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Systemic administration of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)-Ig abrogates alveolar bone resorption in induced periodontitis through inhibition of osteoclast differentiation and activation: an experimental investigation

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Running Head: CTLA-4 and bone resorption in periodontitis

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Abstract

Background/Objectives: Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is a critical immunoregulatory molecule expressed on T cells. CTLA-4 also binds to the surfaces of monocytes and macrophages, precursors of osteoclasts. Research on rheumatoid arthritis demonstrated that CTLA-4 suppresses inflammation and bone resorption. However, its effects on alveolar bone have yet to be understood. The purpose of this study was to investigate the role and potential mechanism of CTLA-4 in bone resorption in periodontitis.

Materials and Methods: In vivo, the effects of systemic administration of CTLA-4 immunoglobulin fusion protein (CTLA-4-Ig) on alveolar bone resorption were investigated using a periodontitis mouse model. A total of 20 C57BL/6J mice were randomly assigned to two groups according to the administration modes. Periodontitis was induced by placing a ligature around the left maxillary second molar. The contralateral tooth was left un-ligated. In the CTLA-4-Ig (+) group, CTLA-4-Ig was administered by intraperitoneal injection at 1 and 3 days after the ligature placement. Animals in the CTLA-4-Ig (-) group were given only phosphate-buffered saline each time. At 5 days after ligature placement, bone resorption was assessed by microcomputed tomography and histological examination, and the prevalence of osteoclast-like cells was assessed by tartrate-resistant acid phosphatase (TRAP) staining. In vitro, the effects of CTLA-4-Ig on osteoclasts were evaluated. Viability of RAW 264.7 cells treated with receptor activator of nuclear factor-κB ligand (RANKL) and CTLA-4-Ig was tested by WST-1 assay. Osteoclast-like cells were enumerated by TRAP staining, and osteoclast activity was evaluated by resorption pit assay. Gene expression levels of osteoclast differentiation markers (macrophage-colony stimulating
factor receptor, carbonic anhydrase II, cathepsin K, and Trap) and protein phosphatase 2A (PP2A), a major serine-threonine phosphatase, were assessed by quantitative real-time polymerase chain reaction. The effect of CTLA-4-Ig on the nuclear factor κB (NF-κB) activation was assessed by enzyme-linked immunosorbent assay.

**Results:** *In vivo*, ligature-induced bone resorption and the numbers of osteoclast-like cells were significantly decreased by the administration of CTLA-4-Ig. *In vitro*, treatment with RANKL and CTLA-4-Ig had no effect on cell viability. CTLA-4-Ig significantly reduced the prevalence and activation of osteoclast-like cells and decreased the expressions of osteoclast differentiation markers, compared to the RANKL-treated control. CTLA-4-Ig significantly suppressed RANKL-induced phosphorylation of NF-κB p65 but increased PP2A expression.

**Conclusion:** These results suggest that CTLA-4-Ig abrogates bone resorption in induced periodontitis, possibly via inhibition of osteoclast differentiation and activation. The regulation of the NF-κB pathway and PP2A expression may be one mechanism by which CTLA-4-Ig suppresses osteoclast behavior.
1 Introduction

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is a surface molecule on T cells, which negatively influences co-stimulation process between T cells and antigen-presenting cells (APCs).\textsuperscript{1-3} T-cell activation is modulated by the co-stimulation, and CTLA-4 competes for the binding of CD28 on T cells with the co-stimulation molecules CD80 (B7-1) and CD86 (B7-2) on APCs. Due to higher affinity for CD80/86, CTLA-4 interferes with the co-stimulation and prevents a pathological activation of T cells.\textsuperscript{4} CTLA-4 gene-deficient mice develop marked proliferation of CD4\textsuperscript{+} T cells and severe systemic inflammatory disease and die in the first few weeks of life.\textsuperscript{5,6} Because CD80/86 are expressed on various cell surfaces, the cellular effects of CTLA-4 are drawing increased attention. CTLA-4 can bind to the CD80/86 expressed in monocyte/macrophage lineage cells, which are the origin of osteoclasts.\textsuperscript{3} A previous study showed that $\text{Cd80}^{-/-}$ and $\text{Cd86}^{-/-}$ mice exhibited reduced bone mass and increased numbers of osteoclasts at the bone surface, when compared with wild-type mice.\textsuperscript{7} In vitro experiments from the same study indicated that $\text{Cd80}^{-/-}\text{Cd86}^{-/-}$ osteoclasts resisted the inhibition by CTLA-4.\textsuperscript{7} Interestingly, a recent study reported that CTLA-4 immunoglobulin fusion protein (CTLA-4-Ig) suppressed the osteoclast formation in human monocytes.\textsuperscript{8} Macrophages produce cytokines at sites of inflammation and differentiate into osteoclasts near the mineralized tissue such as bone.\textsuperscript{9} When bone destruction and resorption become dominant over bone formation, it causes diseases such as osteoporosis and rheumatoid arthritis (RA). CTLA-4-Ig has been shown to increase bone mineral density of the femoral neck in RA patients.\textsuperscript{10}
Periodontitis is a multifactorial, inflammation- and immune-mediated disease of dysbiotic polymicrobial etiology. As is the case with RA, bone resorption is the main feature of periodontitis. There are several reports on the potential association between periodontitis and CTLA-4. For instance, under stimulation by the outer membrane antigen of a major periodontal pathogen, *Porphyromonas gingivalis*, T cells from patients with periodontitis showed higher proliferative activities and the prevalence of CTLA-4 expressing cells than those from healthy subjects. In T-cell clones established from the gingival tissues of periodontitis patients, mRNA expression of CTLA-4 was noted. It has also been postulated that CTLA-4 gene variants are related to susceptibility to specific forms of periodontitis. These data suggest potential roles of CTLA-4 in the progression of periodontal diseases. However, information is scarce concerning the role of CTLA-4 in alveolar bone resorption in periodontitis.

With regard to RA, it has been reported that CTLA-4-Ig reduces gene expression of nuclear factor-κB (NF-κB) in human macrophages. NF-κB is one of the most ubiquitous transcription factors and promotes osteoclast progenitor differentiation by receptor activator of nuclear factor-κB ligand (RANKL). In periodontitis, influx of inflammatory cells induces the production of RANKL, which promotes bone resorption through osteoclasts. RANKL-mediated osteoclastogenesis plays a critical role in inflammatory bone resorption in periodontitis. Increased activation of NF-κB has been reported in patients with periodontitis. Given these information, it is possible that, if CTLA-4 plays a certain role in the regulation of alveolar bone resorption in periodontitis, NF-κB pathway would be one of the molecular cascades involved.
2 Materials and Methods

2.1. Animals

Eight to ten-week-aged male C57BL/6J mice were used (weight 20-30g; Sankyo Labo Service, Tokyo, Japan). The mice were individually kept in cages in a temperature-controlled facility and were given water and regular chow pellets. This study conformed to the institutional guidelines for the animal experiment (approval no. 202204) and the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines).

2.2. In vivo experimental design

C57BL/6J mice underwent anesthesia by intraperitoneal injection of sodium pentobarbital at a dose of 30 mg/kg. Periodontitis was induced by the ligation method. Briefly, a 6-0 silk suture (Mani, Utsunomiya, Japan) was placed around the left maxillary second molar for 5 days. The contralateral tooth was left un-ligated.

To investigate the effects of CTLA-4, CTLA-4-Ig (Abatacept; Bristol-Myers Squibb, Princeton, NJ, USA) was used. A total of 20 mice were randomly assigned to two groups according to the administration modes (n = 10 per group). Randomization was performed using computer-generated allocation, by one of the coauthors who was not involved in the assessment. In the CTLA-4-Ig (+) group, CTLA-4-Ig [50 mg/kg; diluted with phosphate-buffered saline (PBS; pH 7.4)] was administered by intraperitoneal injection at 1 and 3 days after the ligature placement. This administration protocol was based on the previous studies using mouse arthritis models. Animals in the CTLA-4-Ig (-) group were given only PBS each time. The following analyses were performed at 5 days after ligature placement.
2.3. Microcomputed tomography analysis

At 5 days, the animals were deeply anesthetized with sodium pentobarbital and received cardiovascular perfusion with 4% paraformaldehyde. The maxilla was retrieved and analyzed by microcomputed tomography (micro-CT) (R-micro-CT; Rigaku, Tokyo). The presence and extent of bone resorption was evaluated by the previously reported method\textsuperscript{20}: the distance between cemento-enamel junction (CEJ) and alveolar bone crest (ABC) was measured at six sites in the palatal side of the maxillae. The distance at each site was summed up to obtain total CEJ-ABC distance. The bone resorption was determined by the following equation: The total CEJ-ABC distance for the ligated side was subtracted by that of the contralateral control side in each mouse. The measurements were done using Image J software (Ver. 1.52a, National Institute of Health, Bethesda, MD, USA).

2.4. Histological and histomorphometric analyses

Following fixation in buffered 4% paraformaldehyde for 24 hours, the maxilla was decalcified in 10% ethylenediaminetetraacetic acid disodium salt (EDTA-2Na, pH 7.0; Muto Pure Chemicals, Tokyo) for 1 week, and the specimens were paraffin-embedded, cut into 5 μm thick slices coronal planes using a microtome for hematoxylin and eosin (H-E) staining. The specimens were observed under a light microscope (UPM Axiophot 2; Carl Zeiss Japan, Tokyo) with a software (Axio Vision 4.7; Carl Zeiss Japan, Tokyo). The distance from CEJ to ABC was measured as the extent of alveolar bone resorption in the palatal side of the second molar using Image J.

Additionally, the number of osteoclast-like cells was assessed by staining with
tartrate-resistant phosphatase (TRAP) using a staining kit (Cosmo Bio, Tokyo). Briefly, after deparaffinization and rinsing, an aliquot of 50 µl staining solution was applied on each section. Following incubation and rinsing, the sections were counterstained with hematoxylin for 1 min. TRAP-positive cells with three or more nuclei and contact with the bone were considered as osteoclast-like cells. Osteoclast-like cells on the alveolar bone crest surface at the palatal root were enumerated according to a previously reported method, under a light microscope (Carl Zeiss Japan). The counts were summed for each sample and averaged for each group. Data were expressed as the mean number of cells per 1.0 mm of alveolar bone.

The micro-CT and histomorphometric data were evaluated by one examiner who was blinded to the experimental grouping.

2.5. Cell culture condition and viability assay

RAW 264.7 mouse monocyte/macrophage cell line (ATCC Number: TIB-71) was used in this study. Cells were cultured in 50 ml culture flasks containing DMEM (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin (Wako, Tokyo) at 37 °C in a humidified atmosphere containing 5% CO₂. Recombinant mouse RANKL (462-TR-010) (100 ng/ml) (R&D Systems, Minneapolis, MN, USA) and various concentrations of CTLA-4-Ig (1, 10, 100, 300 µg/ml) were added after 24 hours of culture.

After 4 days, cell viability was evaluated using the WST-1 (Takara Bio, Tokyo), according to the manufacturer's protocol. Briefly, premixed WST-1 reagent (10 µl; Takara Bio, Tokyo) was added to treated cells (1 x 10³ cells/well) in a 96 well plate, and
incubated for 2h to allow color development. The resulting supernatants were then analyzed for absorbance at 450 nm.

2.6. *In vitro* TRAP staining

RAW 264.7 cells were cultured in 96 well plates (1 x 10^4 cells/well) with RANKL and various concentrations of CTLA-4-Ig. After 5 days, cells were washed with PBS and fixed with 10% formaldehyde for 10 minutes. Then, the cells were stained using the TRAP staining kit. After washing with purified water, TRAP-positive osteoclast-like cells were counted per well by light microscopy (BZ-X700; Keyence, Tokyo).

2.7. Resorption pit assay

Osteoclast activity was assessed by using bone resorption pit assay. Cells (1 x 10^4 cells/well) were cultured in calcium phosphate (CaP)-coated plates (Bone Resorption Assay Plate 24, PG Research, Tokyo) with RANKL and various concentrations of CTLA-4-Ig. After 6 days, cells were lysed with 5% sodium hypochlorite (NaClO), and wells were washed with purified water and air-dried. The substrate surfaces were observed by using a fluorescence microscope (Keyence). The average total resorption area per 1.5 mm^2 of the substrate was measured by Image J.

2.8. Quantitative real-time polymerase chain reaction

Cells were cultured in 24 well plates (1 x 10^5 cells/well) with RANKL and different doses of CTLA-4-Ig for 24-96h. At each time point, the cells were collected, and total RNA was prepared using TRIzol (Invitrogen, Carlsbad, CA). The concentration of total RNA was quantified using a spectrophotometer (ND-1000, NanoDrop Technologies,
Wilmington, DE, USA), and cDNA was generated using a reverse transcription kit (Qiagen, Hilden, Germany). After mixing with gDNA Wipeout Buffer (Qiagen) and RNase free water, Quantiscr ipt Reverse Transcriptase, Quantiscr ipt RT Buffer (Qiagen) and RT Primer mix (Qiagen) were added to the samples and incubated at 42 °C for 15 minutes, followed by incubation at 95 °C for 3 minutes. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using StepOnePlus (Applied Biosystems, Carlsbad, CA) with QuantiFast SYBR Green RT-PCR Kit (Qiagen) according to standard protocols. Specific PCR primers for macrophage-colony stimulating factor receptor (C-fms), carbonic anhydrase II (CaII), cathepsin K (Cat-k), Trap, serine/threonine-protein phosphatase 2A (PP2A), and the internal control glyceraldehyde 3-phosphate dehydrogenase (Gapdh) were used (Table S1). The relative gene expression was determined by threshold comparison.

2.9. Enzyme-linked immunosorbent assay (ELISA)

The expression level of NF-kB p65 (pS536)/Total NF-κB p65 was evaluated by ELISA using Simple Step ELISA™ kit (Abcam, Cambridge, MA, USA), according to the manufacturer’s protocol. Briefly, RAW 264.7 cells were cultured with RANKL and CTLA-4-Ig (300 µg/ml) for 30 minutes. Cells were lysed using Cell Extraction Buffer PTR. Cell lysates and the antibody cocktail were added to each well of the ELISA plate. After incubation for 1h, each well was washed with Wash Buffer PT and blot dried. Then TMB Substrate was added and further incubated for 15 min. The reaction was stopped by the addition of a stop solution, and protein levels were measured at OD 450 nm using a microplate reader. Values of phosphorylated NF-kB p65 (pS536) over total NF-kB were obtained using the standard curve method.
2.10. Statistical analysis

For in vivo experiments, the sample size was set to 10 per group, assuming that the total difference in bone resorption at the 6 sites was 0.6 mm between the periodontitis side and the healthy side, the standard deviation of 0.4 mm, a power of 90%, and a significance level of 0.05%.

Statistical comparisons of the micro-CT images between two groups were made by Mann-Whitney U test. Multiple comparisons for histomorphometric (H-E staining, in vivo TRAP staining) were sought by two-way analysis of variance (ANOVA) with Tukey post hoc test, multiple comparisons for WST-1 assay, in vitro TRAP staining, pit assay, qRT-PCR and ELISA were made by Kruskal-Wallis test with Dunn’s post hoc test. The analyses were performed using a software package (Prism 7, GraphPad Software, San Diego, CA). P < 0.05 was recognized as statistically significant.
3 Results

3.1 CTLA-4-Ig suppressed alveolar bone resorption in ligature-induced periodontitis

At 5 days, all mice appeared healthy, and the ligature was still intact at ligated sites. The results of micro-CT analysis are shown in Figure 1. The ligation induced enhanced bone resorption around the maxillary second molars compared to the non-ligated side as shown in the three-dimensional images (Fig. 1A). Following the injection of CTLA-4-Ig (50 mg/kg; twice during the ligation period), mice showed no signs of discomfort, weight loss, and changes in behavior. Bone resorption on the palatal side of the ligature/CTLA-4-Ig (+) group was significantly lower than that of ligature/CTLA-4-Ig (-) group ($p < 0.05$) (Fig. 1B).

The results of histological analysis are shown in Figure 2. In the non-ligature group, no obvious pathological change was noted in the periodontal tissue (Fig. 2A). In the ligature group, inflammatory cell infiltration was observed. In the ligature/CTLA-4-Ig (+) group, the infiltration of inflammatory cells was less evident compared to the ligature/CTLA-4-Ig (-) group. H-E staining also verified alveolar bone resorption at the palatal root (Fig. 2A). In the quantitative analysis of the histological specimen, the amount of alveolar bone resorption on the palatal side of the ligature/CTLA-4-Ig (+) group was significantly reduced compared to the ligature/CTLA-4-Ig (-) group ($p < 0.01$) (Fig. 2B).

3.2. CTLA-4-Ig reduced the number of osteoclast-like cells in vivo

Tissue sections from the ligated sites were subjected to TRAP for the detection of osteoclast-like cells (Fig. 3A). In the ligature/CTLA-4-Ig (-) group, lacunae containing
large osteoclast-like cells were found on the bone surface in the periodontal ligament side. The multi-nucleated cells with TRAP-positive (stained red) cytoplasm (Fig. 3A, inset) were regarded as osteoclast-like cells. In the ligature/CTLA-4-Ig (+) group, fewer number of osteoclast-like cells were observed compared to the ligature/CTLA-4-Ig (-) group.

In the quantitative analysis, the number of osteoclast-like cells per bone perimeter in the ligated side was significantly higher than that in the non-ligated side of the CTLA-4-Ig (-) group ($p < 0.001$) (Fig. 3B). The ligature/CTLA-4-Ig (+) group showed significantly fewer osteoclast-like cells than the ligature/CTLA-4-Ig (-) group ($p < 0.001$).

**3.3. CTLA-4-Ig did not affect cell viability**

Treatment of RAW 264.7 cells with RANKL and CTLA-4-Ig showed no significant effect on the viability, as assessed by the WST-1 assay (Supporting Material; Fig. S1). Also, no obvious changes in morphology were found in cells treated with CTLA-4-Ig at the concentration range tested (data not shown).

**3.4. CTLA-4-Ig decreased the number of osteoclast-like cells in vitro**

The effect of CTLA-4-Ig on osteoclastogenesis was assessed by culturing RAW 264.7 cells with RANKL and CTLA-4-Ig. Significantly greater numbers of TRAP-positive osteoclast-like cells were observed in the RANKL-treated control compared with CTLA-4-Ig treated group (300 µg/ml) (Fig. 4A). The number of osteoclast-like cells was significantly decreased by the treatment with CTLA-4-Ig in a dose-dependent fashion (Fig. 4B).
3.5. CTLA-4-Ig reduced the osteoclast activity

We further examined whether CTLA-4-Ig influences the ability of osteoclast-like cells to resorb bone. RANKL-treated cells formed a number of resorption pits on the substrate (Fig. 4C). The treatment with CTLA-4-Ig significantly reduced the pit area in a dose-dependent fashion (in the range of 1-100 µg/ml) compared to RANKL-treated control (Fig. 4D).

3.6. CTLA-4-Ig reduced osteoclast differentiation

In qRT-PCR analysis, the treatment of cells with CTLA-4-Ig (300 µg/ml) significantly inhibited the RANKL-induced expression of C-fms \( p < 0.001 \) to the level similar to non-treated cells (Fig. 5A). CatII expression was significantly reduced in CTLA-4-Ig treated cells (1-300 µg/ml) compared to RANKL-treated control \( p < 0.05-0.001 \) (Fig. 5B). Cat-k was also significantly reduced by the CTLA-4-Ig treatment (100, 300 µg/ml) \( p < 0.001 \) (Fig. 5C). CTLA-4-Ig treatment (300 µg/ml) also significantly reduced the expression of Trap \( p < 0.05 \) (Fig. 5D).

3.7. CTLA-4-Ig suppressed NF-κB p65 phosphorylation and increased PP2A expression

We next assessed the effect of CTLA-4-Ig on the level of phosphorylated NF-κB p65. In the RANKL-treated control, the level of phosphorylated NF-κB p65 was significantly increased compared to the non-treated cells \( p < 0.05 \) (Fig. 6A). The treatment of cells with CTLA-4-Ig (300 µg/ml) reduced the level of phosphorylated NF-κB p65 \( p < 0.05 \).
In addition, the involvement of PP2A in the mechanism by which CTLA-4-Ig inhibits osteoclast differentiation was examined. As shown in Figure 6B, CTLA-4-Ig (300 µg/ml) treated cells demonstrated higher gene expression of PP2A compared to RANKL-treated control (p < 0.01).
4 Discussion

In the present study, we investigated the role and potential mechanism of CTLA-4 in alveolar bone resorption. We have shown, for the first time, that the CTLA-4-Ig administration significantly inhibits alveolar bone resorption in induced periodontitis, and osteoclast differentiation and activation. Such effects are considered to be exerted via the suppression of NF-κB p65 phosphorylation and increased expression of PP2A. These findings have salient implications for the regulation of alveolar bone resorption in periodontitis.

CTLA-4-Ig is a fusion protein of CTLA-4 with a modified Fc portion of human IgG1. A CTLA-4-Ig agent, Abatacept, is used for the treatment of refractory RA in adults and juvenile idiopathic arthritis. In a pilot, dose-finding, clinical study with RA patients, it was confirmed that 10 mg/kg CTLA-4-Ig was required to achieve an optimum response, and this dosage is widely used clinically. In the experiments with mice, 10 mg/kg CTLA-4-Ig has been used as low dose, 50 mg/kg as high dose, and 250 mg/kg as extremely high dose. Based on these studies, we used 50 mg/kg of CTLA-4-Ig in the in vivo experiments. In an in vitro study, human peripheral blood monocytes were treated with CTLA-4-Ig with concentrations ranging from 1 to 100 µg/ml. Because the effects of the concentration of chemical substances differ depending on the cell type, the effect of CTLA-4-Ig (up to 300 µg/ml) on the viability of RAW 264.7 cells was assessed by WST-1 (Supporting Material; Fig. S1). Upon finding no cytotoxic effect, we selected the concentration range of 1 to 300 µg/ml in the subsequent in vitro experiments.

Bone resorption is a process initiated by the proliferation of osteoclast precursor cells, differentiation into osteoclasts, and finally by the degradation of bone by mature
In RA, CTLA-4 is thought to be involved not only in inflammation but also in suppressing bone resorption. In a rat collagen-induced arthritis model, prophylactic administration of CTLA-4-Ig significantly reduced the inflammatory mediators, and bone and joint destruction. It has been suggested that inhibition of CD28 co-stimulation by CTLA-4-Ig promotes osteogenesis and increases bone mass in osteoporotic conditions. Balancing inflammatory and regulatory T-cell responses plays an important role in periodontitis. In a mouse model of periodontitis, bacterial invasion led to the generation of Th17 cells that evoke immune responses and induce bone destruction. It has been shown that treatment with abatacept reduced the proportion of Th17 cells and activated regulatory T cells. In the current study, the administration of CTLA-4-Ig suppressed ligature-induced bone resorption and decreased the number of osteoclast-like cells. It is possible that the suppression of pathological T-cell activation by co-stimulation interference may have played a role in the observed effect on alveolar bone.

Alternatively, CTLA-4-Ig could have exerted a direct effect on osteoclast precursors, via binding to the CD80/CD86 on the surface of monocytes/macrophages. In order to further investigate the mechanisms that may account for the effect of CTLA-4-Ig on bone, a series of in vitro experiments were conducted. Treatment of RAW 264.7 cells with RANKL induces differentiation into osteoclasts, which are TRAP-positive cells. In the present in vitro experiments, CTLA-4-Ig significantly reduced the number of RANKL-induced TRAP-positive, osteoclast-like cells. These results suggested that CTLA-4-Ig induces inhibitory effect on osteoclastogenesis. Bone resorption by osteoclasts occurs by two steps: the first is the recruitment of new osteoclasts, and the second is the activation of mature osteoclasts. Therefore, in analysis of bone resorption, it is necessary to evaluate not only the presence but also the activity of...
osteoclasts. In this study, we assessed osteoclastic activity by using the resorption pit assay. The area of pits in the CTLA-4-Ig treated cells was significantly reduced compared to the RANKL-treated control. This result suggests that CTLA-4-Ig exerts an inhibitory effect on mature osteoclast function. Because bone resorption is a sequel of enhanced osteoclast activity,\(^{39}\) suppression of osteoclast activation by the treatment with CTLA-4-Ig may help to control the progression of periodontitis.

In order to analyze molecular mechanisms involved in the effect of CTLA-4 on bone resorption, we assessed osteoclast differentiation markers in the RANKL-treated RAW 264.7 cells. C-fms is a receptor for M-CSF which is an osteoclast survival factor secreted by osteoblasts. C-fms is expressed in cells at late stages of osteoclastogenesis.\(^{40}\) CaII is characterized by high expression in mature osteoclasts,\(^{41}\) and provides an acidic environment in the extracellular space adjacent to bone.\(^{42}\) This acidification of the extracellular environment initiates the degradation of bone mineral components and activates Cat-k.\(^{43-44}\) Cat-K is the major cysteine proteinase of osteoclasts and is involved in the hydrolysis of extracellular bone matrix proteins during acid lysis of the inorganic matrix.\(^{9}\) Furthermore, Trap is one of the important markers for osteoclast differentiation. In the present study, CTLA-4-Ig inhibited the gene expressions of C-fms, CaII, Cat-k, and Trap. These data indicate that CTLA-4-Ig suppresses osteoclast differentiation. It should be noted that CTLA-4-Ig had no significant effect on cell viability (Supporting Material; Fig. S1). It is suggested that the low prevalence of osteoclast-like cells by the CTLA-4-Ig treatment was not caused by the cytotoxic effect but by the inhibition of osteoclast differentiation.

To investigate the signaling mechanism behind the regulation of osteoclast functions by CTLA-4-Ig, we focused on the NF-κB pathway. RANKL stimulation of RAW 264.7
cells resulted in p65 phosphorylation, whereas treatment with RANKL and CTLA-4-Ig did not alter the degree of p65 phosphorylation. It has been reported that CTLA-4-Ig reduces gene and protein expressions of NF-κB in human macrophages. In addition, in RA patients, binding of CTLA-4 to CD80/86 on B-cells did not induce the phosphorylation of NF-κB p65. It is conceivable that CTLA-4-Ig may regulate the activation of the NF-κB pathway upon binding to CD80/86 and suppress osteoclast differentiation.

It has been reported that the inhibition of PP2A affects the activation of the IKKα/IκBα/NF-κB pathway. This led us to consider the possible involvement of PP2A in the suppression of NF-κB activation by CTLA-4. PP2A is a ubiquitous multifunctional protein phosphatase in cells that regulates activated protein kinases. PP2A-dependent p65 dephosphorylation inhibited the activation of NF-κB in epithelial cells and renal cells. In osteoclasts, dauricine, an isoquinoline alkaloid with anti-inflammatory activity, suppressed lipopolysaccharide-induced bone resorption via the ROS/PP2A/NF-κB pathway. In the present study, CTLA-4-Ig with high dose induced increased gene expression of PP2A. Regulation of PP2A expression may be one of the mechanisms by which CTLA-4-Ig suppresses osteoclast differentiation.

There are several limitations to the present study. The present study did not fully clarify the effects of CTLA-4-Ig on osteoclastogenesis. Further in vitro studies using primary cells are necessary. Whether CTLA-4-Ig influences the formation or function of osteoblasts in periodontitis remains to be proven. We focused mainly on the NF-κB pathway in relation to CTLA-4-Ig, but other signaling pathways need to be investigated. Despite the limitations, our data provide a better understanding of the role of CTLA-4 in periodontitis and the regulatory mechanism of alveolar bone resorption. Whether
these findings can be translated into clinically beneficial treatment of periodontitis is worth further investigation.

5 Conclusion

Our findings suggest that the systemic administration of CTLA-4-Ig suppresses bone resorption in experimental periodontitis, possibly through the inhibition of osteoclast differentiation and activation. The regulation of NF-κB pathway and PP2A expression may be one of the mechanisms by which CTLA-4-Ig influences osteoclast behavior. Further investigations, especially in relation to the action mechanisms of CTLA-4-Ig on the osteoclastogenesis, are required.

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Conflict of Interest and Source of Funding

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Figure legends

FIGURE 1 Micro-CT analysis of alveolar bone resorption.

(A) Representative micro-CT images of the maxillary second molars (palatal side) with or without ligature placement for 5 days. Original magnification ×20, Scale bar = 1500 µm. CTLA-4-Ig (50 mg/kg) or PBS was administered at 1 and 3 days after the ligature placement. (B) Quantification of alveolar bone resorption on micro-CT images in the ligature group. The distance between CEJ and alveolar bone crest (ABC) was measured at 6 sites around the second molars. The distance at each site was summed up to obtain total CEJ-ABC distance. The bone resorption in the ligated side relative to the contralateral non-ligated side was determined as follows: The total CEJ-ABC distance for the ligated side was subtracted by that of the contralateral non-ligated side in each mouse. Scatter plots showing individual data with mean (middle line) and standard deviation (error bars). (n = 10, each group). *p < 0.05 vs CTLA-4-Ig (-) group, by Mann-Whitney U test.

FIGURE 2 Histological analysis.

(A) Representative photomicrographs of H-E stained sections around the palatal root of the second molars. Original magnification ×100, bar = 100µm. White arrowheads indicate cemento-enamel junction (CEJ), and gray arrowheads indicate alveolar bone crest (ABC). (B) Quantification of alveolar bone resorption. The distance from CEJ to ABC was measured as the amount of alveolar bone resorption. Scatter plots showing individual data with mean (middle line) and standard deviation (error bars). (n = 10). *p < 0.05, **p < 0.01 and ***p < 0.001, by two-way analysis of variance with Tukey post hoc test.
FIGURE 3 *In vivo* evaluation of the prevalence of osteoclast-like cells.

(A) TRAP-stained histological sections around the palate root of the second molars. Original magnification ×400, bar = 100 µm. Arrowheads indicate osteoclast-like cells. Inset in the ligature/CTLA-4-Ig (-) group shows the enlarged image of an osteoclast-like cell (magnification ×1200). (B) The number of osteoclast-like cells by TRAP staining. Scatter plots showing individual data with mean (middle line) and standard deviation (error bars). (n=10) *p < 0.05, ***p < 0.001, by two-way analysis of variance with Tukey post hoc test.

FIGURE 4 *In vitro* evaluation of the prevalence of osteoclast-like cells and osteoclast activity.

RAW 264.7 cells were treated with RANKL and various concentrations of CTLA-4-Ig and further incubated. (A) Representative TRAP-staining images of cells treated with RANKL and CTLA-4-Ig (300 µg/ml) at 5 days after the treatment. Original magnification ×20, bar = 100 µm. (B) Quantification of the number of osteoclast-like cells. (C) Representative microscopic images of CaP coated plates at 6 days. The black area show the pits where mature RAW 264.7 cells (osteoclast-like cells) dissolved CaP substrate. Original magnification×10, bar = 200 µm. (D) Total pit areas were summed using image J. Results are shown as the percent control. Data are expressed as mean ± SD (n = 6). *p < 0.05, **p < 0.01, and ***p < 0.001, by Kruskal-Wallis test with Dunn’s post hoc test.
FIGURE 5 qRT-PCR analysis of the mRNA expressions of osteoclast differentiation markers.

RAW 264.7 cells were treated with RANKL and various concentrations of CTLA-4-Ig and further incubated. (A) mRNA level of C-fms at 72 hours after the treatment. mRNA levels of CaII (B), Cat-k (C), and Trap (D) at 96 hours after the treatment. Data are shown as mean ± SD (n = 7). *p < 0.05, **p < 0.01, ***p < 0.001 by Kruskal-Wallis test with Dunn’s post hoc test.

FIGURE 6 Evaluation of the expression levels of NF-kB p65 (pS536)/Total NF-κB p65 by ELISA.

RAW 264.7 cells were treated with RANKL and various concentrations of CTLA-4-Ig and further incubated. (A) Percentage of phosphorylated p65 protein in total p65 protein at 30 minutes. Data are expressed as mean ± SD (n = 6). *p < 0.05 by Kruskal-Wallis test with Dunn’s post hoc test. (B) mRNA level of PP2A at 24 hours after the treatment. Data are shown as mean ± SD (n = 6). **p < 0.01 vs RANKL-treated control by Kruskal-Wallis test with Dunn’s post hoc test.
Supporting Material

Table S1. Sequences of real-time PCR primers used in the study.

Figure S1. *In vitro* assessment of cell viability by WST-1.
Following 24 hours of culture, RAW 264.7 cells were treated with RANKL (100 ng/ml) and various concentrations (1, 10, 100, 300 µg/ml) of CTLA-4-Ig. After 4 days, WST-1 was used to evaluate cell viability. Data are expressed as mean ± SD (n = 6).
Figure 1

(A) CTLA-4-Ig (-)  CTLA-4-Ig (+)

non-ligature

ligature

(B) Bone resorption (mm)

CTLA-4-Ig (-)  CTLA-4-Ig (+)

*
Figure 2

(A) CTLA-4-Ig (-) vs CTLA-4-Ig (+)

(B) CEJ-ABC distance (mm)

Comparison of CEJ-ABC distance between non-ligature and ligature conditions for CTLA-4-Ig (-) and CTLA-4-Ig (+) groups.
Figure 3

(A) Comparison of CTLA-4-Ig (-) and CTLA-4-Ig (+) conditions in non-ligature and ligature conditions.

(B) Graph showing the number of osteoclast-like cells (mm) with statistical significance indicated by asterisks.

**Figure 3**
Figure 4
Figure 5
Figure 6

(A) 

% Phospho p65 of total p65

<table>
<thead>
<tr>
<th>RANKL</th>
<th>-</th>
<th>+</th>
<th>+</th>
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<tbody>
<tr>
<td>CTLA-4-Ig (µg/ml)</td>
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<td>-</td>
<td>300</td>
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(B) 

Relative mRNA expression (fold)

<table>
<thead>
<tr>
<th>RANKL</th>
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<th>+</th>
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<tbody>
<tr>
<td>CTLA-4-Ig (µg/ml)</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>10</td>
<td>100</td>
<td>300</td>
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PP2A

**
Supporting Material

Table S1. Sequences of real-time PCR primers used in the study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Product size (bp)</th>
<th>Accession number</th>
</tr>
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</table>
| C-fms   | Forward: GGTTGTAAGCCCGGGTGAAA  
Reverse: AAGAGTGGGGGCAGATCTTTG | 233               | NM_001037859.2   |
| CaII    | Forward: GACCCAGGTGTCTCATGTTG  
Reverse: GACGCCAGTTGTCACCACATC | 243               | NM_009801.5      |
| Cat-k   | Forward: CTCAACAGCAGGATGGTTG  
Reverse: TGCAGGCTTTCTCGTCCC | 719               | NM_007802.4      |
| Trap    | Forward: AGGACGTGTTCTCTGACCG  
Reverse: CGCAAAACGTTAGTAAGGG | 148               | NM_007388.3      |
| PP2A    | Forward: ATGGACGAGAAGTGTTCAC  
Reverse: GACGCCATGAGACAAAG | 508               | XM_036196547.1   |
| GAPDH   | Forward: AAGCAACCCCTTCTGACCA  
Reverse: TCCAGCAGCATACTCAGCAC | 191               | NM_001289726.1   |

C-fms; macrophage-colony stimulating factor receptor, CaII; carbonic anhydrase II, Cat-k; cathepsin K, Trap; tartrate-resistant phosphatase, PP2A; serine/threonine-protein phosphatase 2A, GAPDH; glyceraldehyde 3-phosphate dehydrogenase
Figure S1. *In vitro* assessment of cell viability by WST-1.
Following 24 hours of culture, RAW 264.7 cells were treated with RANKL (100 ng/ml) and various concentrations (1, 10, 100, 300 µg/ml) of CTLA-4-Ig. After 4 days, WST-1 was used to evaluate cell viability. Data are expressed as mean ± SD (n = 6).