Increased Interleukin-18 in the Gingival Tissues Evokes Chronic Periodontitis after Bacterial Infection

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Periodontal disease is a chronic inflammatory disease that results in the breakdown of the tooth-supporting tissues, and can ultimately lead to resorption of the alveolar bone. Recently, several studies have shown a close relationship between increased interleukin-18 (IL-18) levels and the pathogenesis of chronic periodontitis, a major cause of tooth loss. However, it has yet to be shown whether chronic periodontitis results from or causes an increase in IL-18 after bacterial infection. In the present study, we investigated how IL-18 overexpression relates to periodontal disease using IL-18 transgenic (Tg) mice. IL-18Tg and wild-type mice were inoculated intraorally with *Porphyromonas (P.) gingivalis*, which has been implicated in the etiology of chronic periodontitis. Seventy days after *P. gingivalis* infection, alveolar bone loss and gingival cytokine levels were assessed using histo-morphological analysis and enzyme-linked immuno-absorbent assay, respectively. Periodontal bone loss was evoked in IL-18Tg mice, but not in wild-type mice. Interestingly, levels of bone-resorptive cytokines, including IL-1α, IL-1β, tumor necrosis factor-α, and IL-6, were unchanged in the gingival tissues of IL-18Tg mice infected with *P. gingivalis*, although levels of interferon γ (a proinflammatory T-helper 1 cytokine) decreased. RT-PCR analysis showed elevated expression of mRNAs for receptor activator of nuclear factor kappa-B ligand (a key stimulator of osteoclast development and activation) and CD40 ligand (a marker of T cell activation) in the gingiva of IL-18Tg mice infected with *P. gingivalis*. We conclude that increased IL-18 in the gingival tissues evokes chronic periodontitis after bacterial infection, presumably *via* a T cell-mediated pathway.

Introduction

Periodontal disease is a chronic inflammatory disease that results in the breakdown of the tooth-supporting tissues, and can ultimately lead to resorption of the alveolar bone. Chronic periodontitis is the most common form of this disease, and is a major cause of tooth loss. A multitude of pathogens are known to have a role in the development of chronic periodontitis, but *Porphyromonas (P.) gingivalis*, a Gram-negative anaerobic bacterium, has been particularly strongly implicated in the etiology of this disease (Sun et al. 2010; Hajishengallis et al. 2012). Not all individuals are equally susceptible to bone resorption when infected with this bacterium (Griffen et al. 1998), but this finding suggests that host factors are important in the induction and progression of the disease.

One such host factor is the interleukin cytokine, IL-18, which has recently been reported to be higher in both gingival tissue and gingival crevicular fluid in patients with periodontitis when compared with healthy subjects (Orozco et al. 2006; Figueredo et al. 2008; Pradeep et al. 2009). Similarly, serum IL-18 was significantly elevated in patients with chronic periodontitis relative to that in healthy subjects (Sánchez-Hernández et al. 2011), and increases in IL-18 in gingival biopsy tissues were correlated directly with pocket depth (Johnson and Serio 2005). Thus, several studies have shown that the levels of IL-18 in patients with periodontitis differ from those in healthy subjects. These findings imply a close relationship between increased IL-18 levels and the pathogenesis of chronic periodontitis.

IL-18, a member of the IL-1 family, was originally discovered in the *Propionibacterium acnes*-induced toxic shock model as an interferon (IFN)-γ-inducing factor, which induces both Th1 and Th2 cytokines, proinflammatory cytokines, chemokines, and IgE and IgG1 production (Hoshino et al. 1999, 2000). Additionally, it has been reported that overexpression of IL-18 in the skin aggravates allergic and non-allergic cutaneous inflammation (Kawase et al. 2003). These findings suggest that excessive IL-18 in the gingival tissues could be a key stimulator of periodontal disease. However, there is no direct evidence indicating whether IL-18 overexpression can evoke periodontitis after bacterial infection *in vivo*. Therefore, we examined the role of IL-18 in periodontal bone loss, using IL-18 transgenic (Tg) mice.

Materials and Methods

Mice

All experiments complied with the Guidelines for Care and Use of Laboratory Animals in Tohoku University. IL-18Tg mice with a C57BL/6N background (8-10 weeks old) were kindly provided by T. Hoshino (Kurume University, Kurume, Japan). In these mice, keratinocytes express mouse IL-18 fused to the signal peptide of the mouse immunoglobulin κ-chain, under the control of the human keratinocyte K5 promoter (Kawase et al. 2003). Age-matched wild-type (WT) C57BL/6N mice were purchased from Charles River Japan (Yokohama, Japan). The mice were bred in the animal facility of Tohoku University Graduate School of Dentistry (Sendai, Japan) and were maintained under pathogen-free conditions. Animals were matched for age and gender (all female) in all studies.

Infection with P. gingivalis

P. gingivalis W83, a pathogenic strain isolated from a case of human periodontitis, was initially grown on tryptic soy broth agar plates with 5% sheep blood, and subsequently in mycoplasma broth under anaerobic conditions (37°C, 80% N_2, 10% H_2, and 10% CO_2). The cells were harvested by centrifugation at 7,000×g for 15 min and resuspended in pre-reduced, anaerobically sterilized Ringer's solution (PRAS). The final concentration of *P. gingivalis* W83 was determined spectrophotometrically, and adjusted to 1×10^{10} cells/ml in phosphate-buffered saline containing 2% methylcellulose. Periodontal infection of mice with this *P. gingivalis* culture was carried out as previously described (Baker et al. 1999, 2000a, b; Sasaki et al. 2004b).

Prior to infection, all animals received antibiotic treatment (Sulfatrim suspension; 20 ml/100 ml of drinking water) for 4 days to reduce the original oral flora, followed by 3 days of no antibiotics. Each animal was infected with 1×10^9 *P. gingivalis* W83 delivered into the oral cavity and esophagus three times from day 0 to day 6 at 2-day intervals. Non-infected mice (negative controls) were given methylcellulose gavage without *P. gingivalis*. To confirm *P. gingivalis* colonization, the oral cavity of each animal was sampled on day 14 after the initial bacterial administration using a sterile cotton swab immersed in PRAS. Swab samples were vigorously mixed in 500 μl of PRAS. A 50-μl aliquot was plated, in triplicate, onto tryptic soy broth agar plates and incubated anaerobically for 3-4 weeks to identify *P. gingivalis* by monitoring black pigmentation and Gram staining. Animals without measurable *P. gingivalis* colonization were

excluded from the infection group.

Sample preparation

Animals were killed by CO_2 inhalation on day 70 after the initial round of *P. gingivalis* infection. The mandible and maxilla was removed from each animal, and the mandibles hemisected (the right and left mandibular hemisections being used for histological analysis and protein extraction, respectively). Maxillae were used for RNA extraction. Gingival tissues were isolated under a surgical microscope and stored at −70°C until further analysis.

The left hemisected mandible was subsequently de-fleshed, bleached, and mounted on a microscope slide for bone loss measurements. For protein extraction, gingival tissue was ground, using a sterile tissue homogenizer, in 1 ml of lysis buffer, as previously described (Sasaki et al. 2000, 2004a, b). The mixture was incubated at 4°C for 1 h, and the supernatant collected after centrifugation and stored at −70°C until assayed. Total gingival RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), with genomic DNA contamination eliminated using DNA-free™ reagent (Ambion, Austin, TX, USA) according to the manufacturer's protocols.

Histological analysis

The right mandibular hemisections were fixed at 4°C in 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer, pH 7.4, for 20 h. The tissue specimens were decalcified for 12 h in Kalkitox™ (Wako Chemical, Osaka, Japan) then soaked in 5% sodium sulfate solution (Wako Chemical) for 24 h at 4°C. After decalcifying, the tissue specimens were stored overnight in a 10% sucrose solution, then in a 20% sucrose solution for 12 h. Serial cryostat sections of each mandible were cut in the sagittal plane at a thickness of 10 μm using a Leica cryostat CM 3050S (Leica Microsystems, Solms, Germany), then mounted on glass slides as described previously (Kawamoto 1990; Kawamoto and Shimizu 2000).

For histopathological analysis, thawed tissue sections were stained with hematoxylin and eosin. For immunohistochemistry analysis, the sections were incubated with rabbit polyclonal anti-mouse IL-18 overnight at 4°C, then treated with peroxidase blocking reagent (DAKO, Glostrup, Denmark) for 20 min and with a secondary antibody (goat anti-rabbit Simple Stain Mouse MAX-PO(R) (Nichirei, Tokyo, Japan)). The chromogen used was 3′,3-diaminobenzidine tetrahydrochloride (DAKO). Sections were counterstained with hematoxylin. As a negative control, rabbit immunoglobulin (DAKO) was used.

Bone loss measurements

Images of molar teeth and alveolar bone were captured using digital microscopy (Leica MZ6 and Leica DFC295 system) and saved as TIFF files. The area of periodontal bone loss was determined using Adobe Photoshop™ (Adobe Systems, San Jose, CA, USA). The polygonal area enclosed by the cementoenamel junction, the lateral margins of the exposed tooth root, and the alveolar ridge were measured on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/), as previously reported (Sasaki et al. 2004b). An image of a ruler was captured at the same magnification and used for calibration. Results are expressed in mm^2.

Cytokine ELISA assay

For non-infected WT mice and non-infected IL-18Tg mice,

blood samples were obtained from the lateral tail vein, and serum was separated by centrifugation at 2,000 × g at 4°C, and stored at −70°C until use. Serum IL-18 levels were assayed using an IL-18 enzyme-linked immunoabsorbent assay (ELISA) kit (Medical and Biological Laboratories Company, Nagoya, Japan), according to the manufactur-

er's instructions.

Other commercially available ELISA assay kits were used to measure cytokines in tissue extracts; kits for IL-1α, IL-1β, and tumor necrosis factor (TNF)-α were obtained from BD Biosciences (San Jose, CA, USA), while kits for IFN-γ, IL-12, IL-6, IL-4, and IL-10
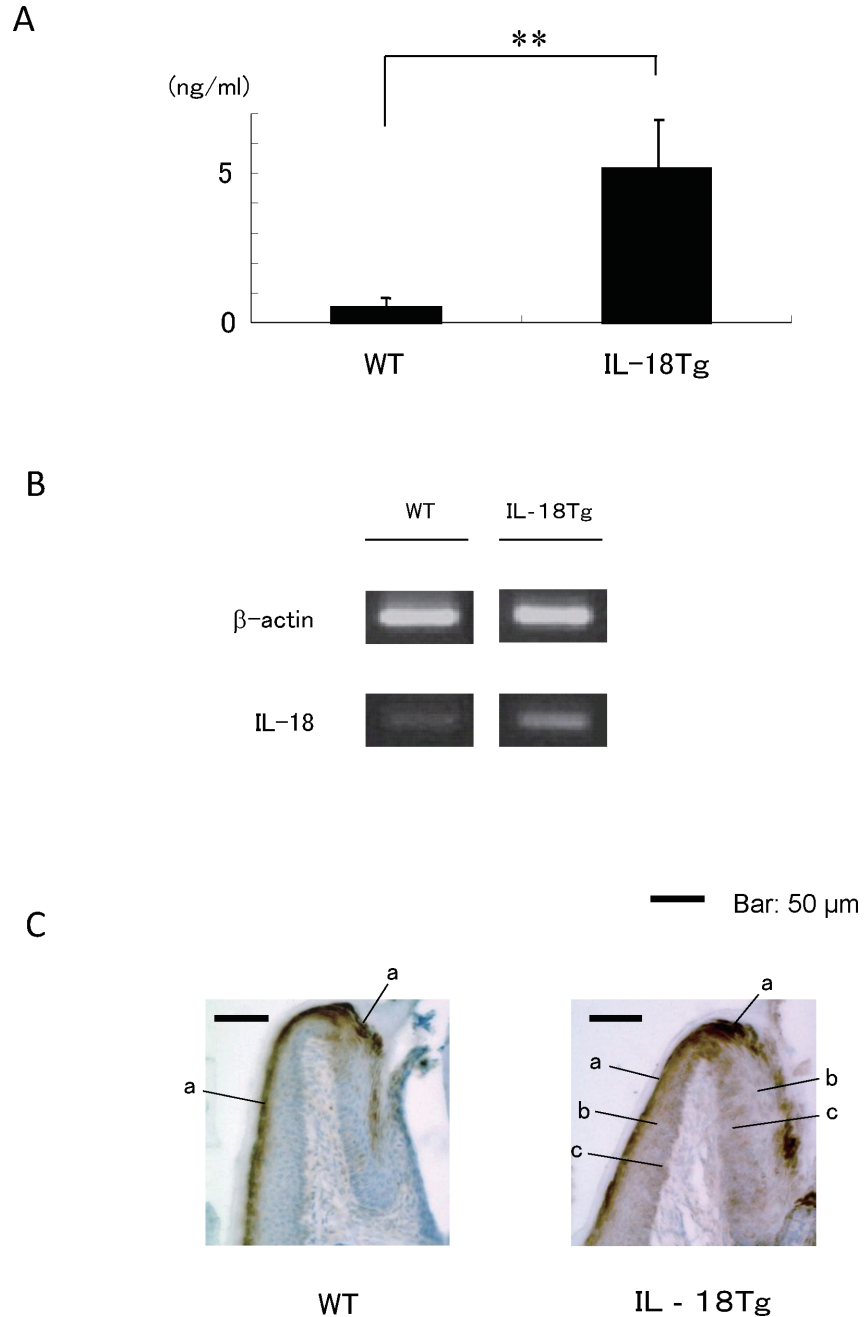
Fig. 1. Serum IL-18 levels and IL-18 expression in gingival tissues.
(A) Mean serum IL-18 levels in non-infected WT and IL-18Tg mice (*n* = 6). Data represent the mean ± s.d. of serum IL-18 levels in ng/ml. **P < 0.01. (B) Expression of IL-18 in gingival tissues. Total RNA was extracted from gingivae on day 70 after infection of mice with *Porphyromonas gingivalis* and was analyzed by semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR). *β*-Actin was used as housekeeping gene. (C) Immunohistological analysis if IL-18 distribution within epithelial tissues. Cryosections of murine gingival tissues from a non-infected WT mouse and an IL-18Tg mouse were stained with anti-IL-18 monoclonal antibody 25-2G and counterstained with hematoxylin (blue). The presence of IL-18 is visible as a brown coloration in the epithelium of gingival tissues (a, granular cell layer; b, spinous cell layer; c, basal cell layer of the epithelium). WT, wild-type mice; IL-18Tg, IL-18 transgenic mice.

were purchased from BioSource International (Camarillo, CA, USA). All assays using commercial kits were performed according to the manufacturers' instructions. Results were expressed as picograms of cytokine per milligram of periodontal tissue (pg/mg).

Gene expression analysis

Gene expression in gingival tissues was determined on day 70 after the initial round of infection using reverse-transcription polymerase chain reaction (RT-PCR). cDNA was reverse-transcribed with SuperScript™ II RT and an oligo-dT$_{12-18}$ primer (both from Invitrogen). cDNA was amplified using the HotStarTaq System (Qiagen, Valencia, CA). Sequences of specific primer sets were as follows: β-actin (382 bp): sense 5′-AGTACCCCATTGAAC ATGGC-3′, antisense 5′-TCGGTCAGGATCTTCATGAG-3′; IL-18 (434 bp): sense 5′-ACTGTACAACCGCAGTAATACGG-3′, antisense 5′-AGTGAACATTACAGATTTATCCC-3′; receptor activator of nuclear factor kappa-B ligand (RANKL; 812 bp): sense 5′-GGTCGG GCAATTCTGAATT-3′, antisense 5′-GGGAATTACAAAGTGC ACCAG-3′; osteoprotegerin (284 bp): sense 5′-GAAAGACCTGCA AATCGAGC-3′, antisense 5′-AAACAGCCCAGTGACCATTC-3′; CD40L (802 bp): sense 5′-TCAGTCAGCATGATAGAAAC-3′, antisense 5′-GACAGCGCACTGTTCAGAGT-3′; CD23 (228 bp): sense 5′-CACTGGGAAACGGAGAAG-3′, antisense 5′-CCTTAG ATCCTCCTGGAGT-3′. An optimized protocol of thermal cycling was used, comprising 95°C for 15 min, followed by 27 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The number of cycles was in the linear range of amplification for all PCR products.
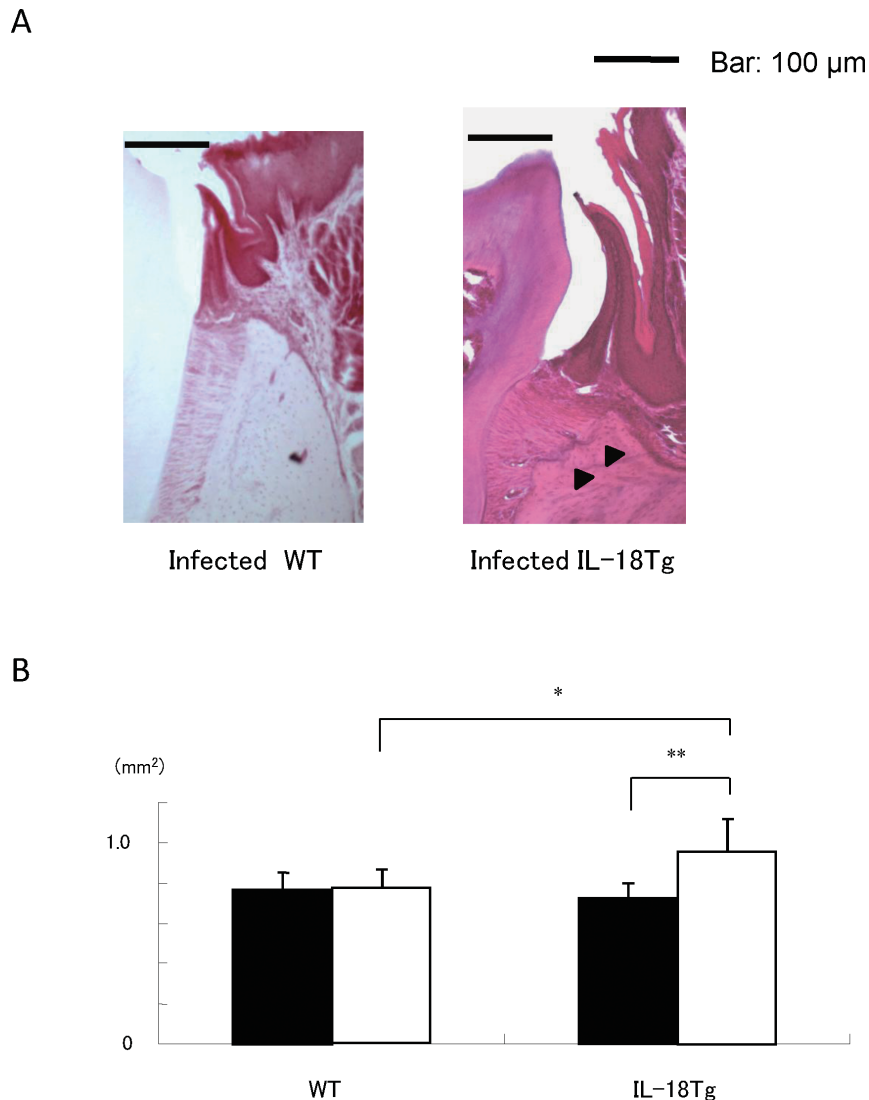

Fig. 2. Effect of *Porphyromonas gingivalis* infection on alveolar bone loss.
(A) Cryosections of gingival tissue from *P. gingivalis*-infected WT and IL-18Tg mice. Tissue was stained with hematoxylin and eosin and is shown at 100× magnification. The arrowheads indicate the position of alveolar bone resorption. (B) Effect of *P. gingivalis* infection on alveolar bone loss in WT and IL-18Tg mice. Data represent the mean area ($\pm$ s.d.) of periodontal bone loss (mm^2) for the non-infected group ($n = 8$ each, solid shading) and the infected group ($n = 8$ each, no shading). **$P < 0.01$, infection effect; *$P < 0.05$, genotype effect. The area of exposed cementum in the non-infected animals represents the physiologic attachment site of gingival tissue. WT, wild-type mice; IL-18Tg, IL-18 transgenic mice.

Statistical analysis

Results are expressed as mean ± standard deviation (S.D.). Statistical analyses were performed using Stat View 5.0 software (SAS Institute Japan, Tokyo, Japan). Differences in bone loss measurements and cytokine levels in gingival tissues from WT mice and IL-18Tg mice were analyzed using the Tukey-Kramer method after analysis of variance. Differences in serum IL-18 levels between non-infected WT mice and non-infected IL-18Tg mice were analyzed using Student's *t*-test. In these tests, *P*-values less than 0.05 ($P < 0.05$) were considered to be significant.

Results

K5-dependent IL-18 transgene elevates gingival IL-18 expression

As shown in Fig. 1, serum IL-18 levels were significantly higher in non-infected IL-18Tg mice than in non-infected WT mice (Fig. 1A). Moreover, gene expression of IL-18 in the gingivae was up-regulated in IL-18Tg mice compared with WT mice (Fig. 1B).

Cryosections of murine gingival tissues, stained to indicate the presence of IL-18, from non-infected WT and IL-18Tg mice, are shown in Fig. 1C. IL-18 expression in non-infected WT mice was detected only in the granular cell layer and a part of the spinous cell layer of the oral epithelium. In contrast, in non-infected IL-18Tg mice, IL-18 was detected in the granular, the spinous and the basal cell layers of the epithelium.

Effect of P. gingivalis infection on alveolar bone loss

In IL-18Tg mice, *P. gingivalis*-infection induced apparent alveolar bone resorption (Fig. 2A), while no histopathologic changes were observed in WT mice after *P. gingivalis* infection. As shown in Fig. 2B, *P. gingivalis*-infected IL-18Tg mice exhibited greater alveolar bone loss ($P < 0.01$) than both infected WT mice and non-infected IL-18Tg mice. In contrast, *P. gingivalis* infection did not cause significant bone loss in WT mice.
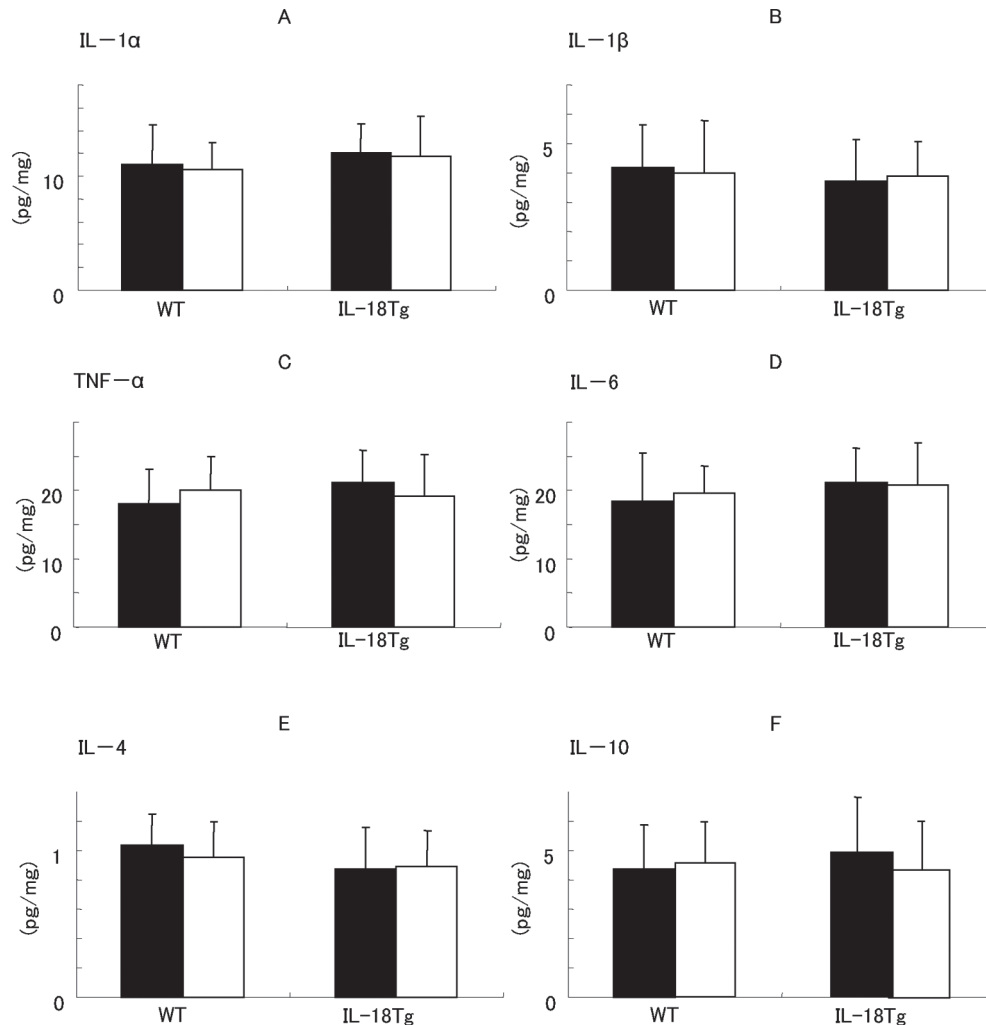

Fig. 3. Bone-resorptive cytokines and Th2 cytokine levels in gingival tissue.
Data are the mean ± S.D. (in pg/ml) of the levels of IL-1α (A), IL-1*β* (B), TNF-α (C), IL-6 (D), IL-4 (E) and IL-10 (F) in gingival tissues in WT and IL-18Tg mice (*n* = 6 for each). Solid shading: non-infected group; no shading: infected group. WT, wild-type mice; IL-18Tg, IL-18 transgenic mice.
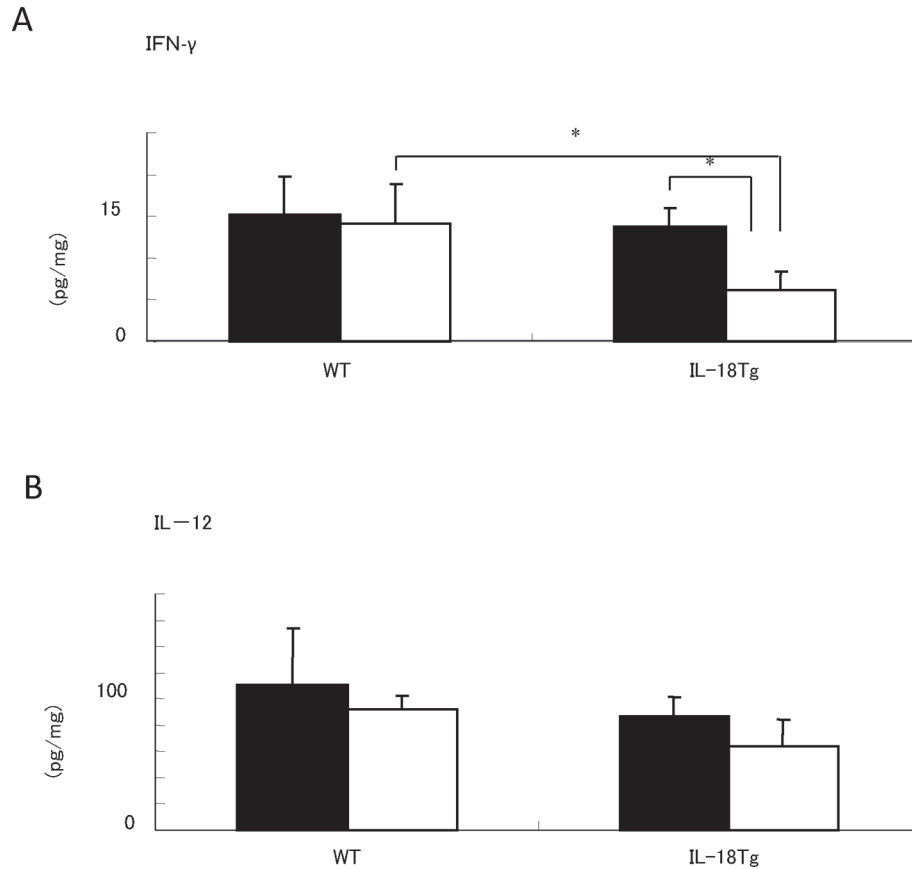
A

IFN-γ

B

IL－12

Fig. 4. Th1 cytokine levels in gingival tissues.
Data are the mean ± S.D. (in pg/ml) of the levels of the Th1-secreted cytokines IFN-γ (A) and IL-12 (B) in gingival tis-
sues in WT and IL-18Tg mice (*n* = 6 each). Non-infected group, solid shading; infected group, no shading. *P* < 0.05,
infection effect and genotype effect. WT, wild-type mice; IL-18Tg, IL-18 transgenic mice.

*Levels of bone-resorptive cytokines and regulatory cyto-
kines in gingival tissues*

P. gingivalis-infection did not affect the levels of bone-
resorptive cytokines (Fig. 3A-D) or Th2 cytokines (Fig. 3E,
F) in either IL-18Tg or WT mice. However, the levels of
IFN-γ were significantly (*P* < 0.05) decreased in IL-18Tg
mice, but not in WT mice, following infection. A genotype
effect was clear in the infected group (*P* < 0.05), but not in
the non-infected group (Fig. 4A). As shown in Fig. 4B,
levels of IL-12 were non-significantly decreased (by 27%)
in IL-18Tg mice after *P. gingivalis* infection.

Expression of cell activation markers

The gene expression profiles of several cell activation
markers were compared in WT and IL-18Tg mice by
RT-PCR. As shown in Fig. 5, gingival RANKL and CD40L
(a marker of T cell activation) were up-regulated by *P. gin-
givalis* infection in both mouse strains, but by a greater
magnitude in IL-18Tg mice. Gingival OPG, which acts as
a decoy receptor for RANKL, was not changed by *P. gingi-
valis* infection in either mouse strain. The IgE receptor,
CD23, which is expressed by activated B cells and macro-
phages, was up-regulated by *P. gingivalis* infection in
IL-18Tg mice, but not in WT mice.

Discussion

We have demonstrated here that, within 70 days of
infection, *P. gingivalis* induces periodontal bone loss in
IL-18Tg mice, but not in WT mice. Analyses of cell-asso-
ciated molecules showed that infection with *P. gingivalis*
up-regulated the expression of RANKL (a key stimulator of
osteoclast development and activation) and CD40L (a
marker of activated T cells) in IL-18Tg mice to a greater
degree than in WT mice. The results of the ELISA assay,
however, showed no concomitant up-regulation of the
bone-resorptive cytokines IL-1α, IL-1β, TNF-α, and IL-6.
These data indicate that bone loss in IL-18Tg mice corre-
lates with the expression of RANKL, but not with the
expression of the bone-resorptive cytokines. These results
are consistent with those of Sasaki et al. (2004b), who
determined that the pathway that stimulates bone loss is
independent of bone-resorptive cytokines. It has been
reported that RANKL is induced directly on activated T
cells (Kong et al. 1999; Gravallese et al. 2000; Kotake et al.
2001) and on osteoblasts and fibroblasts stimulated by IL-1
and TNF-α (Fujihashi et al. 1996; Gravallese et al. 2000;
Takayanagi et al. 2000a). The present study suggests that
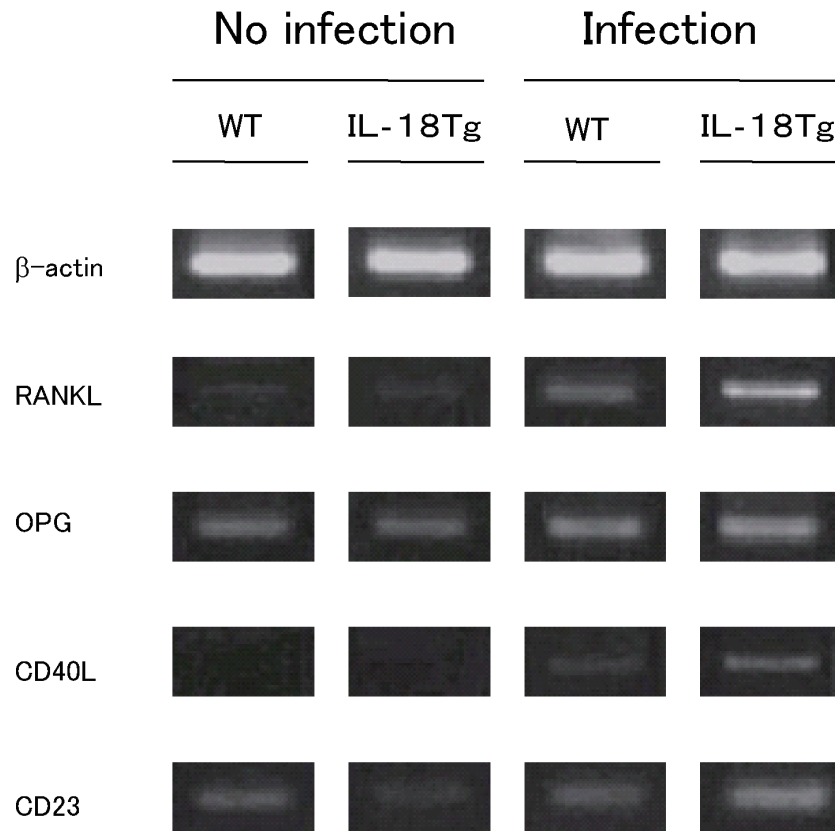RANKL produced by activated CD4^{+} T cells could have

Fig. 5. Expression of cell activation markers in gingival tissues.
  Total RNA was extracted from mouse gingivae at 70 days after infection with *Porphyromonas gingivalis* and analyzed by semi-quantitative RT-PCR. *β*-actin served as a housekeeping gene. Images shown are representative of at least three independent repeats. RANKL, receptor activator of nuclear factor kappa B ligand; OPG, osteoprotegerin; CD40L, CD40 ligand. WT, wild-type mice; IL-18Tg, IL-18 transgenic mice.

pathogenic consequences, in keeping with evidence that it can increase joint destruction in immune complex arthritis (Kong et al. 1999; Kotake et al. 2001) and enhance alveolar bone destruction *in vivo* (Teng et al. 2000).

In this study, the levels of IFN-*γ* were reduced by *P. gingivalis* infection in IL-18Tg mice, but not in WT mice, whereas no genotype effect was observed in the non-infected group. This result indicates that a reduction in IFN-*γ* levels could result in bone loss in *P. gingivalis*-infected IL-18Tg mice. It has been reported that the balance between the levels of RANKL and IFN-*γ* may regulate osteoclast formation (Takayanagi et al. 2000b). The effect of IFN-*γ* involves accelerated degradation of the RANK adaptor protein, tumor necrosis factor receptor-associated factor 6 (TRAF6). For example, during acute immune reactions, enhanced production of IFN-*γ* counterbalances the increased expression of RANKL and reduces aberrant osteoclast formation. In chronic synovitis of rheumatoid arthritis, however, this balance may be skewed in favor of RANKL expression (Takayanagi et al. 2000b). Thus, low levels of IFN-*γ* and enhanced expression of RANKL may contribute to the activation of osteoclastogenesis. Further study is required to clarify the mechanism and physiological significance of this phenomenon.

We have demonstrated here that the levels of IL-12 were decreased by 27% in IL-18Tg mice after *P. gingivalis* infection (Fig. 3B). This result is consistent with a report by Johnson and Serio (2005) that IL-12 is negatively correlated with the gingival sulcular depth. IL-12 alone was reported to cause a shift from a Th2 to a Th1 cellular profile (Pope and Shahrara, 2013), which suggests that the inverse correlation between IL-12 and bone loss in our study could be another important factor in a defective Th1-Th2 shift in periodontal bone loss.

Conflict of Interest

The authors declare no conflict of interest.

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