Effect of Periodontopathic Bacteria *Fusobacterium Nucleatum* on Intestinal Immune Cells

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**Abstract**

This study examined mucosal immune responses in the lower gastrointestinal tract after oral intake of *Fusobacterium 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.

**Keywords**: inflammation, intestinal immunity, oral infection, *Fusobacterium nucleatum*

**Introduction**

The human gastrointestinal tract is estimated to be colonised by over 10¹⁴ bacteria, approximately 10-fold of the total number of cells in the human body (1). Disruptions to the microbiome have been associated with severe pathologies of the host, including metabolic disease, cancer and inflammatory bowel disease (2–4).

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 (5). 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 (6). 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 (5).

*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, respira-
tory tract infections, organ abscesses, rheumatoid arthritis, and Alzheimer's disease (7). It has been demonstrated that *F. nucleatum* biofilm plays an important role in these diseases.

Therefore, in this study, we focused on *F. nucleatum* and 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.

**Materials and Methods**

**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% H2, 80% N2, and 10% CO2 for 3-5 days. Cultures were then inoculated into brain-heart infusion (Difco Laboratories, Detroit, MI, USA) for 2 days until OD660nm=0.8 was reached, corresponding to \(10^{9}\) CFU/ml. The cultured cells were centrifuged at 8000 x g for 20 min at 4°C and resuspended in 5% carboxymethyl cellulose (CMC) for oral infection.

**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).

**Oral infection**

Mice were randomly divided into two groups (n=6 per group). The first group was orally challenged with live *F. nucleatum* \(1 \times 10^9\) 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.

**Isolation of mononuclear cells**

Mononuclear cells from the lamina propria (LP) of small and large intestines were isolated, as described previously (8). In brief, after removing the Peyer's patches, the small and large intestines were cut into pieces, which were incubated in 0.3 mg/mL collagenase type IV (Sigma, St. Louis, MO, USA) in RPMI 1640 medium (Wako Pure Chemical Industries Ltd., Osaka, Japan) at 37°C for 20 min with stirring. Mononuclear cells were enriched using discontinuous Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) and re-suspended in RPMI 1640 medium supplemented with HEPES buffer (15 mM), L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 mg/mL), and 10% fetal calf serum (FCS), referred to as complete medium.

**Flow cytometry analysis**

For intracellular staining, cells \(1.0 \times 10^6\) cells) were labeled with allophycocyanin (APC)-conjugated anti-mouse CD4 monoclonal antibody (mAb) (eBioscience, San Diego, CA, USA) at 4°C 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 GACS 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.

**Histological analysis of small and large intestines**

Day 1 and 30 after the last infection, mice were sacrificed and the small and large intestine was removed and fixed in 4% paraformaldehyde in PBS for 24 h. The samples were then embedded in paraffin and 4 µm-thick serial sections were then prepared and stained with hematoxylin and eosin.

**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.
Results

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. 1A 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 day 1 except for IFN-γ producing cells, but the number of cells in the infected group tended to be higher than in the control group.

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. 2A and B). On the other hand, in CD4⁺ RoRγt⁺ Th17 cells, there was no significant difference between F. nucleatum-challenged mice and control mice.

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. 3A 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.

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. 4A and B). On the other hand, CD4⁺ RoRγt⁺ 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.

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

The lymphoid follicle in LiLP was expanded on day 1 after F. nucleatum challenge compared to control group, and this trend continued on day 30 after administration (Fig. 5A). 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 administra-
tion, but decreased on day 30, which was almost the same as the control group (Fig. 5B).

**Discussion**

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 (9), obesity (10), and inflammatory bowel disease (11). *Fusobacterium* is a bacterium that mainly lives in the upper digestive tract, especially in the oral cavity (12). 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 (13). 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 (14). This result strongly suggests that this enteric bacterium, which has been reported to be involved in the carcino- 
genic process of colon cancer, is derived from the oral cavity, that is, F. nucleatum in the oral cavity is involved 
in colon cancer.

In this 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) (15). 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 (16), and that dysfunction of NOD2, an intracellular receptor for bacterial components, is involved in CD development (17). As a result, the role of enteric bacteria has been highlighted. The onset of IBD involves the Th reaction in the intestine (18). 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 (19).

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 (20). 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 expansion 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. In conclusion, 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 suggest that the persistence and spread of such inflammation by *F. nucleatum* may contribute to IBD and colon cancer.

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