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The potential role of interleukin-17 in the immunopathology of periodontal disease

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Abstract

Background: Interleukin-17 (IL-17) is exclusively produced by activated T cells, and this cytokine can induce inflammatory responses, support immune responses (Th1), and stimulate osteoclastic bone resorption in combination with receptor activator of NF-κB (RANK) and RANK ligand (RANKL). These biological functions are relevant to the aetiopathogenesis of periodontitis, and thus we sought to investigate whether IL-17 is produced in periodontal lesions and to assess the relationship of gene expression between IL-17 and other cytokines, and to determine the effect of IL-17 on IL-6 production in human gingival fibroblasts (HGF).

Materials and Methods: IL-17 was detected and measured in periodontal tissues obtained as biopsy samples during periodontal surgery and in the cell-free culture supernatants cultured ex vivo, by using Western immunoblotting and enzyme-linked immunosorbent assay, respectively. IL-17 and other cytokine gene expression were investigated by the reverse transcription-polymerase chain reaction (RT-PCR) method. The contribution of IL-17 to IL-6 production by HGF was studied.

Results: IL-17 protein was moderately detected in periodontal tissues. In contrast, IL-17 mRNA was expressed only in nine of 23 periodontitis tissue samples by RT-PCR. The IL-17 mRNA-positive samples simultaneously expressed mRNAs encoding interferon (IFN)-γ, IL-2, RANK, and RANKL, but not IL-4. IL-10 (Th2 cytokine) was detected more frequently in the samples than IFN-γ and IL-2 (Th1 cytokine). Recombinant human IL-17 induced IL-6 production from HGF in a dose- and time-dependent fashion.

Conclusions: These results indicate that IL-17 is produced in periodontal lesions, which may be involved in Th1 modulation and enhance inflammatory reactions via gingival fibroblast-derived mediators in periodontal disease. Thus, IL-17, together with other cytokines, has a potential role in the aetiopathogenesis of periodontal disease.

Key words: cytokine; interleukin-17; periodontal disease; T cells

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Periodontitis is a chronic infectious inflammatory disease characterized by a heavy lymphocytic infiltration into the periodontal lesions, resulting in the secretion of a variety of cytokines, which ultimately leads to the destruction of periodontal tissues including alveolar bone (Williams 1990). The role of T cells in the pathogenesis of periodontal disease has been widely investigated from different standpoints, such as conversion from T-cell lesions to B-cell lesions (Seymour et al. 1979), altered CD4/CD8 T-cell ratios (Kinane et al. 1989), autologous mixed lymphocyte reactions (Kimura et al. 1991), polyclonal B-cell activation (Tew et al. 1989), antigen-restricted immune responses (Ohyama et al. 1998), and Th1/Th2 cytokine profiles (Taubman & Kawai 2001, Berglundh et al. 2002).

Th1 cells produce interleukin-2 (IL-2) and interferon (IFN)-γ, which induce cellular immunity and production of pro-inflammatory cytokines (Yamamura et al. 1991). In contrast, Th2 cells produce IL-4, IL-6, and IL-10 and favour B-cell-mediated humoral immunity (Modlin & Nutman 1993). Despite a high number of T cells in periodontal lesions, concentrations of the T-cell cytokines IL-2, IFN-γ, and IL-4 are quite low (Fujihashi et al. 1996, Roberts et al. 1997). In periodontitis tissues, Th2 cells are predominant (Fujihashi et al. 1993, Yamazaki et al. 1995, Tokoro

et al. 1997), and their common precursor, Th0 cells, can also be found (Gemmell & Seymour 1998), although recent studies suggest that Th1 cells play a crucial role in the bone resorption that occurs in the periodontium (Takeichi et al. 2000, Taubman & Kawai 2001). Thus, the exact role of T cells in the destruction of the periodontium is still controversial.

IL-17, a pro-inflammatory cytokine, is exclusively produced by activated $CD4^+$ T cells (Yao et al. 1995, Aarvak et al. 1999), although it has recently been reported that neutrophils also secrete this cytokine (Ferretti et al. 2003, Witowski et al. 2004). It has been reported that T cells are involved in the bone destruction via IL-17 production in rheumatoid arthritis (Chabaud et al. 1999, 2000). IL-17 has been shown to stimulate epithelial, endothelial, and fibroblastic cells to produce IL-6, IL-8, and PGE_2 (Fossiez et al. 1996). In addition, IL-17 induces RANKL production by osteoblasts (Kotake et al. 1999). As IL-17 shares properties with IL-1 and TNF-α, it may affect osteoclast-mediated bone resorption (Kotake et al. 1999, Van bezooijen et al. 1999). Therefore, we hypothesized that T cells in periodontal lesions produce IL-17 and exacerbate inflammatory periodontal disease by activating gingival fibroblasts to produce inflammatory mediators. In addition, it has been reported that T cells can be directly involved in bone metabolism via T-cell-derived cytokines, receptor activator of NF-κB ligand (RANKL), and RANK (Kong et al. 1999, Takayanagi et al. 2000, Teng et al. 2000). To test this hypothesis, we investigated IL-17 production and its expression pattern compared with that of other T-cell cytokines, RANKL and RANK in periodontal tissues by using the reverse transcription-polymerase chain reaction (RT-PCR), Western blotting, and enzyme-linked immunosorbent assay (ELISA) techniques. We also studied the effect of IL-17 on IL-6 production by human gingival fibroblasts (HGF) in vitro.

Materials and Methods

Reagents

Recombinant human IL-17 (rhIL-17) and goat anti-IL-17 antibody were purchased from R&D Systems (Minneapolis, MN, USA). This antibody has no effect on the action of IL-1, TNF-α, GM-CSF, or monocyte CSF. α-modified essential medium (α-MEM) was purchased from GIBCO BRL Technologies (Rockville, MD, USA), and foetal bovine serum (FBS) from GIBCO BRL Technologies. Penicillin and streptomycin were obtained from Wako (Osaka, Japan). The restriction enzyme *Hin*fI was purchased from Nippon Gene Co (Toyama, Japan).

Subjects and sample collection

Tissue samples were obtained from periodontitis tissues resected during periodontal surgery from 23 patients with periodontitis (eight males and 15 females, aged 38–68 years, average 50.3 years) who visited Meikai University School of Dentistry. All patients had a history of chronic periodontitis for more than 5 years and were diagnosed according to the criteria as described previously (Takahashi et al. 2001). Written informed consent was obtained from all patients and controls, in line with the Helsinki declaration, before inclusion in this study. A total of 39 tissue samples were taken from the 23 patients in which one sample was taken from each of seven patients and two samples were taken from different sites of each of 16 patients. Twenty-three tissue samples from each of 23 patients were used for RT-PCR analysis and the other 16 samples that were taken from the 16 patients in which two samples were taken were used for Western blotting. Pocket depths in all biopsy sites were greater than 5 mm and the sites bled on probing. Eight clinically healthy gingiva were obtained from eight volunteer dental students and staff (three males, five females, aged 23–42 years, average 28.1 years). Obtained tissue samples were then used for various analyses. The protocol was reviewed and ethical approval was given by our institutional review board.

Ex vivo tissue culture

Sixteen gingival tissue samples as described above were cultured for 48 h in RPMI 1640 medium (GIBCO BRL Technologies) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 50 μg/ml gentamicin, and 20 mM Hepes buffer in 24-well plates (Falcon, Oxford, CA, USA) in a final volume of 2 ml/well at 37°C in a 5% CO_2/95% humidified air. Cell-free supernatants were harvested and frozen at −80°C until they could be measured for their IL-17 levels, and the tissues were analysed by Western immunoblotting.

Measurement of IL-17

IL-17 levels in the culture supernatants from cultured gingival tissue samples were determined using a solid-phase sandwich enzyme immunoassay kit (R&D) according to the manufacturer's guidelines. The minimum significant detection level of the assay was 15 pg/ml of IL-17.

Western immunoblotting

IL-17 proteins in cultured tissue samples were detected by Western immunoblotting. Proteins were extracted by the method of Guo et al. (2000). In brief, precipitated proteins were resolved by 10% SDS-PAGE and then electroblotted onto nitrocellulose membranes, and the membranes were blocked with 5% skimmed milk prepared in PBS with 0.05% Tween-20 (PBS-T). Thereafter, they were incubated for 1 h with biotinylated goat anti-IL-17 antibody, washed with PBS-T, and reacted for 1 h at room temperature with avidin–horseradish peroxidase conjugate (Pierce Chemical Co, Rockford, IL, USA) at 2 μg/ml in PBS-T including 10% milk. The membrane was washed extensively (five times for 15 min) in PBS-T, and the blot was developed for horseradish peroxidase activity by the enhanced chemiluminescence method (Amersham Life Sciences Inc., Arlington Heights, IL, USA). Quantification of protein concentrations in cellular extracts was determined by using a modification of the micro-Lowry method (protein assay kit; Sigma, St. Louis, MO, USA).

Culture of HGF

HGF were obtained from biopsies of periodontally healthy gingiva from volunteers (one male, two females, average 18.7 years). HGF were identified by their characteristic spindle shapes under a phase-contrast microscope, and their collagen synthesis activity, as described previously (Takahashi et al. 1994a). Primary cells were grown to confluence in 100 cm^2 tissue culture flasks in α-MEM containing 100 U/ml penicillin (Wako), 200 μg/ml kanamycin (Wako) and 10% FBS (GIBCO

BRL Technologies). All experiments were performed with cultures that were between the fifth and twelfth cell passages. The cells were stimulated for various periods (see ''Results'') with different concentrations of IL-17 (0.005, 0.05, 0.5, 5, 50 ng/ml). After stimulation, the HGF were cultured for 24 h. Cell-free supernatants were then collected, and the IL-6 levels were measured by using a commercially available ELISA kit (R&D) according to the manufacturer's instructions.

RT-PCR

Approximately 100 mg of tissue from each of the specimens was immediately prepared for total cellular RNA isolation by using TRIzol reagent (GIBCO BRL Technologies). After purification, the RNA concentration was calculated from the absorbance at 260 and 280 nm. cDNA was synthesized from 100 ng of total RNA by extension of random primers with 200 U of Superscript II (GIBCO BRL Technologies). PCR of the cDNA was performed in a final volume of 25 μl containing 2.5 mM $MgCl_2$, 2.5 U of AmpliTaq Gold (Perkin-Elmer, Norwalk, CT, USA), and specific primers at 1 μM by using the geneAmp 2400 PCR system (Perkin-Elmer). Information on the primers used is listed in Table 1. Thirty-five amplification cycles were carried out in PCR reaction under optimal conditions as shown in Table 1. One-fifth of the synthesized PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. For calibration, a 100 bp DNA ladder was purchased from GIBCO BRL Technologies.

Nucleotide sequencing of amplified PCR products

PCR products were also confirmed by sequencing or restriction enzyme (*Hin*fI) treatment. In brief, the cDNA fragment amplified by PCR was excised from the gel and purified with a gel extraction kit (Funakoshi, Tokyo, Japan). The cDNA fragments were cloned by using a TA cloning kit (Invitrogen Co., Carlsbad, CA, USA) and were sequenced using a DNA sequencing kit (Perkin-Elmer) based on the dideoxy chain termination method. An automated DNA sequencer (ABI PRISM 310, Perkin-Elmer) was used.

Statistical analysis

The differences in the distribution of the data among subject groups were analysed with Student's *t*-test. The computer package STATVIEW (Abacus Concepts Inc., Berkeley, CA, USA) was used to perform the statistical analysis.

Table 1. Characterization of primers, method for confirming the DNA sequences, and annealing temperatures

	Size (bp)	Primers	Confirmation of DNA	AT
β-actin	642	F: ATGACCCAGATCATGTTTGAG R: AGGAGCAATGATCTTGATCTTCA	RF (*Hin*fI)	58
IL-17	468	F: ATGACTCCTGGGAAGACCTCATTG R: TTAGGCCACATGGTGGACAATCGG	DS	55
IFN-γ	355	F: AGTTATATCTTGGCTTTTCA R: ACCGAATAATTAGTCAGCTT	DS	55
IL-2	266	F: ACTCACCAGGATGCTCACAT R: AGGTAATCCATCTGTTCAGA	DS	50
IL-4	317	F: CTTCCCCCTCTGTTCTTCCT R: TTCCTGTCGAGCCGTTTCAG	DS	62
IL-10	352	F: ATGCCCCAAGCTGAGAACCAAGACCCA R: TCTCAAGGGGCTGGGTCAGCTATCCCA	DS	50
RANKL	642	F: ATAGAATATCAGAAGATGGCACTC R: TGCTGTTCCTACAAAGTTTACG	RF (*Hin*fI)	62
RANK	432	F: CTTTGAGTGCTTTAGTGCGTG R: TAAGGAGGGGTTGGAGACCTCG	DS	62

bp, base pair; AT, annealing temperature; RF, restriction enzyme; DS: direct sequencing; RANK, receptor activator of NF-κB; RANKL, RANK ligand; IL, interleukin; IFN, interferon.
References for primers: β-actin, Takahashi et al. (1994b); IL-17, Fossiez et al. (1996); IFN-γ, IL-2, IL-4, IL-10, Yamamura et al. (1991); RANKL, RANK, Sakata et al. (1999).

Results

IL-17 protein

IL-17 protein was detected in 10 of 16 periodontitis tissue samples by Western blotting. This cytokine could also be detected in five of 10 cell-free culture supernatants of periodontitis tissues by ELISA in which IL-17 could be detected by Western blotting.

IL-17 and other cytokine mRNA expression

Figure 1 shows an agarose gel containing PCR products amplified from periodontitis tissue samples of IL-17, IFN-γ, IL-2, IL-4, IL-10, RANKL, and RANK. The PCR products were also purified, sequenced, and confirmed as human IL-17 (Table 1). Likewise, other molecules were also confirmed from the sequencing or restriction enzyme digestion method, as shown in Table 1. IL-17 mRNA was detected in nine of 23 periodontitis tissue samples (Table 2). IL-4 mRNA was detected in one of 23

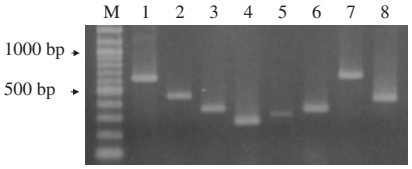

Fig. 1. Specific detection of interleukin-17 (IL-17) mRNA expression. M, position and sizes of molecular markers; lane 1, β-actin; lane 2, IL-17; lane 3, interferon-γ; lane 4, IL-2; lane 5, IL-4; lane 6, IL-10; lane 7, RANKL; lane 8, RANK.

Table 2. Frequency of detection of IL-17 and other cytokines obtained from periodontitis and healthy sites

	% positive samples	
	periodontitis	healthy
IL-17	39.1	0
IFN-γ	30.4	37.5
IL-2	34.8	12.5
IL-4	4.3	0
IL-10	47.8*	0
RANKL	17.3	0
RANK	52.2*	0
β-actin	100	100

*Significant difference: $p < 0.05$ Fisher's exact test. Periodontitis ($n = 23$), healthy ($n = 8$). IL-4 and IL-10 in periodontitis samples were detected in one of 23, 11 of 23, respectively.
IL, interleukin; IFN, interferon; RANK, receptor activator of NF-κB; RANKL, RANK ligand.

Table 3. mRNA expressions of other molecules in IL-17 mRNA-positive samples

Age (years)	Gender	PD (mm)	BL (%)	Th1		Th2		RANKL	RANK
				IFN-γ	IL-2	IL-4	IL-10		
36	M	8	40	+	+	–	+	+	+
48	M	5	60	+	+	–	+	+	+
57	M	8	80	+	+	–	+	–	+
62	M	8	50	+	–	–	–	–	–
48	F	8	50	+	+	–	+	–	+
48	F	9	70	+	+	–	+	–	+
51	F	4	50	–	–	–	–	–	+
51	F	7	100	+	+	+	+	+	+
57	F	3	80	–	–	–	–	–	–

M, male; F, female; PD, pocket depth; BL, bone loss index (%); IL, interleukin; IFN, interferon.

periodontitis samples, whereas IL-10 mRNA was detected in 11 of 23 (Table 2). RANK mRNA was detected more frequently than RANKL mRNA. β-actin was detected in all samples, which shows that cDNA synthesis and amplification was possible in all RNA samples. IL-17 mRNA expression was simultaneously detected in IFN-γ, IL-2, IL-10, and RANK mRNA-expressing samples (Table 3).

Effect of rhIL-17 on IL-6 production by HGF

Figure 2 illustrates the effect of rhIL-17 on IL-6 production in HGF. IL-17 increased IL-6 secretion in a dose- and time-dependent fashion (Fig. 2a, b). HGF secreted a significant level of IL-6 after 4 h of stimulation with IL-17 ($p<0.05$). This effect was completely blocked with anti-IL-17 antibody (Fig. 2c).

Discussion

The current study can be summarized as follows: (1) IL-17 could be detected in biopsy samples from periodontal lesions at both mRNA and protein levels, (2) the IL-17 expression was concomitant with that of other Th1 cytokines such as IL-2 and IFN-γ; (3) IL-17 induced IL-6 production from HGF in a dose- and time-dependent fashion in vitro, and (4) IL-17 was associated with cells producing RANK and RANKL, which may be related to bone resorption activity.

At the mRNA level, IL-17 was detected in the samples from periodontal lesions as well as other Th1 cytokines IL-2 and IFN-γ (Tables 2 and 3). Recently, Oda et al. (2003) have reported that IL-17 mRNA was detected in inflamed gingival tissues and our

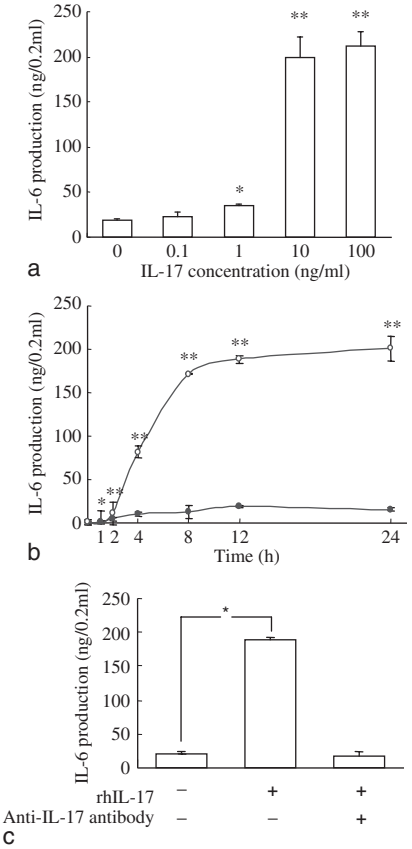

Fig. 2. Effects of recombinant human interleukin-17 (rhIL-17) on IL-6 production by human gingival fibroblasts (HGF). (a) Effect of rhIL-17 at different concentrations on IL-6 production by HGF. HGF were stimulated with different concentrations of rhIL-17 for 24 h. Data were expressed as the mean ± SD from triplicate studies. $^{*}p<0.05$; $^{**}p<0.01$ *versus* control. (b) Time course of rhIL-17-induced IL-6 production. HGF were stimulated with rhIL-17 (10 ng/ml) for different periods of time. Open circles, IL-17; solid circles, unstimulated. $^{*}p<0.05$; $p<0.01$ *versus* control. (c) Anti-human IL-17 antibody (10 μg/ml) completely inhibited IL-17-induced IL-6 production by HGF. $^{*}p<0.001$ *versus* control.

results confirmed theirs. These Th1 cell-derived cytokines may play a crucial role in inflammatory bone resorption (Kawai et al. 2000). It has been proposed that the balance of Th1/Th2 cytokines might be important for immune responses against bacterial infection (Modlin & Nutman 1993). Our results showed that the mRNA expression pattern of IL-17 was correlated with that of Th1 cytokines, IL-2 and IFN-γ, but not with the Th2 cytokine IL-4. This result suggests that IL-17 may be produced by Th1 cells in periodontal tissues, which is consistent with a previous report (Aarvak et al. 1999). Recent evidence shows that PMN also produces this cytokine (Ferretti et al. 2003, Witowski et al. 2004), although it has been reported that IL-17 is restrictedly produced by lymphocytes when we started this study. Actually, we tried to detect IL-17 protein by immunohistochemistry; however, it was not successful. However, in the gingival tissues, few PMN were observed generally, so we at present believe that IL-17 is produced by lymphocytes.

IL-4, the counterpart of IFN-γ, was detected in very low frequency (one of 23, Table 2) and this result was consistent with a previous finding (Fujihashi et al. 1993). Yamamoto et al. (1996) reported that the absence of IL-4 in inflamed gingiva inhibits apoptotic cell death in the accumulated macrophages and hence may contribute to the chronicity of the disease. In contrast, IL-10 was more frequently detected than that of IL-2 or IFN-γ. It has been reported that IL-10 is produced by T cells, B cells, and macrophages. This cytokine may also contribute to the chronicity of periodontal diseases. Therefore, the interaction of IL-17 with other cytokines within the complex cytokine network needs to be elucidated.

In this study, we could detect IL-17 at both mRNA and protein levels in periodontal lesions. Regarding the IL-17 protein, 10 of 16 gingival explants from periodontitis patients examined produced IL-17. These results support the possibility that IL-17 may be constantly produced by T cells in the periodontal tissues, even though IL-17 mRNA expression may not be readily detected. However, this is also true of IL-4 (Yamazaki et al. 1994, 1997). IL-17 mRNA is only transitory and very short-lived similar to IL-4 mRNA. The short half-life of some mRNAs is typical of those that contain AU-rich

elements, as does the IL-4 gene, which means that the results do not necessarily reflect the actual cytokine production. The IL-17 gene also contains AU-rich elements (Yao et al. 1995), and thus may also be degraded shortly after its formation. In our preliminary study, we could consistently detect IL-17 mRNA from all PHA-stimulated PBMC examined (data not shown).

It was reported that T cells could regulate Aa-induced periodontal inflammation and alveolar bone destruction through RANKL on T cells and RANKL-mediated osteoclast activation (Teng et al. 2000). Our results indicate that RANKL and RANK are produced in periodontal lesions where IL-17 is produced by activated gingival T cells. It was also reported that RANKL is expressed on T cells (Horwood et al. 1999, Kong et al. 1999) and periodontal ligament cells (PDL) (Kanzaki et al. 2001), whereas RANK is present on HGF (Sakata et al. 1999) and PDL (Wada et al. 2001). These results, and previous findings, suggest that T cells in periodontal lesions can regulate inflammatory reactions and bone resorption by making contact with other cell types.

IL-17 was undetectable in the GCF samples tested from periodontitis patients (data not shown), whereas it could be frequently detected by Western blotting and ELISA of cultured periodontal tissues from them. This discrepancy can be explained in several possible ways: (1) IL-17 produced by activated T cells in periodontal lesions is bound to IL-17 receptor-positive cells, (2) IL-17 may be degraded in the gingival crevice, as is known to occur for IgA1 (Takahashi et al. 1997), by both bacteria- and host cell-derived proteases, and (3) the amount of IL-17 in GCF is relatively low. Our results suggest that IL-17 produced by gingival T cells may be bound by membrane-bound IL-17 receptors in periodontal tissues. Therefore, further study is required to investigate IL-17 receptor-positive cells and the possible existence of soluble IL-17 receptors in the periodontium to better understand the role of IL-17 in the lesions.

The function of IL-17 as a pro-inflammatory T-cell-derived cytokine produced in the periodontium led us to the hypothesis that this cytokine could also be involved in inflammation and bone destruction. We have previously reported that IL-6 was produced from gingival fibroblasts both in in vivo and in vitro assays (Takahashi et al. 1994b, Takigawa et al. 1994). Therefore, we have investigated the kinetics of IL-6 production from IL-17-stimulated HGF (Fig. 2). We accordingly investigated the effect of rhIL-17 on the production of the inflammatory cytokine IL-6 by HGF in vitro. rhIL-17-stimulated HGF to produce IL-6 in a dose- and time-dependent fashion (Fig. 2). These results indicate that HGF express the IL-17 receptor and that T cells regulate the HGF response through this receptor. Thus, IL-17 produced in periodontal lesions may exacerbate the inflammatory response in part via inflammatory cytokines from HGF as well as other fibroblasts (Fossiez et al. 1996, Zhou et al. 1998).

This study highlights that IL-17 is locally produced by T cells in periodontal lesions and that this cytokine may exacerbate inflammatory reactions both directly and indirectly via inflammatory mediators from HGF in periodontal tissues. In addition, these findings have contributed to our understanding of the molecular basis of bone destruction and immunology and further elucidates the pathogenesis of other inflammatory diseases that induce bone destruction.

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