Effect of periodontopathic bacteria *Fusobacterium nucleatum* in oral and intestinal inflammation

（歯周病原細菌 *Fusobacterium nucleatum* による
口腔および腸管における炎症への影響）

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Title: Effect of periodontopathic bacteria *Fusobacterium nucleatum* in oral and intestinal inflammation

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Contents

1. Preface.

This article is constructed with a main reference paper "Involvement of *Fusobacterium nucleatum* in bone resorption and periodontal tissue inflammation" and a reference paper "Effect of periodontopathic bacteria *Fusobacterium nucleatum* on intestinal immune cells" in International Journal of Oral-Medical Sciences.

2. Abstract

Fusobacterium nucleatum is a gram-negative anaerobic bacterium that is resident in the oral cavity and causes various infectious diseases including periodontal disease and has recently been linked to colorectal cancer. In this study, we investigated the immune response of inflammatory periodontal tissue in mice to elucidate how *F. nucleatum* affects the immune responses of oral immune system. *F. nucleatum* was suspended in 5 % carboxymethyl cellulose (CMC), and mice were inoculated intraorally 5 times for 3 weeks. Mononuclear cells were isolated from gingival tissues 1 and 30 days after the last oral inoculation, followed by flow cytometry using fluorescently labeled antibodies, DNA extraction, and quantitative PCR. The histopathological examination of gingiva was performed with hematoxylin and eosin staining. The mandibles were collected and the alveolar bone resorption was measured using micro CT. In the group inoculated with *F. nucelatum*, infiltration of inflammatory cells was observed under the gingival mucosa, and remarkable horizontal bone resorption of alveolar bone was observed. In addition, real-time PCR analysis revealed that pro- interleukin (IL)-18 expression and receptor activator of nuclear factor kappa-B ligand (RANKL) / osteoprotegerin (OPG) ratio increased in gingival tissues. Furthermore, flow cytometry analysis confirmed that $CD4^+RANKL^+$ cells gradually increased in the gingival inflammation lesion. These results suggested that oral inoculation of *F. nucleatum* caused inflammation of the gingival tissue and activated osteoclasts via $CD4^+RANKL^+$ cells, resulting in alveolar bone resorption.

In the next study, we examined mucosal immune responses in the lower

gastrointestinal tract after oral intake of *F. nucleatum* to elucidate how chronic periodontal disease affects the immune response of the intestinal tract immune system. Mice were orally administered live *F. nucleatum* 5 times a week for 3 weeks and sacrificed 1 and 30 days after the final oral inoculation. Mononuclear cells were isolated from the small intestinal lamina propria (SiLP) and large intestinal lamina propria (LiLP), and these cells and tissues were used for immunological and histological analysis. On day 1 after the final oral administration of *F. nucleatum*, $CD4^+$ T cells producing IFN- γ, IL-17, and IL-10, and T cells with transcription factor Foxp3 significantly increased in LiLP, and in particular, IFN-γ-and IL-17- producing $CD4^+$ T cells and $Foxp3^+$ T cells tended to increase even on day 30. On the other hand, in SiLP, a slight increase in IFN-γ- and IL-17- producing $CD4^+$ T cells was observed on day 1, while IFN-γ- and IL-10- producing $CD4^+$ T cells were significantly increased on day 30. Histological analysis showed continuous observation of lymphocyte accumulation in LiLP. These results suggest that oral inoculation of *F. nucleatum* affects the dynamics of effector T cells involved in maintaining homeostasis of the lower gastrointestinal mucosa. In particular, it is suggested that effector T cells are activated not in the small intestine but in the large intestine, thereby disrupting the balance of the intestinal mucosal immune system.

3. Introduction

Periodontal disease is a chronic inflammatory disease that affects many adults. It is also a major cause of tooth loss and is characterized by chronic infection by gram-negative anaerobic bacteria in dental biofilms. It leads to irreversible destruction of the tissues that support the teeth and can be detected clinically by loss of periodontal pockets and alveolar bone (1, 2). Periodontitis often causes bacteremia, and the relationship between periodontitis and systemic diseases caused by periodontal pathogens has been investigated (3). Recently, it has been suggested that intestinal dysbiosis due to periodontal pathogens is a potential cause of various diseases (4). Periodontitis is a multifactorial disease, and the genetic background of the patient, the presence of pathogenic bacteria and immune mechanisms are important factors. During the process of inflammation in response to the plaque bacterial species, gingival fibroblasts produce interleukin (IL) -1 and IL-18 (5). Cytokine secretion at the site of inflammation is initially protective in the removal of infectious bacteria, but excessive or persistent pro-inflammatory cytokine production is associated with periodontal destruction, accompanied by loss of periodontal attachment and alveolar bone resorption caused by receptor activator of nuclear factor kappa-B ligand (RANKL) / osteoprotegerin (OPG) signaling pathway. Gingival crevicular fluid (GCF) and salivary cytokine levels are higher in patients with aggressive periodontitis than in healthy individuals and decrease after periodontal treatment (6). Macrophages are an important source of inflammatory cytokines such as IL-1β and TNF-α. In pathogenic dysregulation, these

cytokines contribute to host tissue destruction. Therefore, understanding the mechanism of immune cell signaling induced by periodontal pathogens can provide useful information for the prevention and treatment of periodontitis.

F. nucleatum is a gram-negative anaerobic gonococcus resident in the oral cavity, which causes various infections including periodontal disease, as well as being associated with GI disorders such as colorectal cancer and inflammatory bowel disease and adverse pregnancy (7). The bacteria also associated with atherosclerosis, respiratory tract infections, organ abscesses, rheumatoid arthritis, and Alzheimer's disease (7). In oral diseases, *F. nucleatum* is associated with a variety of periodontal diseases, including gingivitis and advanced chronic periodontitis, periodontitis including localized aggressive periodontitis and systemic aggressive periodontitis (8). It increases with disease severity, and rate of progress in inflammation and depth of pocket (8).

F. nucleatum has been reported to produce inflammatory cytokines such as IL-1 and IL-6 in gingival epithelial cells and macrophages in vitro (9, 10). However, there is no report that links the inflammation caused by *F. nucleatum* and the pathology of periodontal disease. Therefore, in the first study, we investigated the effects of oral *F. nucleatum* infection on bone resorption and periodontal inflammation in mice.

The human gastrointestinal tract is estimated to be colonised by over 10^{14} bacteria, approximately 10-fold of the total number of cells in the human body (11). Disruptions to the microbiome have been associated with severe pathologies of the host, including metabolic disease, cancer and inflammatory

bowel disease (12-14). The oral cavity and colon are separate anatomical regions, but both are highly established by distinct microbiota. However, oral bacteria can spread to the colon (15). This is mostly evident in the destruction of oral microbiomes, such as periodontitis, where certain bacteria such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis* have a pathogenic profile (16). In the colon, these bacteria change the composition of the residual microbiota in a complex biofilm situation, causing intestinal symbiosis. This oral driven disruption promotes abnormal immune and inflammatory responses and ultimately leads to colorectal cancer (CRC) tumorigenesis (15).

Therefore, in the second study, we examined the effects of oral infection with this bacterium on immunocompetent cells in the small and large intestines and the inflammation state of the intestinal tissue.

4. Materials and methods

4. 1. Bacterial Strain

F. nucleatum (ATCC 23726) was cultured on anaerobic blood agar plates (Becton Dickinson, Franklin Lakes, NJ, USA) in a model 1024 anaerobic system (Forma Scientific, Marietta, OH, USA) with 10% H_2, 80% N_2, and 10% CO_2 for 3-5 days. Cultures were then inoculated into brain-heart infusion (Difco Laboratories, Detroit, MI, USA) supplemented with 5% fetal bovine serum for 2 days until OD_{660nm} =0.8 was reached, corresponding to 10^9 CFU/mL. The cultured cells were centrifuged at 8000 x *g* for 20 min at 4℃ and resuspended in 5% carboxymethyl cellulose (CMC) for oral infection.

4. 2. Mice

Eight-week-old female BALB/c Cr Slc (BALB/c) mice, obtained from Sankyo Laboratories (Tokyo, Japan), were provided regular mouse feed and water *ad libitum*. The mice were maintained under specific-pathogen-free conditions on temperature-controlled clean racks with a 12-h light-dark cycle. All animals experiments were performed in accordance with the guidelines of the Bioscience Committee of Nihon University and were approved by the Institutional Animal Care and Use Committee of Nihon University (Approval number: AP19MAS006-1).

4. 3. Oral infection

Mice were randomly divided into two groups (n=8 per group). The first group was orally challenged with live *F. nucleatum* (1×10^9 CFU/100 μL with 5% CMC/mouse) once per day for 15 days. The second group consisted of sham-infected mice that received 100 μL of 5% CMC.

4. 4. Histological analysis of gingival tissue, small and large intestines and the measurement of alveolar bone loss

Thirty days after the last infection, mice were sacrificed and the skin of the lower jaw was removed and fixed in 4% paraformaldehyde in PBS for 24 h. The lower jaw was decalcified by incubation in 150 mM EDTA in PBS for 5–7 days at 4℃ and then embedded in paraffin. Four-micrometer-thick serial sections were then prepared and stained with hematoxylin and eosin. Horizontal bone loss around the maxillary molars was assessed using a morphometric method, as described previously (17). 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 cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was measured at 14 buccal sites per mouse (18). Measurements were made under a dissecting microscope (×20) fitted with a video image marker measurement system standardized to provide measurements in millimeters. In the small intestine and large intestine, the mice were removed after sacrifice at 1 and 30 days after the last infection. Samples were then processed and stained with hematoxylin and eosin in the same manner as described above.

4. 5. Flowcytometric analysis

At 1, 7, 15, and 30 days after the last *F. nucleatum* infection, mice were sacrificed and gingival tissues from the upper and lower jaws were carefully removed using microsurgical tweezers under a stereomicroscope. Cells from gingival tissues were prepared by gently teasing the tissue using sterile stainless steel screens, followed by an enzymatic dissociation procedure with 0.3 mg/mL of collagenase (Nitta Gelatin Co. Ltd., Osaka, Japan) in RPMI 1640 (Wako Pure Chemical Industries Ltd., Osaka, Japan) (19). GMCs were purified on discontinuous Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) and resuspended in RPMI 1640 supplemented with HEPES buffer (15 mM), L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% fetal bovine serum (Biofill, Victoria, Australia) (complete medium). GMC-enriched populations (2 x 10^5 cells) were stained with a combination of fluorescence-conjugated or biotinylated monoclonal antibodies, including anti-CD4, and -RANKL (BD Pharmingen, San Diego, CA, USA). The samples were subjected to flow cytometry analysis (FACSCalibur™; BD Biosciences).

Mononuclear cells from the lamina propria (LP) of the small and large intestine are isolated in the same manner as described above, concentrated using a discontinuous Percoll gradient (Pharmacia Fine Chemicals, Uppsala, Sweden), HEPES buffer (15 mM), L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), and complete medium Resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum(Biofill, Victoria, Australia) (complete medium). after that, for intracellular staining, cells (1.0×10^6 cells)

were labeled with allophycocyanin (APC)-conjugated anti-mouse CD4 monoclonal antibody (mAb) (eBioscience, San Diego, CA, USA) at 4℃ for 20 min. Cells were fixed, permeabilized with BD Cytofix/Cytoperm reagents, and then stained with phycoerythrin (PE)-labeled anti-mouse IFN-γ, IL-17, and IL-10 mAbs (BD Bioscience). For analysis of transcription factors, cells were incubated with APC-conjugated anti mouse CD4 or CD25 monoclonal antibodies (mAbs) and were then intracellularly stained with the Foxp3 or RORγt mAbs (BD Bioscience). Samples were analyzed by FACS flow cytometer (BD Biosciences) equipped with CellQuest software (BD Biosciences Pharmingen, San Diego, CA, USA). A minimum 10,000 cells in the lymphocyte gate (forward scatter/side scatter) were acquired and cell percentages were obtained using a dot-plot with a quadrant marker. Isotype controls were used as compensation controls and to confirm antibody specificity.

4. 6. Analysis of gene expression in GMCs

At 1 and 30 days after the last *F. nucleatum* infection, total RNA from gingival tissue samples was extracted using a 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) in accordance with the manufacturer's protocol.

All reactions were carried out in a total volume of 25 μL, containing 30 ng of reverse-transcribed cDNA, 12.5 μL of 2x SYBR Green PCR Master Mix (Takara Bio Inc.), and each primer at 100 nM. The primer sequences were as follows:

pro-IL-1β forward (5'-TGACTGACTACGCCTGCCTG-3') and

reverse (5'-TCTTTGAAGTTGACGGACCC-3');

pro-IL-18 forward (5'-CAGGCCTGACATGTTGTGCAA-3') and

reverse (5'-TCTGACATGGCAGCCATTGT-3');

RANLK forward (5'-CAGGTCCAGCGCAATATAA-3') and

reverse (5'-CTGGACATGTGCCACTGAGAA-3');

OPG forward (5'-AAACAGCCCGTGACCTTCCTA-3') and

reverse (5'-AGGTTGGTGCACAGCTTCACAA-3');

GAPDH forward (5'-TGTGTCCGTCGTGGATCTGA-3') and

reverse (5'-TTGCTGTTGAAGTCGCAGGAG-3').

PCR was performed in duplicate for each gene. PCR was performed using the following protocol: 95˚C for 30 s, followed by 50 cycles of 95˚C for 5 s, 60˚C for 30 s. Next, a dissociation curve analysis was performed to confirm specificity. The amplification of each gene and melting curve analysis were performed in triplicate. Target mRNA levels were normalized to that of GAPDH mRNA.

4. 7. Statistical analysis

All results are presented as means ± the standard errors of the mean (SEM), and experimental groups were compared with controls using an unpaired non-parametric Mann-Whitney U test in Statview software.

5. Result

5. 1. F. nucleatum-challenge induces gingival inflammation and alveolar bone loss

Oral infection of BALB/c mice with *F. nucleatum* induced inflammatory cell infiltration and connective tissue thickening compared to the control group treated with CMC on 30 days after the final administration (Fig. 1A). In the infected group, non-thinning of the alveolar bone was also observed.

Furthermore, in the measurement of alveolar bone loss, the distance from CEJ to ABC in 14 locations of the upper jaw was measured, and the average value of the total values for each mouse was shown. The distance of the control group was 213 ± 15.0 μm, while the average value of the *F. nucleatum* infected group was 329 ± 11.9 μm. Therefore, oral infection with *F. nucleatum* induced significant bone resorption of alveolar bone compared to the control group on 30 days after the final administration (Fig. 1B and C).

5. 2. F. nucleatum-challenge downregulates the expression of pro-inflammatory- and bone resorption-related factors

Inflammatory effects on bone resorption are mediated by inflammatory cytokines (20), and RANKL and OPG are involved in osteoclastogenesis (20). Therefore, we next examined the expression of gingival inflammatory cytokine genes and the ratio of RANKL / OPG gene expression. Oral infection of BALB/c mice with *F. nucleatum* showed increased expression of pro-IL-18 in the gingiva compared with the control group treated with CMC on 30 days after the final administration. We also observed a significant increase in the

RANKL / OPG ratio due to *F. nucleatum* infection (Fig. 2A and B). On the other hand, oral infection with *F. nucleatum* did not significantly increase the expression of pro-IL-1β in the gingiva compared to the control group treated with CMC.

5. 3. F. nucleatum-challenge induce CD4⁺RANKL⁺T cells in GMC

T cells play a central role in regulating immune responses, and many T cells are infiltrated in periodontitis tissues. Since RANKL, an osteoclast differentiation factor, has been confirmed to be produced in immune cells by inflammatory reactions (21), we next examined the increase or decrease in the ratio of $CD4^+RANKL^+$T cells in GMC. Oral infection of BALB/c mice with *F. nucleatum* showed a significant increase ($p<0.05$) in the ratio of $CD4^+RANKL^+$T cells in GMC compared to the control group treated with CMC on 30 days after the last administration（Fig. 3）.

5. 4. Analysis of CD4⁺ T cells producing IFN-γ, IL-17 and IL-10 in LiLP

The frequencies of $CD4^+$ T cells producing IFN-γ, IL-17 and IL-10 in LiLP were counted by flow cytometry day 1 and 30 after the final infection (Fig. 4A and B). Oral infection with *F. nucleatum* significantly increased $CD4^+$ T cells producing IFN-γ, IL-17 and IL-10 compared with the control group on day 1. The increase in these cells decreased on day 30 compared to day1 except for IFN-γ producing cells, but the number of cells in the infected group tended to be higher than in the control group.

5. 5. Th17 and regulatory T cell frequencies in LiLP

The frequencies of Th17 and Treg cells in LiLP $CD4^+$ T cells were counted by flow cytometry day 1 and 30 after the final infection. $CD25^+$ $Foxp3^+$ regulatory T (Treg) cells were significantly increased in *F. nucleatum*-challenged mice compared with the control group on day 1 and 30 (Fig. 5A and B). On the other hand, in $CD4^+RoR\gamma t^+$ Th17 cells, there was no significant difference between *F. nucleatum*-challenged mice and control mice.

5. 6. Analysis of $CD4^+$ T cells producing IFN-γ, IL-17 and IL-10 in SiLP

The frequencies of $CD4^+$ T cells producing IFN-γ, IL-17 and IL-10 in SiLP were counted by flow cytometry day 1 and 30 after the final infection (Fig. 6A and B). Oral infection with *F. nucleatum* significantly increased $CD4^+$ T cells producing IFN-γ and IL-17 compared with control group on day 1. However, these increases were significantly lower compared to those in LiLP. On the other hand, on day 30, an increase in IL-10 producing $CD4^+$ T cells in addition to IFN-γ was observed.

5. 7. Th17 and Treg cell frequencies in SiLP

The frequencies of Th17 and Treg cells in SiLP $CD4^+$ T cells were counted by flow cytometry day 1 and 30 after the final infection. $CD25^+Foxp3^+$Tregs decreased slightly on day 1, but increased on day 30 compared to controls (Fig. 7A and B). On the other hand, $CD4^+$ $RoR\gamma t^+$ Th17 cells in mice treated with *F. nucleatum* increased slightly on day 1 and decreased slightly on day 30 compared to control mice, but there was no significant difference.

5. 8. Changes in histological features of the large and small intestine after administration of F. nucleatum

The lymphoid follicle in LiLP was expanded on day1 after *F. nucleatum* challenge compared to control group, and this trend continued on day 30 after administration (Fig. 8A). However, in SiLP, the lymphoid follicle was expanded in *F. nucleatum*-challenged group as in LiLP compared to the control group on day 1 after administration, but decreased on day 30, which was almost the same as the control group (Fig. 8B).

6. Discussion

Periodontal disease is a chronic inflammatory disease caused by infection with anaerobic bacteria in the oral cavity, and a disease which bacteria in the biofilm cause inflammation in the gums and eventually dissolve the jaw bone that supports the teeth. *F. nucleatum* is one of the periodontopathic bacteria and plays a central role in dental plaque formation, and forms a biofilm by coaggregating with other bacteria (22).

In this study, *F. nucleatum* challenge induced gingival inflammation and alveolar bone loss. Inflammatory cytokines have been shown to play an important role in the regulation of inflammatory bone resorption. It has been reported that IL-1, IL-6 and TNF-α promote bone resorption, while IFN-γ and IL-4 suppress (20, 23). Further, one of the pattern recognition receptors, NLR family, pyrin domain containing 3 (NLRP3) forms a complex called NLRP3 and induces the production of IL-1β and IL-18, which are inflammatory cytokines. Both processing and pyroptosis of IL-1β and IL-18 precursor induced by inflammasome activation, are deeply involved in immune system activation and defense against infection (24), and play an important role in maintaining periodontal tissue homeostasis. On the other hand, their excessive activation leads to destruction of periodontal tissue (25). Our results indicated that oral infection of mice with *F. nucleatum* increased expression of pro-IL-18 in the gingiva. However, the expression of pro-IL-1β, a precursor of IL-1β, did not increase with *F. nucleatum* infection. In future, it will be necessary to search for changes in IL-1β expression over time and to search for trends in other inflammatory cytokines such as IL-6 and TNF-α.

RANKL is a member of the TNF superfamily and is expressed on the surface of osteoblasts and binds to the receptor activator of NFκB (RANK) on osteoclast precursor cells. The binding of RANK to RANKL activates a transcription factor downstream of TNF receptor associated factor 6 (TRAF6) which is adapter protein of RANK, and osteoclast differentiation proceeds (26). Osteoprotegerin (OPG) acts as a decoy receptor for RANKL and suppresses osteoclast formation (27). In our experiment, oral infection of mice with *F. nucleatum* increased the ratio of RANKL / OPG genes in the gingiva. In fact, *F. nucleatum* infection increased RANKL gene expression and decreased OPG gene expression (data not shown). This suggests that the increase in RANKL is deeply involved in osteoclast formation while OPG acting to suppress osteoclast formation is decreased.

RANKL has also been confirmed to be produced not only by mesenchymal cells such as osteoblasts and fibroblasts, but also by immune cells such as T cells and B cells by inflammatory reactions (4, 28). Thus, it has become clear that RANKL plays an important role in inflammatory bone resorption. In our results, oral infection of mice with *F. nucleatum* induced an increase in $CD4^+$ $RANKL^+$ T cells in GMC. In periodontal tissues that induced LPS-induced bone resorption, infiltration of T cells and RANKL positive cells were confirmed, and these increases were observed as bone resorption increased (29). It has also been confirmed that T cells infiltrating human periodontal tissues expressed RANKL (30). These results suggest that in periodontal tissues, T cells may be involved in promoting bone resorption at the lesion site.

In the second experiment, we examined the effect of oral administration of *F. nucleatum* on the intestinal tract. The human body is inhabited by hundreds of fungal species and more than 100 trillion intestinal bacteria, which plays an important role in maintaining the homeostasis of the body. Recently, the intestinal flora has been attracting attention because it has been reported to be associated with various diseases such as cancer (31), obesity (32), and inflammatory bowel disease (33). *Fusobacterium* is a bacterium that mainly lives in the upper digestive tract, especially in the oral cavity (34). However, in recent years, it has been reported that *F. nucleatum* is frequently detected in colon cancer tissues and may affect the progression of colorectal cancer (35). More surprisingly, it's been found that when *F. nucleatum* was isolated and analyzed from the affected tissue and saliva of colorectal cancer patients, more than 40 % of the patients had common strains in cancer tissue and saliva (36). This result strongly suggests that this enteric bacterium, which has been reported to be involved in the carcinogenic process of colon cancer, is derived from the oral cavity, that is, *F. nucleatum* in the oral cavity is involved in colon cancer.

In the next study, we investigated the effects of oral challenge of *F. nucleatum* on the intestinal immune system. Oral administration of *F. nucleatum* to mice resulted in infiltration and activation of effector cells in the lower gastrointestinal tract. These phenomena were activated more rapidly and more markedly in the large intestine than in the small intestine. These findings suggest that oral *F. nucleatum* may affect inflammatory

lymphocytes of large intestine and may eventually spread to inflammatory lymphocytes of small intestine.

Recently, *F. nucleatum* has also attracted attention as a causative agent of Crohn's disease (CD) (37). Inflammatory bowel diseases (IBD) such as ulcerative colitis and CD have been considered autoimmune diseases. However, it is clear that spontaneous enterocolitis model mice such as IL-10 KO mice do not develop enteritis under aseptic conditions (38), and that dysfunction of NOD2, an intracellular receptor for bacterial components, is involved in CD development (39). As a result, the role of enteric bacteria has been highlighted. The onset of IBD involves the Th reaction in the intestine (40). The Th reaction consists of Th1, Th2, and Th17, and in the normal intestine, the balance is maintained with Treg cells that control the immune response, but their regulatory function has broken in the IBD intestine, and Th1 and Th17 reaction in CD has enhanced (41).

In our results, the increase of Th1 and Th17 cells in LiLP was significantly observed on day 1 after oral administration of *F. nucleatum* to mice, and it tended to persist even on day 30. Furthermore, although their degree in SiLP was lower than that in LiLP, a similar tendency was observed. On day 30, an increase in IL-10$^+$ T cells was observed similar to that in Th1 cells. On the other hand, transcription factor Foxp3$^+$ T cells were significantly increased in LiLP on day 1 and 30, and in SiLP on day 30 after oral administration of *F. nucleatum*. Recently, the conversion of Treg into Th17 cells has been reported, and differentiation from regulatory T cells to Th17 cells has also been reported in human peripheral blood (42). Therefore, the increase of Foxp3$^+$ T

cells by oral infection with *F. nucleatum* may be a pre-stage of Th17 differentiation. Further study is necessary in the future.

In the histology of LiLP, the expansion of lymphoid follicles was remarkably observed in the *F. nucleatum* challenged group as compared with the control group, and this expamsion continued even on day 30. Furthermore, immunostaining at the same site revealed infiltration of $CD4^+$ T cells (data not shown). On the other hand, in SiLP, the expansion of lymphoid follicle was observed on day 1 and there was a tendency to shrink on day 30.

7. Conclusion

In conclusion, our results have shown that oral administration of *F. nucleatum* induces gingival inflammation and alveolar bone loss. These results suggest that *F. nucleatum* causes gingival inflammation through activation of inflammasome, activates osteoclasts through the increase of $CD4^+$ $RANKL^+$ T cells, and leads to alveolar bone resorption. In the second experiment, our results indicate that oral challenge of *F. nucleatum* promotes the increase of inflammatory cells in the lower gastrointestinal tract, particularly the large intestine, and expands inflammation. These results suggests that the persistence and spread of such inflammation by *F. nucleatum* may contribute to IBD and colon cancer.

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Figure Legend

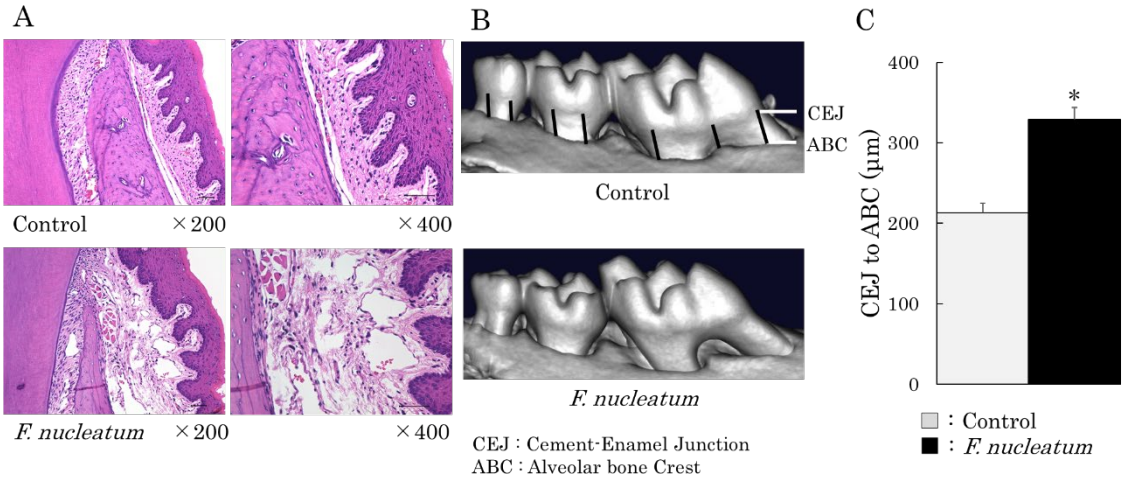

Fig. 1 *F. nucleatum* -induced gingival inflammation and alveolar bone loss. (A) Histochemical analysis of gingival tissue. Thirty days after the last infection with *F. nucleatum*, mouse lower jaws with gingival tissue were stained with hematoxylin and eosin. (B) Thirty days after the last infection, the distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) at 14 predetermined sites in the defleshed maxilla were measured and totaled for each mouse. (C) Bone measurements were performed a total of three times by two evaluators using a random and blinded protocol. All values are presented as the means ± SEM of six mice per group: *$p<0.05$.

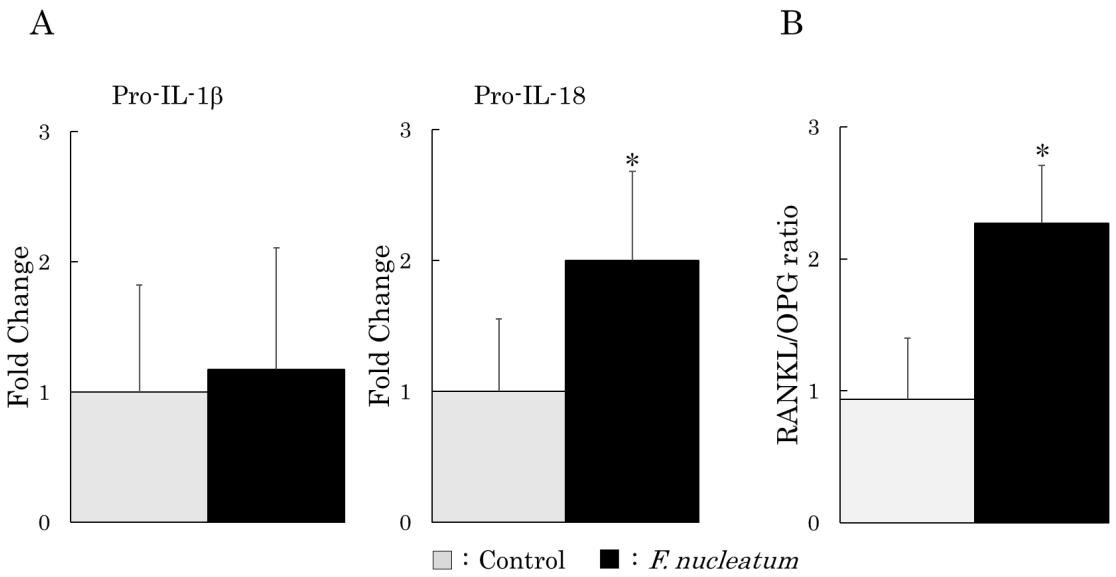

Fig. 2 Gene expressions of pro-inflammatory-(A) and bone resorption-related (B) molecules in mouse gingival tissue following *F. nucleatum* challenge. Relative mRNA levels normalized to GADPH were determined with real-time RT-PCR. Data are expressed as fold-change in mRNA levels compared to controls. All values are presented as the means ± SEM of six mice per group: *$p<0.05$.

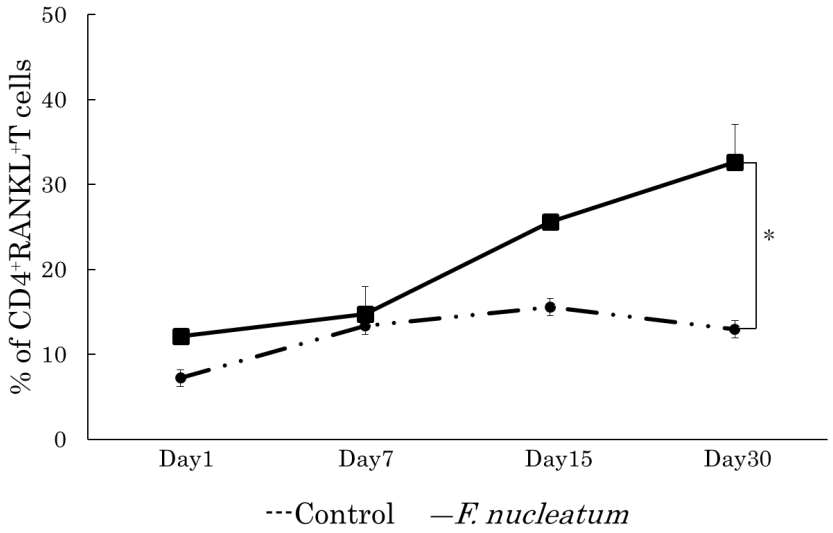

Fig. 3 RANKL expression by $CD4^+$ T cells in mice orally infected by *F. nucleatum*. Thirty days after the last infection with *F. nucleatum*, the frequency of $RANKL^+$ $CD4^+$ T cells was examined by FACS Calibur™. All values are presented as the means ± SEM of six mice per group: *$p<0.05$.

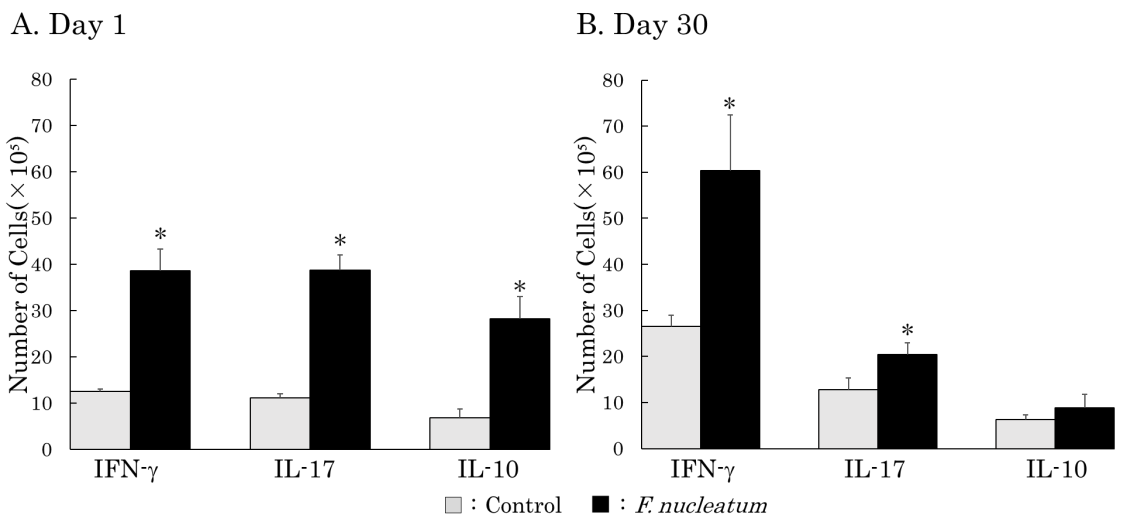

Fig. 4 Comparison of effector T cells in LiLP after *F. nucleatum* challenge. Day 1 (A) and 30 (B) after the final bacterial infection, mice were sacrificed and their large intestines were removed. Mononuclear cells from the lamina propria (LP) of the large intestine (LiLP) were isolated and stained with allophycocyanin (APC)- CD4 and the PE- IFN-γ, PE- IL-17 or IL-10 antibodies (Abs) and then detected through flow cytometry. Values shown are the mean ± standard error of the means ± SEM of six mice per group: *$p<0.05$.

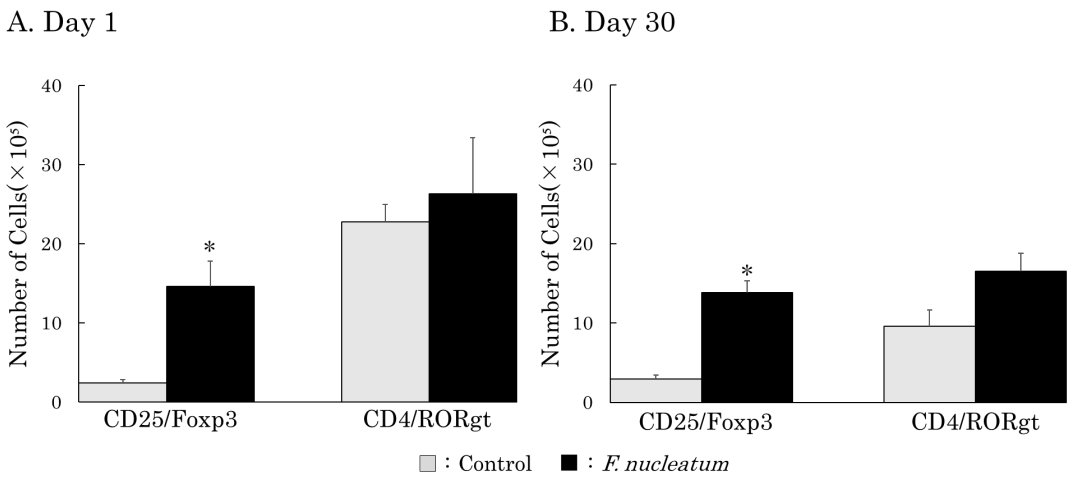

Fig. 5 Comparison of Treg / Th17 cells in LiLP after *F. nucleatum* challenge. Day 1 (A) and 30 (B) after the final bacterial infection, mice were sacrificed and their large intestines were removed. Mononuclear cells from the lamina propria (LP) of the large intestine (LiLP) were isolated and stained with allophycocyanin (APC)- CD4 or APC-CD25 and the PE-Foxp3 or PE-RoRγt antibodies (Abs) and then detected through flow cytometry. Values shown are the mean ± standard error of the means ± SEM of six mice per group: *$p<0.05$.

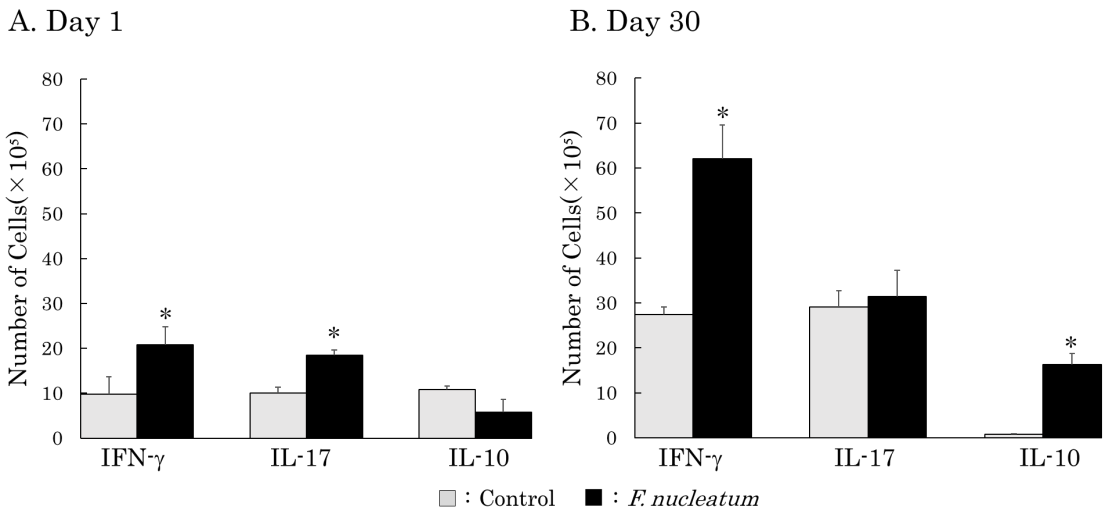

Fig. 6 Comparison of effector T cells in SiLP after *F. nucleatum* challenge. Day 1 (A) and 30 (B) after the final bacterial infection, mice were sacrificed and their small intestines were removed. Mononuclear cells from the lamina propria (LP) of the small intestine (SiLP) were isolated and stained with allophycocyanin (APC)- CD4 and the PE- IFN-γ, PE- IL-17 or IL-10 antibodies (Abs) and then detected through flow cytometry. Values shown are the mean ± standard error of the means ± SEM of six mice per group: *$p<0.05$.

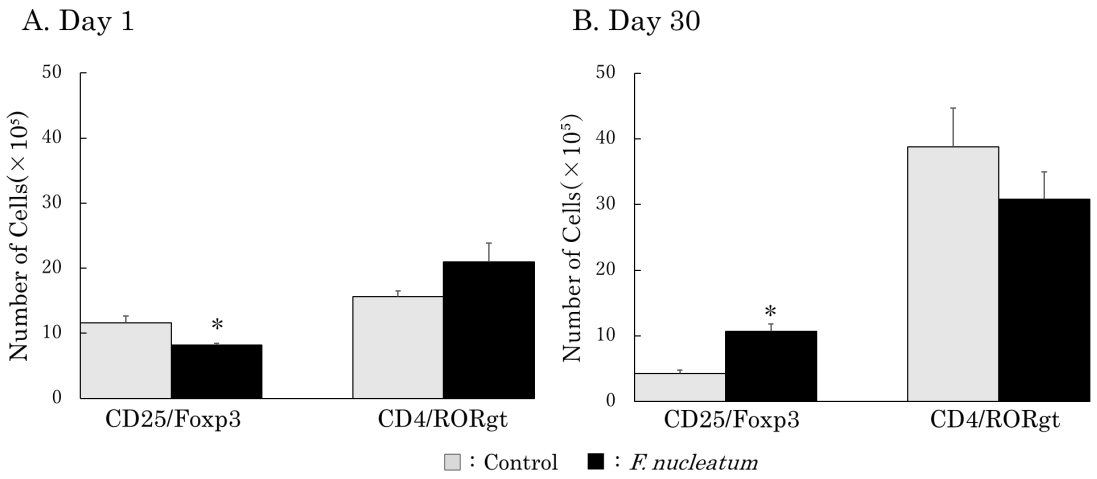

Fig. 7 Comparison of Treg / Th17 cells in SiLP after *F. nucleatum* challenge. Day 1 (A) and 30 (B) after the final bacterial infection, mice were sacrificed and their small intestines were removed. Mononuclear cells from the lamina propria (LP) of the small intestine (SiLP) were isolated and stained with allophycocyanin (APC)- CD4 or APC-CD25 and the PE-Foxp3 or PE-RoRγt antibodies (Abs) and then detected through flow cytometry. Values shown are the mean ± standard error of the means ± SEM of six mice per group: *$p<0.05$.

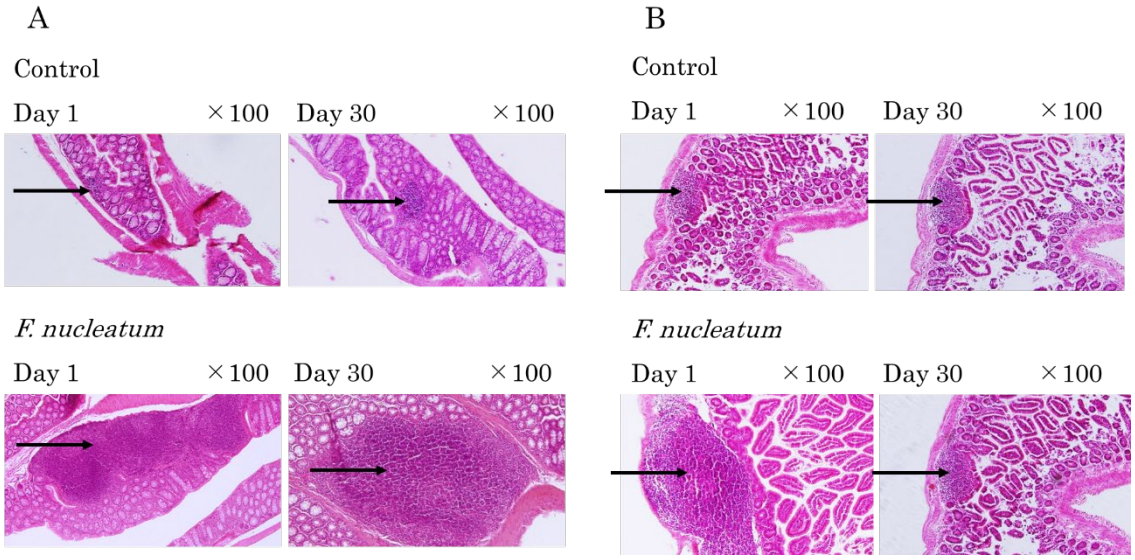

Fig. 8 Histological image of large and small intestine after *F. nucleatum* challenge. Day 1 and 30 after the last infection, mice were sacrificed and the large (A) and small (B) intestines were removed and fixed in 4% paraformaldehyde in PBS for 24 h. Four-micrometer-thick serial sections were then prepared and stained with hematoxylin and eosin.