Lacking Interleukin-10 Regulates the Inflammasome-driven Alveolar Bone Loss

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Original Article

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Introduction

Alveolar bone, a component of periodontal tissues, supports teeth, and protects them from biting forces. Bone remodeling is a dynamic process in which osteoclast and osteoblast work together to resorb and synthesize bone.

Keywords:
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Abstract

Periodontitis triggers inflammation by pathogenic factors such as periodontal pathogenesis, which is exacerbated by the alveolar bone resorption, causing the teeth to fall out. It is known that inflammatory factors such as inflammatory cytokines spread throughout the body through blood circulation and other means and become a risk factor for developing systemic diseases such as diabetes and arteriosclerosis. Immune responses play an essential role in defending against invasion by pathogenic microorganisms and maintaining and improving homeostasis. IL-10, an inhibitory cytokine produced by immune cells, plays an essential role in preventing the development of autoimmune diseases caused by excessive immune responses by directly acting on immune cells and inhibiting their activation. However, the role in the steady-state remains unclear. In this study, we investigated the effects of IL-10 on periodontal tissue under steady-state conditions using IL-10 knockout (KO) mice. Significant alveolar bone resorption was observed in IL-10KO mice. The localization of osteoclast-like cells in the alveolar fossa by TRAP staining. Real-time PCR revealed an increased RANKL/OPG ratio, confirming that bone resorption occurred compared with wild type mice.

Moreover, significant inductions of pro-IL-1β, pro-IL-18, and IL-17A specific mRNA were detected, and protein levels were also induced in these mice’s gingiva. Furthermore, a significant increase in the expression of NLRP3, a representative marker of the inflammasome, was also observed in IL-10KO. These results indicate that IL-10 deficiency leads to alveolar bone resorption even under steady-state conditions. In particular, the markedly formed inflammasome was suggested to induce not only periodontitis but also systemic diseases.

These two events are closely coupled and play a role in maintaining anatomical integrity.

Under the steady condition, osteoclast proceeds by acidification and proteolytic digestion, but once the osteoclast leaves the site of bone resorption, osteoblast migrate and secrete mineralized osteoids to begin new bone formation. The amount of resorbed bone is equal to the amount of regenerated bone (1, 2). Periodontitis causes inflammation of the gingiva and the destruction of the al-
veolar bone and other periodontal tissues (3–6). As a result, the continued inflammatory responses can cause tooth loss by activating osteoclast and resulting in bone resorption’s progression (3–6).

It is well known that various cytokines activate osteoclast and are involved in alveolar bone resorption. Interleukin (IL)-10 is a crucial suppressive cytokine; lacking IL-10 has caused severe periodontitis and accelerate bone loss due to infection with Priopathogenic bacteria (7). However, the regulatory effect of IL-10 on the effects of OPG and RANKL has not yet been defined. IL-10 is known to inhibit TLR signaling. Here, processing the cytokine precursors (e.g., pro-IL-1β, pro-IL-18, and potentially pro-IL-33) into functionally active mature forms is essential for forming the inflammasome, such as caspase-1 activity (8). NLRP3, an NLR family member activation of caspase-1, acts in association with its adaptors protein ASC, leading to the secretion IL-1β and IL-18 (8). These cytokines influence the progression of inflammatory bone diseases such as arthritis (9, 10), suggesting that the inflammasome has been implicated as a factor in developing the inflammatory disease.

We hypothesize that the inhibitory cytokine IL-10 forms the inflammasome under steady-state conditions and induces periodontitis by inducing osteoclast differentiation through proinflammatory cytokines. Investigation of periodontitis pathogenesis, focusing on IL-10, will be useful in elucidating the involvement of various cytokines in the pathogenic mechanisms of periodontitis.

**Materials and Methods**

**Mouse strains**

Five-week-old female BALB/c mice, obtained from CLEA Japan, Inc (Tokyo, Japan) were provided regular mouse feed and water ad libitum. IL-10 KO BALB/c mice were purchased from The Jackson Laboratory (ME, USA).

Animals were maintained in the AIST under pathogen-free conditions. Age-matched wild-type BALB/c mice were used as controls. The mice were maintained under specific-pathogen-free conditions on temperature-controlled clean racks with a 12-h light-dark cycle. The Animal Welfare Committee approved animal experiments of AIST.

**Tissue sample preparation**

Animals were sacrificed at 13 weeks old. Mandibles were removed and hemisected. Gingival tissues were isolated under a surgical microscope and stores at -70°C for later RNA extraction. Alveolar bone was decalcified by 150 mM EDTA in PBS for 5–7 days at 4°C and then embedded in paraffin. Total gingival RNA was extracted using TRIzol reagent (ThermoFisher Scientific, CA, USA), and genomic DNA contamination was eliminated using the DNA-free kit (ThermoFisher Scientific, CA, USA) following the manufacturer’s instructions.

**Measurement of alveolar bone loss**

The preparation of the lower jaw as described above. Horizontal bone loss around the maxillary molars was assessed using morphometric methods, as described previously (11). Briefly, after removing gingival tissue, skulls were immersed overnight in 3% hydrogen peroxide, pulsed for 1 min in bleach, and stained with 1% methylene blue. The distance from the cement-enamel junction to the alveolar bone crest was measured at 14 buccal sites per mouse. Measurements were made under a dissecting microscope (×20) fitted with a video image marker measurement system standardized to provide measurement in millimeters.

**Histological analysis of gingival tissue and alveolar bone**

Four-micrometer-thick serial sections were then prepared and stained with hematoxylin and eosin (HE). For the detection of tartrate-resistant acid phosphatase (TRAP) activity, the section was incubated with TRAP staining solution and counterstained with hematoxylin. Sections were stained with tartrate-resistant acid phosphate (TRAP), and TRAP-positive multinucleated (>3 nuclei) cells were counted as osteoclast. Cytochemical staining for TRAP was used to identify osteoclast cells, as previously described.

**Quantitative real-time PCR**

Total RNA from gingival tissue samples was extracted using the RNeasy Mini kit and treated with DNase I (Qiagen, Germantown, MD, USA) according to the manufacturer’s instructions. Aliquots of RNA were then reverse-transcribed with oligo(dT) primers using SuperScript® reverse transcriptase (Invitrogen Corp, Tokyo, Japan) to generate cDNA. Quantitative real-time
RT-PCR analyses were performed using a Thermal Cycler Dice real-time PCR system (Takara Bio Inc., Otsu, Japan) by the manufacturer’s protocol. All reactions were carried out in a total volume of 25 µL, containing 30 ng of reverse-transcribed RNA, 12.5 µL of 2X SYBR Green PCR Master Mix (Takara Bio Inc., Otsu, Japan), and each primer at 100 nM. Specific primer for pro-IL1β, -17A, -18, NLRP3, RANK, RANKL, OPG, and GAPDH were supplied by Takara Shuzo (Kyoto, Japan). PCR was performed using the following protocol: 95°C for 30 s, followed by 50 cycles of 95°C for 5 sec and 60°C for 30 sec. Next, a dissociation curve analysis was performed to confirm specificity. The amplification of each gene and melting curve analysis was performed in triplicate. Target mRNA levels were normalized to that of GAPDH mRNA. qPCR was performed in the Dice cycler (Takara Bio Inc., Otsu, Japan), and the results were analyzed by using the ΔΔCT methods normalizing against GAPDH. The sequences of the primers for PCR were as follows:

pro-IL-1β forward (5’-TGACTGACTACGCCTGCTGTGG-3’) and
reverse (5’-TCTTTGAAGTTGACGGACCC-3’);
pro-IL-18 forward (5’-CAGGCCTGACATGTTGTGCAA-3’) and
reverse (5’-TCTGACATGGCAGCCATTGT-3’);
IL-17A forward (5’-ATCCCTCAAGCTCAGCGTTGC-3’) and
reverse (5’- TTCCTTCAAAAGCTCAGCGTTCG-3’);
RANK forward (5’-AGCCGTTACGGATAGGTTGTC-3’) and
reverse (5’-GATGAGATGTCTCGGCTGCTTG-3’);
RANKL forward (5’-CAGGTCCAGCGCAATATAA-3’) and
reverse (5’-CTGGACATGTGCCACTGAGAA-3’);
OPG forward (5’-AAACAGCCCGTGACCTTCCTA-3’) and
reverse (5’-AGGTTGGTGCACAGCCTGACCAAC-3’);
NLRP3 forward (5’-ACTGAAGCACCTGCTGTGGGAC-3’) and
reverse (5’-AAAAGCCTTGCTCTGACTGACAC-3’); and
GAPDH forward (5’-TGTGTTGAGCTGAGTCGCCAGG-3’) and
reverse (5’-TTGCTGTGAGCTGAGTCGCCAGG-3’).

Protein preparations and ELISA
Gingival tissue was homogenized using in CelLytic buffer (Sigma, St Louis, MO), according to manufacturer’s protocols. Briefly, frozen gingival tissue was ground using a precooled sterile mortar and pestle, and the tissue fragments were dispersed in 200 µL lysis buffer consisting of 10 µg tissue. The supernatant was collected after centrifugation and stored at -80°C until assay. Assay for cytokine in extracts employed commercially available ELISA kit obtained from following sources: IL-1β (ThermoFisher Scientific, CA, USA), IL-17 (R&D Systems, MN, USA), and IL-18 (MBL, Nagoya, Japan), all assays were conducted by the manufacturer’s instructions. The cytokine concentration was calculated from a standard curve that was constructed using recombinant cytokines provided with each kit. Results were expressed as picogram (pg) cytokine/mg gingival tissue.

Statistical analysis
All results are presented as means ± the standard errors of the mean (SEM), and experimental groups were compared with controls using unpaired non-parametric Mann-Whitney U test in Statview software. In all cases, p < 0.05 was considered significant.

Results
Effect of IL-10 KO on periapical bone destruction
First, we determined the alveolar bone loss in IL-10 KO mice. When the distances between the CEJ to the ABC at buccal sites were measured, significant alveolar bone resorption was seen in IL-10 KO mice. In contrast, wild type BALB/c mice showed minimal bone loss when compared with IL-10 KO mice (Fig. 1A). Histological analysis revealed no differences were seen in both mice (Fig. 2A). Further, the histological analysis indicated that a significant localization of TRAP-positive cells was detected in the peri-apical site of the alveolar bone crest in IL-10 KO mice compared with control mice (Fig. 2B).

Expression of the Bone resorptive-related gene in gingival tissue.
Because of the significant induction of alveolar bone loss in IL-10 KO, we examined the expression of RANK, RANKL, and OPG in the gingival tissue in these mice. RANK specific mRNA was significantly increased, although the ratio of RANKL/OPG was higher than WT
control mice (Fig. 3A). To elucidate this system’s role, we examined the profile of osteoclast in periodontal tissue in these mice. As seen in figure 2B, the TRAP-positive signal was detected in the apical alveolar bone site in IL-10 KO mice. These results suggested that IL-10 KO mice strongly up-regulate the expression of RANKL, which stimulates osteoclast in periodontal tissue, due to IL-10 deficiency.

Expression of osteoclastogenesis-related cytokine in gingival tissue.

Next, we examined the inflammatory responses in the gingival tissue to elucidate the mechanisms involved in alveolar bone resorption in these mice. Because of the lack of IL-10, a major anti-inflammatory cytokine which lead to prevent inflammation, we hypothesized that induction of inflammasome activation contributes to the development of alveolar bone loss in these mice. To confirm this point, we next examined the pro-inflammatory cytokine followed by inflammasome activation in IL-10 KO mice. Total mRNA was extracted from the gingiva of IL-10 KO mice, and pro-inflammatory-related gene expression was compared in WT mice. In contrast, IL-10 KO mice showed significant inductions of pro-IL-1β, pro-IL-18 mRNA in the gingiva of IL-10 KO mice compared with WT mice (Fig. 3A). To further determine the pre-onset of periodontitis associated with increased IL-1 protein production, gingival tissues were homogenized and examined by ELISA. Significant induction of IL-1β was detected in IL-10 KO mice.

Interestingly, IL-18 protein levels were comparable when compared with control WT mice. Therefore, we examined the inflammasome components by gingival tissue. The significantly elevated level of NLRP3 was detected in IL-10 KO mice when compared to WT mice (Fig. 3A).

Further, IL-17 produced either Th17 cells or innate lymphoid cells, contributed to the pathogenesis of periodontitis in animal models and periodontitis patients (12–14). While IL-1 has been shown to promote the differentiation of naive T cells into Th17 cells (15, 16), significant induction of mRNA and protein levels of IL-17 were detected in these mice. These data highlight the specific role of IL-1 cytokine members in the processes of alveolar bone resorption and indicate to us to examine the role of IL-10 in the formation of inflammasomes.

Discussion

We now show that the enhanced alveolar bone loss
seen in IL-10 knockout mice during steady-state condition is reveals increased expression of inflammasome formation and localized expression of IL-1β and IL-17 at sites of gingival tissue. Our data clarify a role for IL-10 as a negative regulator of the inflammasome and its role in osteoclastogenesis in the steady-state of the periodontal tissue.

IL-10 is suppressive cytokine with essential roles in chronic inflammation and suppressing excessive immune responses (17, 18). IL-10-deficient mice develop periodontitis following the periodontal pathogen’s inoculation and show excessive alveolar bone loss with inflamed gingiva (19, 20). Based on other groups studies, IL-10 may influence IL-1β production through gene transcription and di-
Rect regulating inflammasome activation. IL-10 has been shown as a crucial suppressive cytokine in maintaining homeostasis of the immune system (21). Our studies indicate that significant expression of pre- and pro-inflammatory cytokines such as IL-1β, IL-18, and IL-17A. IL-1β and IL-17A productions were highly elevated, but IL-18 productions were comparable in IL-10 KO mice.

NLRP3, which plays an essential role in the inflammatory responses, is a multi-protein complex that induces maturation of the inflammatory cytokines IL-1β and IL-18 through the activation of caspase-1 (22). It has been reported that NLRP3 proteins are increased in the gingi-
val tissues of patients with periodontitis (23). Previous, our studies suggested that a challenge with periodontal pathogenic bacteria with a lack of pathogenic components could not activate all NLRP3-related molecules in peripheral tissues and less inflammatory cytokines (24). Increased inflammatory cytokines (e.g., IL-1β, IL-6, and tumor necrosis factor-alpha (TNF-α)) in acute or chronic periodontitis may recruit immune cells not only in periodontal tissue but also throughout the body (25).

Under the steady condition, the primary function of immune cells is to maintain biological homeostasis in peripheral tissues, but under inflammatory conditions, they take on effector cells’ properties to be involved in infection protection and wound (26). Further, our previous data demonstrated that IL-17, produced by Th17 cells, contributes to periodontitis onset (27), and macrophages release pro-inflammatory cytokines to accelerate alveolar bone loss in the mouse model (26).

Also, the mechanisms for alveolar bone loss with P. gingivalis-infected and lack of IL-10 may be different. In P. gingivalis-infected periodontitis model, P. gingivalis causes direct damage to the epithelial barrier, which allows for infiltration of immune cells and massive inflammation with IL-6 and TNF-α induction. Moreover, IL-10 production was maintained by the activated T cell (28). Our data indicated that the IL-10 knockout mice showed neither gingival epithelial thickening nor infiltration of mononuclear cells, as seen in P. gingivalis-infected periodontitis mouse model. Suggesting that IL-10 is essential for maintaining periodontal tissue homeostasis under steady-state conditions; however, many unanswered questions remained.

IL-1β is also capable of affecting periodontitis (29).

In light of the result of the role of IL-10 in periodontal tissue homeostasis, it may be possible to focus on its function in periodontal tissue and immune cells. Although further research is needed on this issue, the apparent discrepancy in the role of IL-10 may be influenced primarily by the microbiome, which constitutes the ecosystem. The microbiome may largely influence the apparent discrepancy in the role of IL-10 in the oral cavity, but further research is needed to resolve this issue.

Bone and immune cells are very similar, with each having a common progenitor cell that requires cell signaling, and some of the immune cells have shown the ability to differentiate into osteoclast. For instance, Dendritic cells have been shown to differentiate into osteoclast early development (30). This process is stimulated by cytokines secreted by activating helper T cells (31). In terms of osteoclastogenesis, various systemic or local factors influence osteoclast formation and bone resorption ability. Under the steady-state, osteoclast activity is highly balanced by those factors, whereas under the inflammatory state, osteoclast is activated by pro-inflammatory cytokines produced primarily by activated effector T cells (32). As a cytokine involved in bone metabolism, IL-1 itself activates osteoclast and promotes the production of cytokine receptor activator NF-κB ligand (RANKL), a major positive controller of osteoclast formation (33). Besides, osteogenic factors such as hormones, cytokines, and growth factors are thought to exert significant osteoclast activity by regulating the RANK/RANKL/OPG axis (34, 35, 36). Genetically modified mice have become essential to our understanding of how a particular gene function in vivo. Here, we show that IL-10 negatively regulates the expression of NLRP3 inflammasome components followed by IL-1β family cytokines, which provided alveolar bone loss.

Our findings suggest that IL-10 is one of the mechanisms by which cytokines associated with inflammasome and osteoclastogenesis are regulated. The mechanism of inhibition of inflammasome formation by IL-10 remains unclear. We will further verify which regulatory mechanism is involved.

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