Withaferin A inhibits inflammatory responses induced by *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* in macrophages

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Abstract. Periodontitis is a progressive chronic inflammatory disease and a major cause of tooth loss in humans. As a withanolides, withaferin A (WA) is known to exhibit strong anti-inflammatory activity. The present study examined whether WA inhibited inflammatory responses in macrophages in response to two representative periodontal pathogens, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans*. Murine bone marrow-derived macrophages (BMDMs) were used in this study and cytokine production in culture supernatants was measured by enzyme-linked immunosorbent assays. Western blot analysis was performed to determine the activation of nuclear factor-κB and mitogen-activated protein kinases (MAPKs) and the expression of inducible nitric oxide synthase (iNOS), toll-like receptor (TLR) 2 and TLR4. The production of nitric oxide (NO) was determined by the Griess reaction. WA treatment was shown to decrease interleukin (IL)-6 and tumor necrosis factor (TNF)-α production in BMDMs in response to *F. nucleatum* and *A. actinomycetemcomitans* in a dose-dependent manner. The phosphorylation of IκB-α and MAPKs (p38, extracellular signal-regulated kinases and c-Jun N-terminal kinases) induced by *F. nucleatum* and *A. actinomycetemcomitans* was also inhibited by WA. *F. nucleatum* and *A. actinomycetemcomitans* induced iNOS expression and NO production in BMDMs, which was inhibited by WA in a dose-dependent manner. WA also reduced endogenous and induced expression of TLR2 and TLR4 in these cells. These results suggest that WA may be a potential therapeutic agent or preventive additive for periodontitis control.

Introduction

Diseases of the oral cavity include dental caries, periodontitis, cervical abrasion and halitosis (1). Periodontitis, a bacteria-induced chronic inflammatory disease, is recognized as the most common cause of tooth loss in humans (2,3). Gram-negative bacteria, including *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* are major pathogens involved in periodontitis. At the early stage of infection, these bacteria activate host innate immune responses, which result in the recruitment of neutrophils, macrophages and lymphocytes to the site of infection. The progression to chronic inflammation leads to the formation of a periodontal pocket, ulceration of the gingival surface, destruction of the periodontal ligament and the alveolar bone, and finally tooth loss (4,5). Therefore, it is important to inhibit inflammation in periodontal tissues to maintain tooth health. Recently, there has been an increased interest in the field of herbal medicine as a source of novel therapeutic agents that exhibit anti-inflammatory properties for use in the treatment of periodontal diseases (6).

Withania somnifera is a member of the Solanaceae or nightshade family and its major components are alkaloids and withanolides. The latter consist of a steroid backbone bound to a lactone or one of its derivatives and exert prominent anti-inflammatory activities (7). Withaferin A (WA) is a representative withanolide and has been widely investigated for its anti-inflammatory and anti-cancer effects. A previous study demonstrated that WA reduces lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production by downregulating Akt and nuclear factor (NF)-κB activation in a macrophage cell line (8). In addition, WA inhibits the constitutive or induced

expression of inflammatory mediators, such as cytokines and adhesion molecules (such as intercellular adhesion molecule 1 and vascular cell adhesion protein), in various cells, including epithelial cells (9-12), suggesting that WA has anti-inflammatory effects in a wide range of host cells.

As representative innate immune cells, macrophages are specialized phagocytes and are responsible for the control of the growth of invading bacteria. They can produce inflammatory mediators, such as cytokines, chemokines or NO, via pattern recognition receptor- (PRR-) mediated signaling. Toll like receptors (TLRs) are a representative family of PRRs and are characterized by a cytosolic effector Toll/interleukin (IL)-1R homology (TIR) domain and extracellular leucine-rich repeats (LRRs) that are responsible for the recognition of microbial molecules (13). Several studies have shown that TLR2 and TLR4 are involved in cellular immune responses to periodontal pathogens (14-16). However, thus far the effect of WA on TLR expression in host cells remains unknown. In the present study, the inhibitory effect of WA was examined on the inflammatory responses of macrophages in response to two periodontal pathogens, *F. nucleatum* and *A. actinomycetemcomitans*.

Materials and methods

Animals. Wild-type male C57BL/6 mice (8 weeks old; 21-23 g body weight) were purchased from Koatech (Pyeongtaek, Korea). The animals were housed in an animal room at a constant temperature (22-24˚C) and light-dark cycle with 14 h of light and 10 h dark. Food and water were available *ad libitum*. Mice were acclimatized to the laboratory room for 1-3 weeks prior to the experiment. Mice were sacrificed by cervical dislocation and their femur and tibia were used to prepare macrophages. Animal studies were approved and conducted according to the regulations of the Institutional Animal Care and Use Committee at Konyang University (Daejeon, Korea).

Bacterial culture. *F. nucleatum* (25586; American Type Culture Collection, Mannasas, VA, USA) and *A. actinomycetemcomitans* (43718; American Type Culture Collection) were purchased from the American Type Culture Collection (Manassas, VA, USA). Bacteria were grown on brain heart infusion (BHI) broth containing hemin (5 mg/ml) and vitamin K (10 mg/ml) at 37˚C under anaerobic conditions. Bacteria were allowed to grow to optical density (OD)600 = 0.6, which corresponds to ~10^9 CFU/ml of viable bacteria as determined by serial dilution and plate counts, and frozen aliquots were stored at -80˚C. For bacterial infection, aliquots were thawed and diluted to the desired concentration in phosphate-buffered saline or media.

Preparation and stimulation of murine macrophages. Bone marrow-derived macrophages (BMDMs) were prepared as previously described (17). Bone marrow cells were cultured with Iscove's modified Dulbecco's medium (IMDM; Welgene, Gyeongsan, Korea) containing 30% L929 cell culture supernatant (KCTC, Jeongeup, Korea), 1X minimum essential medium, non-essential amino acids, 1 mM sodium pyruvate, 10% fetal bovine serum and 100 U/ml penicillin/streptomycin (all purchased from Gibco; Thermo Fisher Scientific, Inc.,

Waltham, MA, USA) for 6 days. The cells were seeded in 48-well plates at a concentration of $2x10^5$ cells/well or in 6-well plates at a concentration of $2x10^6$ cells/well, and incubated in a 5% CO_2 incubator at 37˚C. The day after seeding, the cells were infected with *F. nucleatum* and *A. actinomycetemcomitans* at the indicated multiplicity of infection (MOI; presented as macrophage/bacterium ratios) in the absence or presence of WA (50-1,000 nM, Sigma-Aldrich, St. Louis, MO, USA). Culture supernatants were collected 6 h after infection for further analysis.

Measurement of cytokines. The concentrations of IL-6 and tumor necrosis factor (TNF)-α in culture supernatants from *F. nucleatum*- and *A. actinomycetemcomitans*-infected BMDMs were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., Minneapolis, MN, USA).

Measurement of nitric oxide. The NO synthase activity in the culture supernatant of infected cells was determined by measuring the NO accumulation by the Griess reaction as previously described (18).

Immunoblotting. BMDMs were infected with *F. nucleatum* or *A. actinomycetemcomitans* at MOI 10 with or without pretreatment of WA (250 nM) for 2 h, and were lysed at the indicated time points (0, 15, 30 and 60 min). The cells were lysed in a buffer containing 1% Nonidet-P40 supplemented with a complete protease inhibitor cocktail (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) and 2 mM dithiothreitol (Sigma-Aldrich). The extracted protein concentration was measured using a Bio-Rad Protein Assay Dye Reagent Concentrate (cat. no. 500-0006; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Samples of protein (30 μg) were cooled on ice following incubation at 95-100˚C for 10 min. Lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes by electroblotting. The membranes were blocked by incubation with 5% skimmed milk for 1 h at room temperature. The following primary antibodies were incubated with the membrane overnight at 4˚C: Rabbit anti-human polyclonal phosphorylated IκB-α (1:1,000; cat. no. 5209; Cell Signaling Technology, Inc., Danvers, MA, USA); rabbit anti-human polyclonal phosphorylated (p)-c-Jun N-terminal kinase (JNK; 1:1,000; cat. no. 9251; Cell Signaling Technology, Inc.); rabbit anti-human polyclonal p-p38 antibody (1:1,000; cat. no. sc-101759; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); mouse anti-human monoclonal p-ERK antibody (1:1,000; cat. no. sc-7383; Santa Cruz Biotechnology, Inc.); rabbit anti-human polyclonal ERK antibody (1:1,000; cat. no. sc-94; Santa Cruz Biotechnology, Inc.); rabbit anti-human polyclonal anti-β-actin antibody (1:2,000; cat. no. sc-130656; Santa Cruz Biotechnology, Inc.); anti-iNOS (1:1,000; cat. no. ab15323; Abcam, Cambridge, MA, USA); anti-TLR2 (1:1,000; cat. no. IMG-319; Novus Biologicals, LLC., Littleton, CO, USA); and anti-TLR4 (1:1,000; cat. no. ab13556; Abcam). The membrane was then rinsed with Tris-buffered saline with Tween 20 (TBST) three times, each time for 10 min. The membrane was then incubated with secondary horseradish peroxidase-conjugated goat
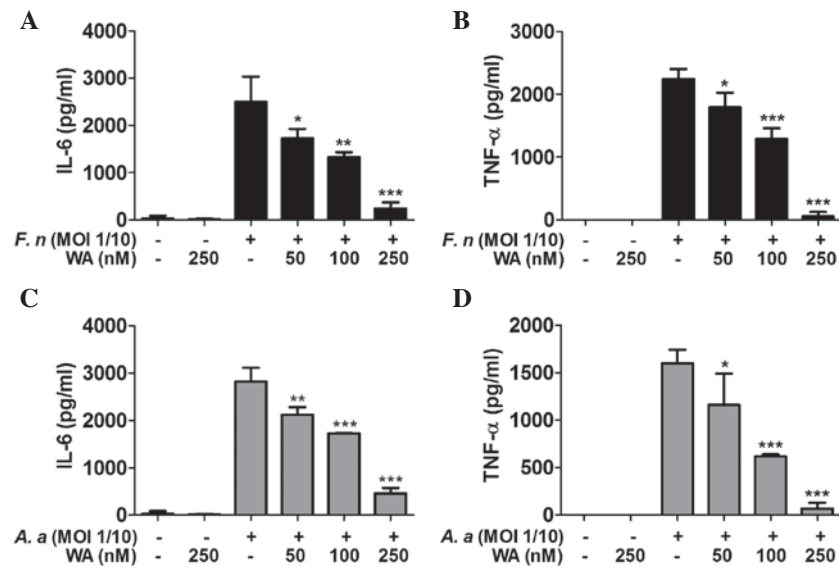
Figure 1. WA reduces the production of IL-6 and TNF-α by BMDMs in response to *F. nucleatum* and *A. actinomycetemcomitans*. BMDMs were infected with *F. nucleatum* (F. n) or *A. actinomycetemcomitans* (A. a) at an MOI of 10 for 6 h with or without pretreatment with WA (50-250 nM) for 2 h. The concentration of (A) IL-6 and (B) TNF-α in culture supernatants infected with *F. nucleatum*, and (C) IL-6 and (D) TNF-α in culture supernatants infected with *A. actinomycetemcomitans* was determined by enzyme-linked immunosorbent assays. Data are presented as the mean ± standard deviation of triplicate samples from one experiment representative of three independent experiments. [*]P<0.05, [**]P<0.01 and [***]P<0.001 vs. *F. n* (+) or *A. a* (+) and WA (-). WA, withaferin A; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; BMDM, bone marrow-derived macrophages; MOI, multiplicity of infection.

anti-rabbit IgG (1:4,000; sc-2301; Santa Cruz Biotechnology, Inc.) or goat anti-mouse IgG (1:2,000; cat. no. 2031; Santa Cruz Biotechnology, Inc.) antibodies for 2 h at room temperature. The membrane was then washed three time with TBST for 10 min. Proteins were detected with SuperSignal West Pico Chemiluminescent Substrate (Gibco; Thermo Fisher Scientific, Inc.). Images of the blots were captured on CP-BU new film (Agfa HealthCare, Mortsel, Belgium) using an Automatic X-ray film processor (JP-33; JPI Healthcare, Seoul, Korea).

Statistical analysis. The differences among the mean values of different groups were assessed. Data are expressed as the mean ± standard deviation. Statistical calculations were performed using one-way analysis of variance followed by the Tukey post test using GraphPad Prism version 5.00 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

WA reduces the production of IL-6 and TNF-α by macrophages in response to F. nucleatum and A. actinomycetemcomitans. To determine the inhibitory effect of WA on cytokine production by macrophages, BMDMs were pretreated with various doses of WA for 2 h and subsequently infected with *F. nucleatum* and *A. actinomycetemcomitans* for 6 h. ELISA results showed that treatment with *F. nucleatum* and *A. actinomycetemcomitans* led to a substantial production of IL-6 and TNF-α by the cells, which was inhibited by WA in a dose-dependent manner (Fig. 1).

NF-κB and MAPK activation is inhibited by WA in macrophages in response to F. nucleatum and A. actinomycetemcomitans. Our previous study demonstrated that NF-κB and MAPKs

(p38, ERK, and JNK) are important for *F. nucleatum*-induced production of IL-6 and TNF-α (15). Induction of cytokine production by *A. actinomycetemcomitans* is also dependent on NF-κB and p38 MAPK signaling (15). Accordingly, it was examined whether WA affects *F. nucleatum*- and *A. actinomycetemcomitans*-mediated activation of NF-κB and MAPKs in BMDMs. *F. nucleatum*-induced phosphorylation of IκB-α and ERK was detected 15, 30, and 60 min after infection, whereas p38 and JNK were phosphorylated at 30 min post-infection (Fig. 2A). This phosphorylation of IκB-α, p38, ERK and JNK was impaired by WA treatment (Fig. 2A). In addition, *A. actinomycetemcomitans* led to activation of NF-κB and MAPKs in macrophages starting 15 min after infection (Fig. 2B). *A. actinomycetemcomitans*-induced ERK phosphorylation was delayed by WA, and was only detected 60 min after infection (Fig. 2B). *A. actinomycetemcomitans*-induced phosphorylation of IκB-α and p38 was markedly inhibited by WA treatment at all time points tested (Fig. 2B). By contrast, WA treatment only led a marginal decrease in JNK phosphorylation in macrophages in response to *A. actinomycetemcomitans* 30 min after infection (Fig. 2B).

WA inhibits iNOS expression and NO production in macrophages in response to F. nucleatum and A. actinomycetemcomitans. NO is a critical factor for the control of bacterial growth and iNOS is a key enzyme catalyzing the NO production from L-arginine. Periodontal pathogens can stimulate macrophages to produce NO (19,20) and a selective iNOS inhibitor, mercaptoethylguanidine, prevents bone destruction in ligature-induced rodent periodontitis (21). Accordingly, it was examined whether WA had an inhibitory effect on iNOS expression and NO production induced by *F. nucleatum* and *A. actinomycetemcomitans* in macrophages. Western blot analysis demonstrated the presence of iNOS
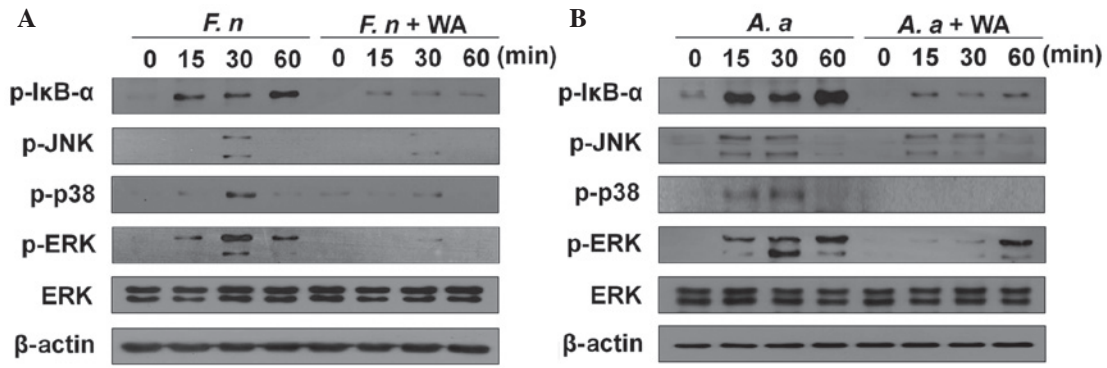
Figure 2. WA inhibits the activation of NF-κB and MAPKs in BMDMs in response to *F. nucleatum* and *A. actinomycetemcomitans*. BMDMs were infected with (A) *F. nucleatum* (F. n) or (B) *A. actinomycetemcomitans* (A. a) at a multiplicity of infection of 10 with or without pretreatment of WA (250 nM) for 2 h and cellular protein was extracted at the indicated time points. Phosphorylation of IκB-α, p38, JNK and ERK was examined by western blotting. Primary antibodies against the regular form of ERK and β-actin were used to confirm the loaded protein amounts. The results shown are from one representative experiment of two independent experiments performed. NF-κB, nuclear factor-κB; MAPKs, mitogen-activated protein kinases; BMDMs, bone marrow-derived macrophages; WA, withaferin A; IκB-α, JNK, c-Jun N-terminal kinases; ERK, extracellular signal-regulated kinases; p-, phosphorylated.
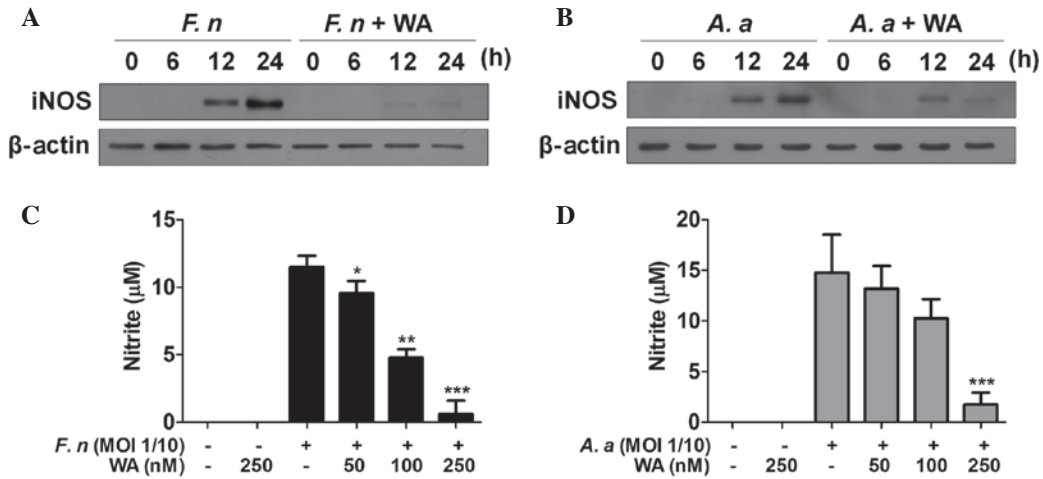

Figure 3. WA inhibits the iNOS expression and NO production induced by *F. nucleatum* and *A. actinomycetemcomitans*. BMDMs were pre-treated with and without WA (250 nM) for 2 h, and then infected with (A) *F. nucleatum* (F. n) or (B) *A. actinomycetemcomitans* (A. a) at an MOI of 10 for the indicated time points. The cellular proteins were harvested at 24 h and the amounts of (A and B) iNOS was determined by western blot analysis. As a separate experiment, the cells were pre-treated with or without WA (50-250 nM) and infected with *F. n* or *A. a* at an MOI of 10 for 24 h. (C and D) NO production was measured by the Griess reaction. The results are from one representative experiment of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. *F. n* (+) or *A. a* (+) and WA (-). iNOS, inducible nitric oxide synthase; WA, withaferin A; BMDMs, bone marrow-derived macrophages; MOI, multiplicity of infection.

protein in *F. nucleatum*-infected macrophages 12 and 24 h after infection, which was mostly impaired by WA treatment (Fig. 3A). *A. actinomycetemcomitans* also induced iNOS expression with similar kinetics to *F. nucleatum* (Fig. 3B). WA inhibited *A. actinomycetemcomitans*-induced expression of iNOS at 24 h, while it had little effect on iNOS expression at 12 h (Fig. 3B). The level of NO in the culture supernatants of BMDMs infected with *F. nucleatum* or *A. actinomycetem-comitans* was detected in the absence or presence of WA. However, NO was undetectable under these conditions regardless of bacterial infection or WA treatment (data not shown), even though *F. nucleatum* and *A. actinomycetemcomitans* could induce expression of iNOS in macrophages. Therefore, the experimental design was altered and cells were also treated with interferon-γ, which is known to enhance NO production in macrophages (22). The results showed that *F. nucleatum* and *A. actinomycetemcomitans* induced NO production in

BMDMs, which was inhibited by WA in a dose-dependent manner (Fig. 3C and D). These findings indicate that WA may effectively inhibit NO production induced by periodontal pathogens in macrophages.

WA reduces the expression of TLR2 and TLR4 in macrophages in response to F. nucleatum and A. actinomycetemcomitans. TLR2 and TLR4 are involved in the production of IL-6 and TNF-α in macrophages in response to *F. nucleatum* and *A. actinomycetemcomitans* (15). Therefore, this study aimed to determine whether WA affects the expression of TLR2 and TLR4 in macrophages. *F. nucleatum* increased the protein expression of TLR2 and TLR4 in macrophages 6 h after infection, and the TLR2 expression level remained increased at 24 h (Fig. 4A). Notably, WA reduced the endogenous and induced expression of TLR2 and TLR4 in *F. nucleatum*-infected macrophages (Fig. 4A). *A. actinomycetemcomitans* also
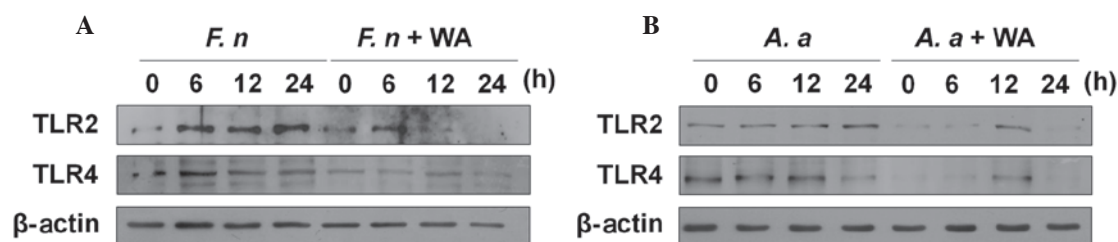
Figure 4. WA reduces the expression of TLR2 and TLR4 in BMDMs in response to *F. nucleatum* and *A. actinomycetemcomitans*. BMDMs were infected with (A) *F. nucleatum* (F. n) or (B) *A. actinomycetemcomitans* (A. a) at a multiplicity of infection of 10 with or without pretreatment of WA (250 nM) for 2 h and cellular protein was extracted at the indicated time points. The expression of TLR2 and TLR4 was examined by western blotting. β-actin served as a loading control. The results are from one representative experiment of two independent experiments performed. TLR, toll-like receptor; BMDMs, bone marrow-derived macrophages; WA, withaferin A.

marginally increased the TLR2 expression in macrophages at 12 and 24 h after infection, whereas the TLR4 expression level was decreased in the cells at 24 h (Fig. 4B). Likewise, WA inhibited both TLR2 and TLR4 expression in *A. actinomycetemcomitans*-infected macrophages (Fig. 4B). These findings suggest that WA may exert its anti-inflammatory effect in macrophages in response to *F. nucleatum* and *A. actinomycetemcomitans* by inhibiting TLR-mediated signaling.

Discussion

Periodontal destruction is the result of the complex interplay between pathogenic bacteria and the host immune responses (23). Bacterial components, such as LPS, and toxic products of periodontal pathogens trigger host immune responses, which results in the destruction of the periodontal tissue. Traditional therapeutics controlling periodontal diseases include dental cleaning, subgingival scaling/root planning and the use of antibiotics. These treatments can reduce the levels of pathogenic bacteria in periodontal pockets (24). In addition to the use of antibiotics, there has been an increased interest in the use of natural products as adjuncts to manage inflammatory disorders, including periodontitis, due to their anti-inflammatory properties (6).

In the present study, the anti-inflammatory effects of WA on *F. nucleatum*- and *A. actinomycetemcomitans*-infected macrophages were investigated. The results showed that WA has inhibitory effects on cytokine production, the activation of NF-κB and MAPKs, and NO production in macrophages in response to the two types of bacteria. This suggests that WA can be used as a natural preventive and therapeutic agent for periodontitis. It is well-known that WA has NF-κB inhibiting activity in a wide range of cell types in response to various stimuli (7). However, the effect of WA on MAPK activation appears to be dependent on the cell type and stimuli. WA alone has been shown to activate p38, ERK and JNK MAPKs in U937 human leukemic cells with different kinetics (25). In addition, WA was shown to induce the phosphorylation of p38 in MCF-7 breast cancer cells within 3 h of treatment (26). In K562 human erythromyeloblastoid leukemia cells, WA induced activation of ERK, although p38 activation was not affected (27). By contrast, WA inhibited TNF-α-induced activation of ERK in human pulmonary epithelial cells, although it did not alter the activation of p38 and JNK (28). In the present study, WA could almost completely inhibit the activation of NF-κB and

MAPKs in macrophages in response to *F. nucleatum* and *A. actinomycetemcomitans*, although its inhibitory effect on *A. actinomycetemcomitans*-induced JNK phosphorylation was not identified to be significant. It remains to be elucidated whether the inhibitory effect of WA on MAPKs is specific to periodontal pathogens and/or macrophages.

TLR2 and TLR4 appears to be critical for periodontal pathogen-induced immune responses although there is controversy regarding the bacterial preparation used and the cell type tested (14-16). In a study using sonicated bacteria, A. actinomycetemcomitans induced IL-8 production in HEK293 cells via TLR2 and TLR4, whereas *F. nucleatum* elicited IL-8 production exclusively via a TLR2-dependent pathway (14). In human periodontal ligament cells, TLR2 and TLR4 are essential for the *F. nucleatum*-induced production of cytokines (16). Likewise, in macrophages, double deficiency of TLR2 and TLR4 leads to decreased production of IL-6 and TNF-α, and delayed IκB-α degradation in response to *F. nucleatum* and *A. actinomycetemcomitans* (15). In the present study, it was demonstrated for the first time that WA inhibits endogenous and induced expression of TLR2 and TLR4 in macrophages, indicating that WA can regulate TLR-mediated signaling. In fact, in the present study, an MOI of 1/10 was used to stimulate macrophages. This dose of *F. nucleatum* and *A. actinomycetemcomitans* could induce the production of substantial levels of TNF-α in TLR2/4 double-deficient macrophages (15) and a high dose of WA (250 nM) completely inhibited TNF-α production in this study. This suggests that WA may inhibit other signaling proteins involved in *F. nucleatum* and *A. actinomycetemcomitans*-induced production of cytokines in addition to TLR2 and TLR4. Previously it was demonstrated that endosomal TLRs are important for cytokine production in TLR2/4 double-deficient macrophages (15), suggesting that WA likely exerts its anti-inflammatory effects by direct inhibition of multiple TLRs. In addition to TLRs, Nod-like receptors (NLRs) participate in the bacteria-induced immune responses in host cells. The first identified NLRs, Nod1 and Nod2, recognize the peptidoglycan motifs meso-diaminophimelic acid and muramyl dipeptide, respectively (29). Periodontal pathogens, including *F. nucleatum* and *A. actinomycetemcomitans*, are known to stimulate Nod1- and Nod2, and their peptidoglycans induce NF-κB activation in Nod1- and Nod2-transfected HEK293 cells (30). A previous study revealed that vimentin, an intermediate filament protein, is an important regulator of Nod2 function (31). WA has been shown to disrupt

the interaction between vimentin and Nod2, and inhibit Nod2-dependent NF-κB activation (31). Although the role of Nod2 in the cytokine production by macrophages in response to *F. nucleatum* and *A. actinomycetemcomitans* remains unknown, it would be useful to clarify whether WA regulates the Nod2-mediated immune response in macrophages in response to periodontal pathogens.

In conclusion, it was demonstrated that WA has inhibitory effects on *F. nucleatum*- and *A. actinomycetem-comitans*-induced immune responses in macrophages by downregulating TLR signaling. These results indicate that WA may have potential as a novel therapeutic and preventive agent for periodontitis.

Acknowledgements

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