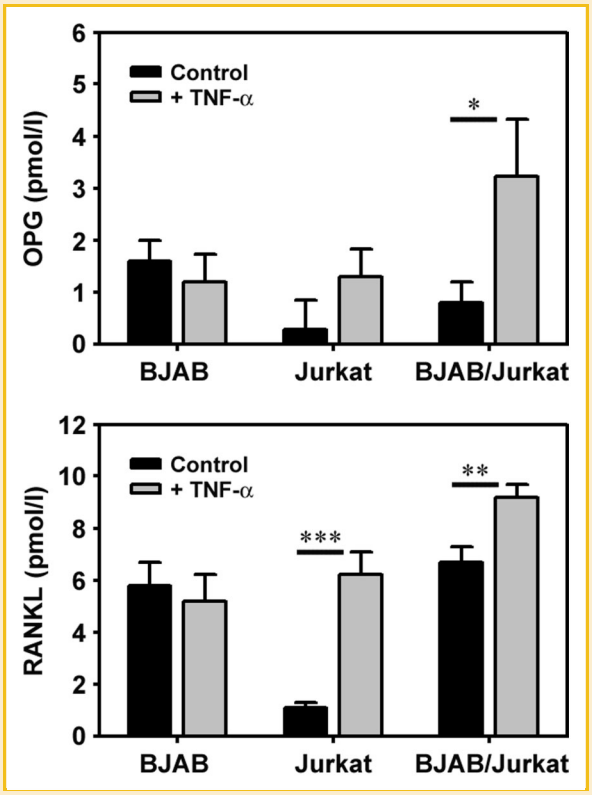

Fig. 5. Exogenous TNF-α increases the secretion of RANKL in Jurkat cells. Single cultures of BJAB and Jurkat cells or the combined cultures of the two cell types were exposed to 10 ng/ml TNF-α for 24 h and then OPG and RANKL levels in the culture supernatants were determined using ELISA kits. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the experiments.

has been suggested that PLF have the potential to prevent bone resorption, although they are also capable of inducing the maturation of osteoclast precursor cells in co-cultures with peripheral blood mononuclear cells [de Vries et al., 2006]. PLF have the capacity to prevent alveolar bone resorption through induction of a high ratio of OPG to RANKL in a normal physiological environment [Kanzaki et al., 2006; Nishijima et al., 2006; Nakao et al., 2007]. These suggest that the default function of PLF could inhibit undesirable bone resorption in physiological conditions without mechanical stimulation. However, it is important to note that the level of RANKL in human GCF was higher than that of OPG, and co-incubation of PLF with 1,25-$(OH)_2D_3$ and Dex increased the level of RANKL only. We suggest that this difference is derived from the presence of other cells such as lymphocytes as well as of stimulators such as several pro-inflammatory cytokines. Immunostaining results also revealed that unlike in the cultured PLF, the protein levels of both OPG and RANKL in the PDL were not differed at a significant level. Collectively, our present results suggest a nature of PLF to produce predominantly OPG more than RANKL, as well as a presence of cellular sources which are responsible for RANKL secretion in and around the PDL.

Orthodontic tooth movement is a process mediated simultaneously by bone deposition on the tension side and bone resorption on the compression side of the PDL [Krishnan and Davidovitch, 2006]. The current findings suggest that the application of tension or compression force significantly reduced osteoclastic differentiation from BMM in co-cultures with PLF and that this occurrence was related predominantly to the tendency of PLF to produce OPG in response to these forces. The results of the current study indicate that various fibroblastic cells naturally produce OPG, thereby supporting the inhibitory roles of fibroblasts on osteoclastogenesis. There are also significant findings showing that mechanical force induces the production of both OPG and RANKL by PLF, while OPG is predominant over RANKL [Yamamoto et al., 2006; Nakajima et al., 2008; Kook et al., 2009]. Importantly, however, the results from GCF analysis showed that the levels of RANKL are naturally higher than those of OPG in this cell type. Incubation of PLF with 1,25-$(OH)_2D_3$ and Dex also stimulated the production of RANKL, but not of OPG, in a time-dependent manner. These results lead us to hypothesize that, at least for in vivo conditions, other RANKL-producing sources which are capable of inducing osteoclastic activation at the compression side will be needed for bone resorption during orthodontic treatment.

Periodontitis is an inflammatory periodontal disease that is triggered by host immune response against periodontal biofilm-associated microorganisms. Rheumatoid arthritis is also a chronic inflammatory disease characterized by the destruction of articular cartilage and bone [Udagawa, 2003]. Accumulated evidence suggests that inflammation-mediated bone resorption can be attributed to the biological actions of B cells [Choi et al., 2001; Harada et al., 2006; Han et al., 2006] and T cells [Teng et al., 2000; Baker et al., 2001]. Orthodontic tooth movement is a process that is accompanied by inflammation and it has been suggested that T and B cells play important roles in bone resorption during this movement. This idea was supported by the current findings using an in vivo experimental model, which showed the marked expression of RANKL, the migration of B and CD4+ T cells into and/or around compression sides, and a concomitant increase in TRAP-positive cells after mechanical force. It has also been shown that peripheral release of vasoactive neurotransmitter after orthodontic force triggers the migration of leucocytes into the capillaries and also activates various types of PDL cells [Davidovitch et al., 1988; Krishnan and Davidovitch, 2006]. Taken together, these results reveal the involvement of B and T cells in the alveolar bone resorption by orthodontic force.

TNF-α promotes RANKL expression by marrow stromal cells and osteoblasts, and augments the responsiveness of osteoclast precursors to RANKL [Takayanagi, 2005; Takayanagi et al., 2005]. TNF-α is also capable of inducing osteoclast differentiation from BMM and bone resorption in the presence of M-CSF, a process that is further facilitated by RANKL [Cho et al., 2010]. In order to verify the roles of TNF-α on lymphocytes in the production of OPG and RANKL, the cultures of Jurkat or BJAB alone, or the co-cultures of these cells were exposed to 10 ng/ml TNF-α. Unlike BJAB cells, Jurkat cells responded sensitively to TNF-α and produced predominantly RANKL rather than OPG. Exogenous TNF-α also apparently stimulated the formation of TRAP-positive cells from BMM and pit formation only in the co-cultures with Jurkat cells. These effects of TNF-α were almost completely suppressed by pre-treating cell cultures with anti-TNF-α antibodies or anti-TNF-α receptor antibodies.

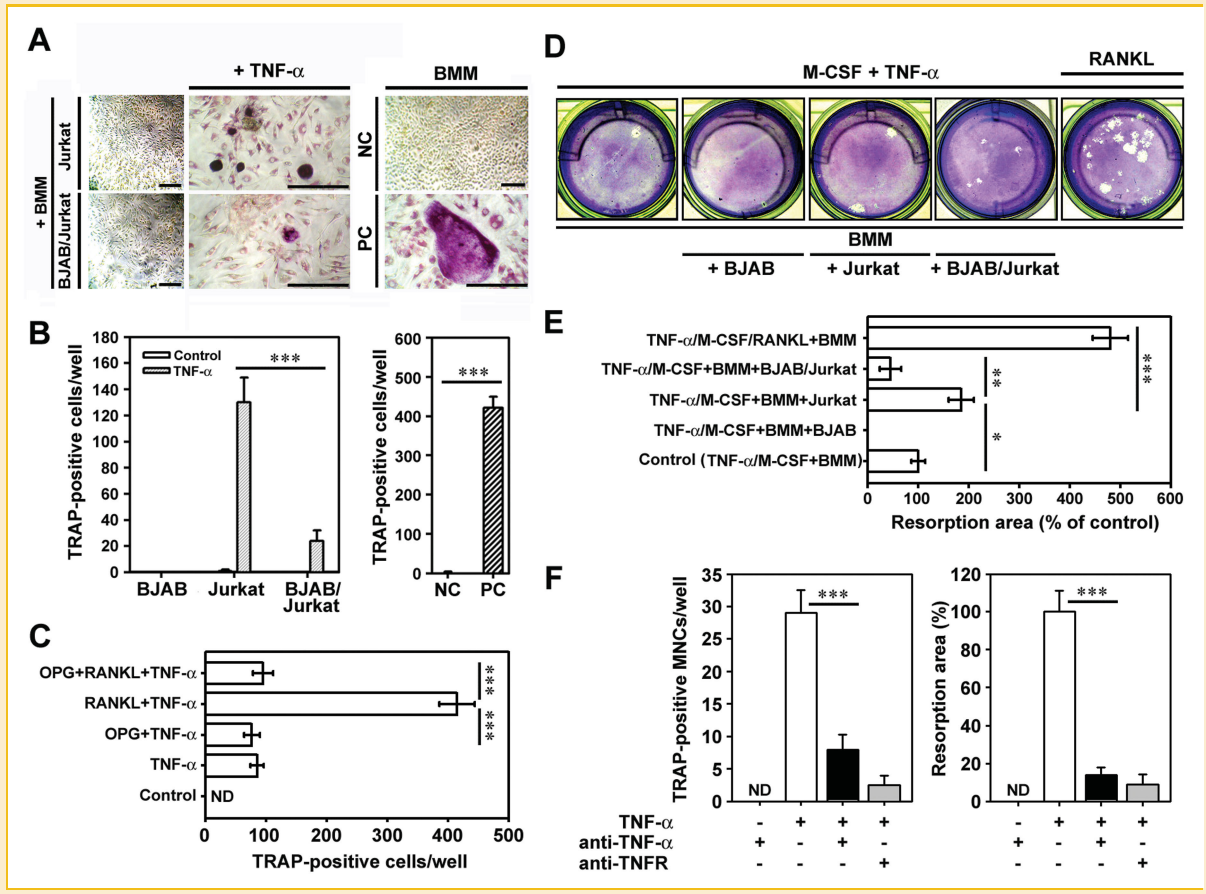

Fig. 6. TNF-α stimulates osteoclastogenesis from BMM in co-cultures with Jurkat cells. A: BJAB and/or Jurkat cells were co-cultured with BMM in the presence of 10 ng/ml TNF-α for 4 days and then processed for TRAP staining. BMM were also incubated in the absence (NC, negative control) or presence of M-CSF, RANKL, and TNF-α (PC, positive control). Bar = 100. B: The number of TRAP-positive cells was counted from triplicate experiments. C: BMM were incubated with the indicated cytokines in the presence of M-CSF and after 7 days of incubation, the cells were processed for TRAP staining. In addition, BMM were cultured with and without BJAB, Jurkat, or in combination with them in the presence of M-CSF, TNF-α, and/or RANKL. D: Pit formation was observed under optic microscopy and (E) resorption area was calculated as relative percentage to the control value. Furthermore, Jurkat cells were co-incubated with BMM in bone-coated 24-well plates in the presence of 10 ng/ml TNF-α, anti-TNF-α antibody (10 μg/ml), and/or a mixture containing anti-TNFR1 (10 μg/ml) and anti-TNFR2 (10 μg/ml) antibodies. F: TRAP-positive MNCs were calculated after 4 days of the incubation, and the resorbed areas were quantified from triplicate experiments after 7 days of the incubation. ND, not detected. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Osteoclasts are derived from hematopoietic precursors of the monocyte/macrophage lineage in response to osteoclastogenic cytokines, including RANKL, M-CSF, and TNF-α. Although RANKL is considered to be the most essential factor for osteoclast formation and bone resorption [Kong et al., 1999; Lam et al., 2000], TNF-α is believed to induce differentiation of osteoclasts from M-CSF-derived macrophages in a RANKL-independent manner [Azuma et al., 2000; Kobayashi et al., 2000]. Our previous study reported that TNF-α induced the formation of TRAP-positive MNCs from BMMs only in the presence of M-CSF. This finding suggested that in the presence of M-CSF, TNF-α plays a critical role in inducing osteoclast formation and bone resorption at the compression side that occurs during orthodontic tooth movement. Similarly, there were numerous investigations to explore the roles of cytokines during orthodontic tooth movement, where TNF-α was believed to play the most important roles [Andrade et al., 2007; Garlet et al., 2007; Kitaura et al., 2008]. The current findings also indicate that TNF-α is produced by PLF and then induces the activation of T cells which are essential sources of RANKL, although the present study does not show a direct evidence on the interaction between TNF-α and lymphocytes. In addition, it is important to consider that TNF-α leads to bone resorption by acting directly on osteoclast differentiation and maturation, and by indirectly exposing the bone matrix, where synergistic interactions between TNF-α and RANKL are able to occur [Assuma et al., 1998].

In summary, the bone and immune systems share numerous cellular signals and regulatory mechanisms. An exact osteoimmunological understanding of bone resorption occurring in alveolar bone will provide strategies for the optimal treatment of orthodontic force as well as of periodontal disease. It is believed that activated T cells induce osteoclast differentiation by directly acting on osteoclast precursor cells under pathological conditions through the expression of RANKL. As the local production of TNF-α is important to T cell activation, the current findings suggest that PLF might secrete TNF-α at the compression side at relatively higher levels than at the tension side, thus leading to RANKL expression by

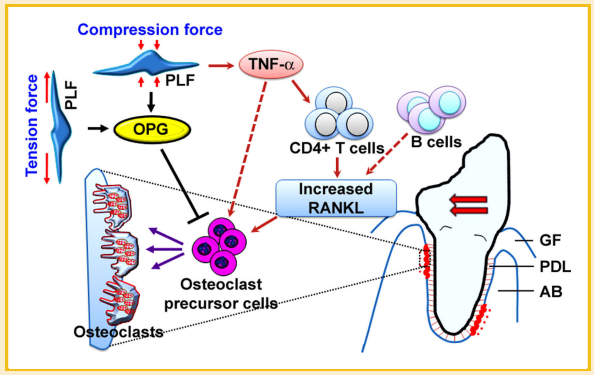

Fig. 7. Schematic drawing of the roles of PLF in the process of alveolar bone remodeling during orthodontic tooth movement. PLF secrete TNF-α at the compression side at relatively higher levels than at the tension side and this imbalance could lead to increased RANKL expression through the activation of CD4+ T cells rather than B cells. TNF-α can also stimulate osteoclastic maturation via synergistic combination with RANKL. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

activating CD4+ T cells (Fig. 7). The PLF-produced TNF-α could also stimulate osteoclast function via cooperation with RANKL.

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