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Xylitol, an Anticaries Agent, Exhibits Potent Inhibition of Inflammatory Responses in Human THP-1-Derived Macrophages Infected With *Porphyromonas gingivalis*

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Abstract

Background—Xylitol is a well-known anticaries agent and has been used for the prevention and treatment of dental caries. In this study, the anti-inflammatory effects of xylitol are evaluated for possible use in the prevention and treatment of periodontal infections.

Methods—Cytokine expression was stimulated in THP-1 (human monocyte cell line)-derived macrophages by live *Porphyromonas gingivalis*, and enzyme-linked immunosorbent assay and a commercial multiplex assay kit were used to determine the effects of xylitol on live *P. gingivalis*-induced production of cytokine. The effects of xylitol on phagocytosis and the production of nitric oxide were determined using phagocytosis assay, viable cell count, and Griess reagent. The effects of xylitol on *P. gingivalis* adhesion were determined by immunostaining, and costimulatory molecule expression was examined by flow cytometry.

Results—Live *P. gingivalis* infection increased the production of representative proinflammatory cytokines, such as tumor necrosis factor- α and interleukin (IL)-1 β , in a multiplicity of infection- and time-dependent manner. Live *P. gingivalis* also enhanced the release of cytokines and chemokines, such as IL-12 p40, eotaxin, interferon γ -induced protein 10, monocyte chemoattractant protein-1, and macrophage inflammatory protein-1. The pretreatment of xylitol significantly inhibited the *P. gingivalis*-induced cytokines production and nitric oxide production. In addition, xylitol inhibited the attachment of live *P. gingivalis* on THP-1-derived macrophages. Furthermore, xylitol exerted anti-phagocytic activity against both *Escherichia coli* and *P. gingivalis*.

Conclusion—These findings suggest that xylitol acts as an antiinflammatory agent in THP-1-derived macrophages infected with live *P. gingivalis*, which supports its use in periodontitis.

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Keywords

Cytokines; inflammation; periodontitis; *Porphyromonas gingivalis*; xylitol

Inflammation is essential for the host defense against invading pathogens and for tissue repair.¹ Periodontitis is a chronic inflammatory disease induced by infection of major periodontopathogens, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*.^{2,3} Chronic inflammation affects the periodontal tissues, resulting in a loss of alveolar bone and teeth.⁴ Periodontopathogens such as *P. gingivalis* have been detected frequently in atherosclerotic plaques, indicating a potential causal relationship between oral inflammatory diseases and various vascular diseases.^{5–8} *P. gingivalis*, an oral black-pigmented anaerobic Gramnegative bacterium, can penetrate the gingival tissue and move into the blood circulation, leading to systemic inflammatory responses, such as atherosclerosis. As a response to infection, inflammatory immune cells, such as monocytes and macrophages, are recruited to the periodontitis tissues and produce cytokines and chemokines, which in turn activate an adaptive immune response.⁹ Therefore, the host innate immune system plays a critical role in the rapid recognition and elimination of invading pathogens through phagocytosis and the induction of inflammation.

Phagocytosis is a key mechanism of innate immunity that is mediated by professional phagocytes, such as peripheral blood mononuclear and macrophage cells.¹⁰ Phagocytes play important roles in the host innate immune defense against infection of pathogens and in inducing adaptive immune responses. The response of a phagocyte to a pathogen involves several steps, including the detection of pathogens through surface receptors and engulfing of pathogens.

Cytokines, small soluble signaling factors, are secreted by immune cells and play essential roles in immune modulation and activation of various immune cells. Although these secreted cytokines play protective roles in the elimination of infected bacteria, overproduction of proinflammatory cytokines might be directly relevant to periodontal breakdown, such as periodontal attachment loss (AL), breakdown of collagen, and alveolar bone resorption.^{11,12} Therefore, understanding these cytokine abnormalities may be beneficial in identifying the pathogenesis of periodontitis and developing an effective therapeutic approach.

Xylitol is a naturally occurring, low-calorie sugar substitute that is considered to be non-fermentable by oral bacteria. It inhibits bacterial growth and metabolism, as well as the polysaccharide production of mutans streptococci, exhibiting both anticariogenic and cariostatic properties.^{13–15} Previously, it was reported that xylitol has an anti-inflammatory effect by inhibiting cytokine production induced by *P. gingivalis* lipopolysaccharide (LPS) in Raw 264.7 cells.¹⁶ However, the anti-inflammatory effects of xylitol on live *P. gingivalis* remains to be elucidated. The immune responses to live bacteria might not be initiated simply by the bacteria cell surface virulence factors. The profiling of cytokines produced by human macrophages secreted by LPS or FimA (Type-1 fimbrial protein, A chain) differed substantially from what was observed when the cells were infected with live *P. gingivalis*.¹⁷ The host defense system senses live bacteria differently from individual bacterial components, and thus it stimulates a different set of immune responses. Thus, the prevention

of periodontitis would be improved if the anti-inflammatory mechanism of macrophages infected with live *P. gingivalis* could be identified.

In the present study, the *P. gingivalis*-induced inflammatory response and the effect of xylitol on the production of inflammatory cytokines and nitric oxide (NO), as well as phagocytosis elicited by live *P. gingivalis* in THP-1-derived macrophages is investigated. The purpose of the present study is to elucidate immunomodulatory effects of xylitol, aiming for the future application of xylitol in chronic periodontitis (CP).

MATERIALS AND METHODS

Bacterial Culture

P. gingivalis (strain 381) were grown in gifu anaerobic medium (GAM)[§] broth, which contained 5 mg/mL hemin^{||} and 0.5 mg/mL 3-phytyl-menadione[¶] (vitamin K) at 37°C in an anaerobic chamber in an atmosphere containing 90% N₂, 5% H₂, and 5% CO₂. An optical density (OD) of 1.0 (650 nm) was determined to correlate to 10⁹ colony forming units/mL. To prepare the bacteria for infection, an overnight culture was diluted to an OD 650 nm of 1.0 in GAM broth. The bacteria were washed once, resuspended in Roswell Park Memorial Institute (RPMI)[#] media, and used to infect the macrophages at a set macrophage/*P. gingivalis* ratio of 1:10, 1:20, 1:50, or 1:100.

Cell Culture

The human monocyte cell line THP-1 was obtained from a commercial source,^{**} and the authors were not able to ascertain the identity of the donors. It was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum,^{††} 100 U/mL penicillin, and 100 µg/mL streptomycin and was incubated at 37°C in a humidified atmosphere of 5% CO₂. THP-1 cells were differentiated into macrophage-like cells with 50 nM phorbol 12-myristate 13-acetate^{‡‡} (PMA). The differentiated cells were pretreated with 3% xylitol for 30 minutes before treatment with live *P. gingivalis*. After incubation of *P. gingivalis* for 90 minutes, cells were washed with phosphate-buffered saline (PBS) and incubated further with 3% xylitol^{§§} for 6 or 24 hours.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

MTT experiments were performed to determine the cell growth rates in the culture media after xylitol treatment. Briefly, the THP-1 cells were plated in a 96-well plate. After 6 or 24 hours of xylitol treatment, cells were incubated with 100 µL of an MTT solution^{||||} of 1 µg/µL at 37°C for 4 hours. The purple formazan crystal was dissolved in 100 µL

[§]Nissui, Tokyo, Japan.

^{||}Sigma-Aldrich, St. Louis, MO.

[¶]Sigma-Aldrich.

[#]Gibco, Thermo Fisher Scientific, Waltham, MA.

^{**}Korean Cell Line Bank, Seoul, Korea.

^{††}Gibco, Thermo Fisher Scientific.

^{‡‡}Sigma-Aldrich.

^{§§}Danisco Sweeteners, Kotka, Finland

^{||||}Sigma-Aldrich.

dimethylsulfoxide and added to the cells. The absorbance was measured using a spectrophotometer at 570 nm.

Measurement of Tumor Necrosis Factor (TNF)- α and Interleukin (IL)-1 β

To determine the amount of TNF- α and IL-1 β released into the culture media after *P. gingivalis* stimulation, the amount was analyzed in accordance with the instructions of the manufacturer using enzyme-linked immunosorbent assay (ELISA) kits.^{¶¶}

Measurement of Cytokines and Chemokines

To determine the quantity of cytokines and chemokines released into the culture media after *P. gingivalis* stimulation, the amount was analyzed according to the instructions of the manufacturer using a multiplex assay kit.^{###} Briefly, standard or sample solution and mixed beads were added to an ELISA well plate. After overnight incubation at 4°C with shaking, detection antibodies were added to the solution and incubated for 1 hour at room temperature. Streptavidin–phycoerythrin was added and allowed to react for 30 minutes. After incubation, the levels of cytokine and chemokine expression were assessed using software.^{***}

NO Measurement

The cell supernatants after *P. gingivalis* stimulation were used to measure the NO concentration using a Griess reagent system.^{†††}

Viable Cell Count

P. gingivalis were harvested, washed, and re-suspended in RPMI medium without antibiotics. The THP-1-derived macrophages were pretreated with 3% xylitol for 30 minutes and then exposed to *P. gingivalis* (multiplicity of infection [MOI] of 50) for 90 minutes at 37°C in a humidified atmosphere of 5% CO₂. To enumerate *P. gingivalis* associated with macrophages, cells were washed three times with chilled PBS to remove the non-adherent bacteria and lysed in sterile distilled water for 20 minutes. The cell lysates were diluted with GAM broth and plated on blood agar supplemented with hemin and vitamin K. The viable cells were counted after incubation in anaerobic conditions for 7 days at 37°C. To count the internalized bacteria, the macrophages were infected with *P. gingivalis* as above and cultured for an additional 1 hour in the presence of 200 µg/mL metronidazole and gentamicin. The cells were then washed and lysed in sterile distilled water, and the bacterial number was counted. *P. gingivalis* colonies were identified by their characteristic black pigment and then enumerated.

Phagocytosis Assay

To measure the effects of xylitol on phagocytic function, the net phagocytosis and response to xylitol were calculated according to the instructions of the manufacturer using a

^{¶¶} eBioscience, San Diego, CA.

^{###} MILLIPLEX MAP Kit, Millipore, Billerica, MA.

^{***} MAGPIX with xPONENT software, Millipore.

^{†††} Promega, Madison, WI.

phagocytosis assay kit.^{†††} Briefly, the THP-1-derived macrophages were treated with 3% xylitol for 30 minutes or 1 hour. After xylitol incubation time, the media were removed, and fluorescein-labeled *Escherichia coli* K-12 bioparticles were added and incubated for an additional 2 hours. After quenching with trypan blue solution, the microplates were read in the fluorescence plate reader using 480 nm and 520 nm.

To determine the effects of xylitol on *P. gingivalis* phagocytic function, *P. gingivalis* were labeled with 10 μ M carboxyfluorescein diacetate succinimidyl ester^{§§§} in PBS containing 0.1% bovine serum albumin (BSA) for 10 minutes at 37°C. THP-1-derived macrophages were treated with 3% xylitol for 30 minutes. After xylitol treatment, the media were removed, and fluorescein-labeled *P. gingivalis* were challenged for 2 hours. After quenching with trypan blue solution, the microplates were read in the fluorescence plate reader^{|||||} using 480 nm and 520 nm.

Immunostaining

THP-1 cells were differentiated into macrophages with 50 nM PMA placed on eight-well chamber slides.^{|||||} THP-1-derived macrophages were pre-treated with xylitol for 30 minutes before stimulation with live *P. gingivalis*, which were labeled with 10 μ M CFSE in PBS containing 0.1% BSA for 10 minutes at 37°C. After 90 minutes of incubation, the cells were washed three times in PBS and fixed with 4% para-formaldehyde^{###} for 10 minutes at room temperature and permeabilized for 5 minutes with PBS containing 0.1% non-ionic surfactant.^{****} The cells were blocked with signal enhancer^{††††} for 30 minutes and then stained with red fluorescent dye^{††††} (1:200 in blocking buffer) for 20 minutes at room temperature. After washing with PBS, the chamber slides were mounted with antifade reagent^{§§§§} with 4',6-diamidino-2-phenylindole (DAPI) and monitored using confocal laser-scanning microscopy.^{|||||||}

Fluorescent Staining and Flow Cytometry

To detect the expression of costimulatory molecules on human monocytes, THP-1 cells were harvested and stained with fluorescent-labeled antibodies against anti-human cluster of differentiation 80 (CD80; B7-1) fluorescein isothiocyanate and antihuman CD86 (B7-2) phycoerythrin^{|||||} as recommended by the manufacturer. Briefly, THP-1 cells were collected with cold PBS, washed in staining buffer (PBS, 2% BSA), and then stained in PBS supplemented with 2% BSA for 1 hour on ice with CD80 and CD86 antibodies. Cells were washed twice with buffer. Samples were acquired using a flow cytometer^{#####} and analyzed using data analysis software.^{*****}

^{†††} Vybrant Phagocytosis Assay Kit, Invitrogen.

^{§§§} CellTrace CFSE, Invitrogen, Thermo Fisher Scientific.

^{|||||} GLOMAX multi-detection system, Promega.

^{|||||} LabTec, Nunc, Thermo Fisher Scientific.

^{###} Electron Microscopy Sciences, Hatfield, PA.

^{****} 0.1% Triton X-100, Amersham, GE Health Care, Little Chalfont, U.K.

^{††††} Image-iT FX signal enhancer, Invitrogen, Thermo Fisher Scientific.

^{††††} Alexa Fluor 633 phalloidin, Gibco, Thermo Fisher Scientific.

^{§§§§} Prolong Gold antifade reagent, Gibco, Thermo Fisher Scientific.

^{|||||||} LSM 700, Carl Zeiss, Jena, Germany.

^{|||||} eBioscience.

^{#####} FACSCalibur, BD Biosciences, San Jose, CA.

Statistical Analyses

Statistically significant differences among samples were analyzed with an unpaired, one-tailed Student *t* test. The data are shown as the mean $\pm$ SD. A *P* value <0.05 was considered statistically significant.

RESULTS

Xylitol Inhibited the Release of TNF- α and IL-1 β Induced by Live *P. gingivalis* Infection

Before performing the experiments to examine the effect of xylitol on *P. gingivalis*-stimulated inflammatory response in THP-1-derived macrophages, an optimal concentration of xylitol was determined. THP-1 cells were treated with different concentrations of xylitol ranging from 0.125% to 24% for 6 or 24 hours, to examine the effects of xylitol on the viability of THP-1-derived macrophage cells. After incubation, the viability of cells was measured by an MTT assay. Compared to the untreated control cells, the viability of THP-1-derived macrophages was reduced by higher concentrations of xylitol (Figs. 1A and 1B). Next, the effects of xylitol concentration on proinflammatory cytokine production induced by live *P. gingivalis* infection were examined. THP-1-derived macrophages were stimulated with live *P. gingivalis* at an MOI of 50. Apparent active release of TNF- α and IL-1 β by live *P. gingivalis* challenge was detected by ELISA in culture supernatants for 6 and 24 hours (Figs. 2A through 2D). The production of these cytokines was inhibited significantly by treatment with 3% xylitol. From these results, 3% xylitol was selected because it does not cause cytotoxicity or have anti-inflammatory effects on THP-1-derived macrophages. The effects of xylitol on proinflammatory cytokine production induced by live *P. gingivalis* infection in an MOI-dependent manner was further analyzed. THP-1-derived macrophages were stimulated with live *P. gingivalis* at an MOI of 10, 20, 50, or 100. Live *P. gingivalis*-induced TNF- α and IL-1 β production was inhibited significantly by xylitol treatment (Figs. 3A through 3D). The results suggest that xylitol negatively regulates the production of proinflammatory cytokines induced by live *P. gingivalis* infection in THP-1-derived macrophages.

Xylitol Inhibited the Production of Cytokines, Chemokines, and NO Induced by Live *P. gingivalis* Infection

To investigate the effects of live *P. gingivalis* infection on the production of various cytokines and chemokines, a multiplex assay was performed in THP-1-derived macrophages. Live *P. gingivalis* infection (MOI of 50 or 100) increased the production of IL-12 p40, eotaxin, interferon γ -induced protein 10 (IP-10; chemokine [C-X-C motif] ligand [CXCL10]), monocyte chemotactic protein-1 (MCP-1; CC chemokine ligand 2 [CCL2]), and macrophage inflammatory protein-1 (MIP-1 α ; CCL3), as well as TNF- α and IL-1 β , for 6 or 24 hours. The production of cytokines and chemokines was inhibited by xylitol pretreatment (Figs. 4A through 4G and 5A through 5G). NO is an important cellular signaling molecule that is generated by macrophages. Next, to further explore the effect of xylitol on NO production, whether live *P. gingivalis* infection increases NO production in

*****FlowJo software, TreeStar, San Carlos, CA.

THP-1-derived macrophages was examined. As shown in Figures 4H and 5H, NO was produced in response to *P. gingivalis* infection (MOI of 50 or 100) for 6 or 24 hours. In addition, NO production stimulated by live *P. gingivalis* infection was reduced by xylitol pretreatment (Figs. 4H and 5H). Overall, these results suggest that xylitol inhibits the production of inflammatory cytokines, chemokines, and NO induced by live *P. gingivalis* infection.

Xylitol Inhibited Adhesion, Internalization, and Phagocytosis of Live *P. gingivalis*

Live *P. gingivalis* was first examined to determine whether it could attach to THP-1-derived macrophages. As shown in Figure 6A, live *P. gingivalis* attached and internalized to THP-1-derived macrophages according to the viable cell count, as described in Materials and Methods. Pretreatment of xylitol inhibited the adhesion and internalization of *P. gingivalis* to THP-1-derived macrophages, although it was statistically insignificant. To visualize the attachment of live *P. gingivalis* on THP-1-derived macrophages, immunofluorescence analysis was next performed with CFSE-stained live *P. gingivalis*. Attachment of live *P. gingivalis* was detected on THP-1-derived macrophages in culture, whereas adhesion was inhibited significantly by xylitol pretreatment (Fig. 6B). Additional experiments were performed to determine whether xylitol regulates the phagocytic activity of THP-1-derived macrophages. Before the examination, the effects of xylitol on phagocytic efficacy against *E. coli* particles were investigated to determine how xylitol acts on the phagocytosis of live *P. gingivalis*. As shown in Figure 6C, xylitol negatively regulates phagocytosis of *E. coli* particles. Next, the effects of xylitol on the phagocytic function of THP-1-derived macrophages against live *P. gingivalis* were observed using CFSE-stained live *P. gingivalis*. The phagocytic function against live *P. gingivalis* at MOIs of 50 and 100 was inhibited by xylitol pretreatment (Fig. 6D). These results suggest that xylitol inhibits *P. gingivalis* attachment and phagocytic function of THP-1-derived macrophages against live *P. gingivalis*. In addition, live *P. gingivalis* stimulated the expression of costimulatory molecules, CD80 and CD86, in THP-1-derived macrophages, as shown in Figures 6E and 6F. Conversely, upregulated CD80 and CD86 expression was not altered by xylitol pretreatment (Figs. 6E and 6F). These results suggest that xylitol does not play a role in the regulation of CD80 and CD86 expression induced by live *P. gingivalis* infection.

DISCUSSION

In this study, the anti-inflammatory effects of xylitol on live *P. gingivalis* infection in THP-1-derived macrophages are examined. Xylitol efficiently inhibited the production of cytokines and chemokines induced by live *P. gingivalis* infection. NO production stimulated by live *P. gingivalis* was inhibited by xylitol pretreatment. Moreover, xylitol had inhibitory effects on the phagocytic activity against *P. gingivalis* and reduced *P. gingivalis* attachment to THP-1-derived macrophages.

Periodontitis is a major cause of adult tooth loss and is closely related to chronic inflammation caused by *P. gingivalis* infection.^{5,6} The host innate immune system produces inflammatory cytokines and chemokines against bacterial infection.¹⁸ Although cytokines produced by bacterial infection play protective roles in the elimination of bacteria, the

overproduction of proinflammatory cytokines might be related directly to periodontal breakdown, such as periodontal AL, breakdown of collagen, and alveolar bone resorption.^{3,6,18} Therefore, the tight regulation of cytokine secretion is required for the treatment of periodontitis.

To the best of the authors' knowledge, it is shown here for the first time that live *P. gingivalis* infection leads to secretion of various proinflammatory cytokines and chemokines, including IL-12, eotaxin, IP-10, MCP-1, and MIP-1 α , as well as TNF- α and IL-1 β in THP-1-derived macrophages. The major secreted proinflammatory cytokines TNF- α and IL-1 β are the markers of periodontitis progression and severity and principle inducers of effector molecules that cause the breakdown of periodontal tissues.^{12,19,20} IL-12 increases natural killer (NK) cell production and is an important factor in CD4⁺ cell differentiation to T-helper 1 (Th1) or Th2 T cells.²¹ The level of IL-12 p40 is reported to be significantly higher in CP sites than healthy sites and is produced by LPS of periodontopathogens.²² Eotaxin is a member of the CC chemokine family with preferential chemotactic capacity for eosinophil.²³ Eotaxin is also expressed strongly in patients with periodontitis, but no relationship has been established between periodontal disease and eotaxin until now.²⁴ IP-10 (CXCL10), an immunoregulatory cytokine, has been discovered in various infectious diseases and mediates leukocyte trafficking.²⁵ It also activates T lymphocytes, B lymphocytes, NK cells, macrophages, and dendritic cells.²⁶ MCP-1 (CCL2) is a CC chemokine responsible for chemotaxis of monocytes and is increased in the crevicular fluids of adult periodontal patients with severe disease.^{27–30} MIP-1 α (CCL3) induces the synthesis and the release of other proinflammatory cytokines, such as IL-1, IL-6, and TNF- α , from macrophages.^{31,32} MIP-1 α is increased in patients with periodontitis.²⁴ In the present study, during stimulation by live *P. gingivalis* infection, macrophages produced and released the inflammatory cytokines, such as TNF- α , IL-1 β , and IL-12, that modulate most of the macrophage functions and cell surface marker expression and the chemokines, such as eotaxin, IP-10, MCP-1, and MIP-1 that contribute to the recruitment of circulating monocytes within tissues. These strongly expressed cytokines and chemokines play a potential role in the breakdown of periodontal tissue. Cytokines and other bacterial products stimulate the expression of NO and enhance the progression of periodontal disease. NO is a multifunctional signaling molecule that regulates important cellular events in inflammation.³³ Overproduction of NO caused by activated macrophages has been linked to the pathogenesis of a number of diverse effects, including direct cellular cytotoxicity and various inflammatory processes and resulting in periodontal tissue breakdown. NO has been linked to etiopathogenesis of inflammatory periodontal diseases and is expressed strongly in the saliva of patients with periodontitis compared with the controls.^{34,35} In this study, it is shown that infection of live *P. gingivalis* increased NO production in THP-1-derived macrophages.

Previously, xylitol was reported to inhibit bacterial growth and metabolism exhibiting both non-cariogenic and cariostatic properties.^{14,15} In addition, xylitol has anti-inflammatory effects by inhibiting the production of cytokines induced by *P. gingivalis* LPS in Raw 264.7 cells.¹⁶ From these studies, it was hypothesized that xylitol could inhibit the production of proinflammatory cytokines induced by live *P. gingivalis* infection. In line with this, to the

best of the authors' knowledge, this study showed for the first time that xylitol effectively inhibits the production of inflammatory cytokines, such as TNF- α , IL-1 β , IL-12, and eotaxin, and chemokines, such as IP-10, MCP-1, and MIP-1 α , and NO, which were induced by live *P. gingivalis* infection in THP-1-derived macrophages. These results suggest that xylitol has an anti-inflammatory effect against live *P. gingivalis* infection, and in turn, the possible use of xylitol as an anti-inflammatory agent for the control of CP is suggested. Additional studies will be needed to determine whether xylitol exerts the anti-inflammatory effects not only on the stabilized cell line but also on peripheral blood monocytes. Therefore, it is also important to examine anti-inflammatory effects of xylitol in animal models to support the role of xylitol in periodontitis in vivo. These results also showed that live *P. gingivalis* can attach to the surface of THP-1-derived macrophages and was internalized into the cells. In line with this, *P. gingivalis* was also shown to be phagocytized into THP-1-derived macrophages. These phenomena were decreased by xylitol pretreatment. Phagocytes first detect bacteria using cell surface receptors and then engulf and kill the bacteria.¹⁰ In addition, phagocytes produce proinflammatory cytokines and chemokines that regulate local and systemic inflammatory responses and direct the development of adaptive immunity. Phagocytic receptors may trigger internalization of bacteria in general non-specific ways. Macrophages activated with soluble LPS, a ligand for Toll-like receptor 4 (TLR4), were shown to exhibit the increases in membrane ruffling, adhesion, and mortality, all of which enhance the binding and uptake of bacteria.^{36–38} These phenomena were suggested to contribute to the innate immune receptors. Stimulation of TLRs increases phagocytosis of Gram-positive *Streptococcus pneumoniae* by microglia, and TLR agonist increases the phagocytosis of fungi and bacteria significantly, suggesting the role of TLRs as phagocytic receptors.^{39,40} Both TLR2 and TLR4 recognize *P. gingivalis*, and these phagocytic receptors can trigger the responses to be effective against the bacteria.^{41,42} *P. gingivalis* are invasive bacteria and can survive in the macrophages. In addition, this study then examined whether heat-killed (HK) *P. gingivalis* induced cytokine production in THP-1-derived macrophages. Infection of HK *P. gingivalis* could not promote the secretion of TNF- α and IL-1 β compared to a live *P. gingivalis* infection (authors' unpublished data), suggesting that live *P. gingivalis* induces strong cellular immunologic responses. To determine how phagocytosis influences *P. gingivalis* infection, cytokine expression after cytochalasin D, an inhibitor of actin-dependent phagocytosis treatment, was also examined. Live *P. gingivalis*-induced cytokine production was inhibited by treatment with cytochalasin D (the authors' unpublished data), suggesting that phagocytosis is required for the secretion of cytokine. Activated phagocytosis of macrophages enhances the production of several proinflammatory cytokines. Therefore, inhibiting phagocytosis during invasive bacterial infection may prevent inflammatory responses. The underlying mechanism of phagocytosis inhibition could be mediated by several ways, such as by regulating expression of phagocytic receptors of macrophages or by modulating the interaction between the bacteria and host receptors. Additional study will be needed to elucidate the detailed mechanism for the inhibition of phagocytosis by xylitol. Moreover, the elucidation of phagocytic response to in vivo infection with live *P. gingivalis* might provide new insight into the treatment of periodontitis, which requires additional studies using animal models.

CD80 and CD86 molecules can regulate inflammation in innate immunity via macrophage contact and are important in macrophage function as antigen-presenting cells and phagocytes. They provide a major costimulatory signal for the activation of T cells through the T-cell counter receptor CD28 molecule.⁴³ Therefore, whether xylitol plays a modulatory role in the expression of the monocyte surface molecules CD80 and CD86 induced by live *P. gingivalis* infection was subsequently examined. Similar to a previous study showing that large numbers of CD80- and CD86-expressing cells were identified in periodontitis lesions,^{44,45} these results showed that live *P. gingivalis* infection stimulated the expression of costimulatory molecules CD80 and CD86, suggesting their role in mediating immune responses to microbial infection. Conversely, this stimulation of CD80 and CD86 expression was unaffected by xylitol pretreatment, suggesting that xylitol does not regulate the expression of costimulatory molecules.

CONCLUSIONS

Live *P. gingivalis* increased the release of cytokines and chemokines, such as TNF- α , IL-1 β , IL-12 p40, eotaxin, IP-10, MCP-1, and MIP-1 α , in THP-1-derived macrophages. The pretreatment with xylitol significantly decreased the production of cytokines, chemokines, and NO induced by live *P. gingivalis* infection. Xylitol also inhibited the attachment of live *P. gingivalis* to THP-1-derived macrophages. Furthermore, xylitol has effects on the antiphagocytic activity against both *E. coli* and *P. gingivalis* in THP-1-derived macrophages. These findings suggest that xylitol acts as an anti-inflammatory agent against live *P. gingivalis* infection, highlighting its potential clinical use in the prevention or treatment of periodontitis.

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Figure 1.

The cytotoxic effects of xylitol on the THP-1-derived macrophages. THP-1-derived macrophages were treated with indicated concentrations of xylitol for 6 (A) or 24 (B) hours, and the cytotoxicity was measured by MTT assay. * $P < 0.05$ compared to control.

Figure 2.

The production of TNF- α and IL-1 β induced by live *P. gingivalis* (Pg) infection was inhibited by xylitol treatment in a concentration-dependent manner. The THP-1-derived macrophages were pretreated with 0.5%, 1%, 2%, and 3% xylitol for 30 minutes and then infected with live *P. gingivalis* (MOI of 50) for 6 hours (A and C) or 24 hours (B and D). Cell culture supernatant was assayed for human TNF- α (A and B) and IL-1 β (C and D) using ELISA. The data represent mean $\pm$ SD values (n = 3). * P < 0.05 compared to control cells. † P < 0.05 compared to *P. gingivalis*-infected cells.

Figure 3.

Xylitol inhibited the dose- and time-dependent release of TNF- α and IL-1 β induced by live *P. gingivalis* (*Pg*) infection. The THP-1-derived macrophages were pretreated with 3% xylitol for 30 minutes and then infected with live *P. gingivalis* (MOI of 10, 20, 50, and 100) for 6 hours (A and C) or 24 hours (B and D). Cell culture supernatants were assayed for human TNF- α (A and B) and IL-1 β (C and D) using ELISA. The data represent mean $\pm$ SD values (n = 3). * P < 0.05 compared to control cells. † P < 0.05 compared to *P. gingivalis*-infected cells.

Figure 4.

Xylitol inhibited the dose- and time-dependent release of cytokines (A,B, and C), chemokines (D,E,F, and G), and NO (H) induced by live *P. gingivalis* (Pg) infection. The THP-1-derived macrophages were pretreated with 3% xylitol for 30 minutes before infection with live *P. gingivalis* (MOI of 50 or 100) for 6 hours. The concentration of cytokines (TNF- α (A), IL-1 β (B), and IL-12 p40 (C)) and chemokines (eotaxin (D), IP-10 (E), MCP-1 (F), and MIP-1 α (G)) in the culture supernatants was analyzed using a multiplex assay kit. For NO (H) concentration, culture supernatant was assayed using Griess reagent system. * P < 0.05 compared to the control cells. † P < 0.05 compared to the *P. gingivalis*-infected cells.

Figure 5.

Cytokines (A,B, and C), chemokines (D,E,F, and G), and NO (H) induced by live *P. gingivalis* (*Pg*) infection were inhibited by xylitol treatment in a dose-dependent manner. The THP-1-derived macrophages were pretreated with 3% xylitol for 30 minutes before infection with live *P. gingivalis* (MOI of 50 or 100) for 24 hours. The cytokines and chemokines concentrations in the culture supernatants were analyzed using a multiplex assay kit. For NO concentration, culture supernatant was assayed using a Griess reagent system. * $P < 0.05$ compared to the control cells. † $P < 0.05$ compared to the *P. gingivalis*-infected cells.

Figure 6.

A) Xylitol inhibited the adhesion and the internalization of live *P. gingivalis* (*Pg*). For viable cell count assay, the THP-1-derived macrophages were pretreated with 3% xylitol for 30 minutes before infection with live *P. gingivalis* (MOI of 50) for 90 minutes (adhesion assay) or 6 hours (internalization assay). **B)** The adhesion of *P. gingivalis* was inhibited by xylitol treatment. The THP-1-derived macrophages were pretreated with 3% xylitol for 30 minutes before infection with CFSE-stained live *P. gingivalis* for 90 minutes and then immunostained with red fluorescent dye. The nucleus was stained with DAPI and observed by confocal microscopy. **C)** Xylitol negatively regulated phagocytic function of THP-1 cells. The THP-1-derived macrophages were treated with 3% xylitol for 0.5 or 1 hour, and phagocytic efficacy for fluorescein-labeled *E. coli* K-12 bioparticles was calculated. **D)** Phagocytosis against *P. gingivalis* was inhibited by xylitol pretreatment. The THP-1-derived macrophages were pretreated with 3% xylitol for 30 minutes before infection with CFSE-stained live *P. gingivalis* (MOI of 50 or 100) for 2 hours. **E** and **F)** Xylitol did not regulate the expression of CD80 and CD86 molecules induced by live *P. gingivalis* infection. THP-1 cells were pretreated with 3% xylitol for 30 minutes before infection with live *P. gingivalis* (MOI of 50) for 90 minutes. **E)** Flow cytometry dot plots shows the percentages of cells expressing CD80 and CD86 molecules. Results represent one of three

individual experiments. F) The numbers represent the percentage of cells, CD80, CD86, and CD80/86 monocytes. The results are expressed as the mean $\pm$ SD of three independent experiments. * $P < 0.05$ compared to the control cells. † $P < 0.05$ compared to the *P. gingivalis*-infected cells. CFU = colony forming units; ns = not significant; Ct = control (without treatment).