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Ursodeoxycholic Acid Inhibits Inflammatory Cytokine Expression in THP-1 Cells Infected with *Aggregatibacter actinomycetemcomitans*

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Background: Periodontitis is an inflammatory disease characterized by the breakdown of tooth-supporting tissues, leading to tooth loss. *Aggregatibacter actinomycetemcomitans* are major etiologic bacterium causing aggressive periodontitis. Ursodeoxycholic acid (UDCA), a hydrophilic gall bladder acid, has been used as an effective drug for various diseases related to immunity. The aim of this study was to investigate the effect of UDCA on the inflammatory response induced by *A. actinomycetemcomitans*.

Methods: A human acute monocytic leukemia cell line (THP-1) was differentiated to macrophage- like cells by treatment with phorbol 12-mystristate 13-acetate (PMA) and used for all experiments. The cytotoxic effect of UDCA was examined by MTT assay. THP-1 cells were pretreated with UDCA for 30 min before *A. actinomycetemcomitans* infection and the culture supernatant was analyzed for various cytokine production by ELISA. The effect of UDCA on bacterial growth was examined by measuring optical densities using a spectrophotometer.

Results: UDCA showed no cytotoxic effect on THP-1 cells, up to 80 μM Ed highlight: Please confirm technical meaning. UDCA pretreatment inhibited the *A. actinomycetemcomitans*-induced IL-1β, TNF-α, and IL-17A secretion in a dose-dependent manner. UDCA also inhibited IL-21 production at 60 μM. The production of IL-12 and IL-4 was not influenced by *A. actinomycetemcomitans* infection.

Conclusion: These findings indicate that UDCA inhibits the production of inflammatory cytokines involved in innate and Th17 immune responses in *A. actinomycetemcomitans*-infected THP-1- derived macrophages, which suggests its possible use for the control of aggressive periodontitis.

Key words: *A. actinomycetemcomitans*, ursodeoxycholic acid, macrophage, IL-1β, TNF-α, IL-17A, periodontitis

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Introduction

Aggressive periodontitis is characterized by the rapid destruction of the periodontal ligament and the surrounding alveolar bone [1] and mainly caused by *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans)* infection [2]. *A. actinomycetemcomitans* is an indigenous oral bacterium that is a non-motile facultative anaerobic gram-negative rod. The population of *A. actinomycetemcomitans* is higher in subgingival plaque and at sites with localized aggressive periodontitis [3, 4]. *A. actinomycetemcomitans* has virulence factors such as lipopolysaccharide, leukotoxin, collagenase, and IgG protease [4]. Moreover, it can invade epithelial cells and provoke inflammatory responses in host immune cells [5, 6]. Macrophages play a critical role by serving as an essential interface

between innate and adaptive immunity during inflammatory responses by secreting various pro-inflammatory cytokines [7, 8]. It is widely accepted that overproduction of inflammatory cytokines plays important roles in the development of periodontitis. Thus, it is crucial to avoid the excessive production of inflammatory cytokines for the control of inflammatory disease.

Recently, there has been increasing interest about the agents derived from natural resource for the treatment of inflammation. In traditional Asian medicine, bear bile extracted from the gallbladder of *Ursus thibetanus* or *Ursus arctos* is considered as a cure for various diseases. With the development of modern medicine, it is found that the major active component in bear bile is ursodeoxycholic acid (UDCA) [9]. Nowadays, synthetic UDCA, which is a safe drug with no side effect is widely used in the treatment of diseases, such as gallstones, primary cirrhosis, autoimmune hepatitis and colon cancer around the world [10]. However, the effect of UDCA on periodontal inflammation has remained to be cleared.

The purpose of this study is to elucidate the effects of UDCA on inflammatory response induced by *A. actinomycetemcomitans* aiming for its possible application for the control of periodontal inflammation.

Materials and Methods

Reagents

The phorbol 12-mystristate 13-acetate (PMA) was purchased from Sigma (St. Louis, MO, USA). Ursodeoxycholic acid (UDCA) was supplied by Dawoong Chemical (Seoul, Korea).

Bacterial culture

A. actinomycetemcomitans (ATCC 33384) was grown in tryptic soy (TBS) broth (BD, Franklin Lakes, USA) with 1% Yeast (LPS solution, Seoul, South Korea) at 37°C in a 5% CO_2 incubator. An optical density (OD) of 0.25 (650 nm) was determined to correlate to $1x10^9$ CFU/ml. *A. actinomycetemcomitans* was harvested by centrifugation at 5000 rpm for 5 min, resuspended in RPMI media (Gibco, Carlsbad, CA, USA) and used to infect the macrophages at a multiplicity of infection (MOI) of 50 for 24 h.

Cell culture and treatment

THP-1 cells, a human monocyte cell line, were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and were cultured at 37°C in 5% CO_2 incubator. THP-1 cells were differentiated into macrophage-like cells with PMA (50ng/ml). The differentiated THP-1 macrophages were infected with *A. actinomycetemcomitans* with or without the pretreatment of UDCA for 30 min.

Cell cytotoxicity assay

To determine the cytotoxicity of UDCA on THP-1 cells, cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. THP-1 cells were seeded in a 96-well plate and treated with UDCA for 24 h. To determine cell viability, cells were incubated with 100 mL of an MTT solution (1 mg/mL) at 37°C for 4 h. The purple formazan crystal was dissolved in 100 mL of dimethylsulfoxide and the absorbance was measured using a spectrophotometer at 570 nm.

Bacteria of growth curve

To determine the effect of UDCA on bacterial growth, *A. actinomycetemcomitans* was cultured with UDCA at various concentrations. *A. actinomycetemcomitans* was cultured to the exponential phase of growth at 37°C in 5% CO_2 incubator. The OD of each well was measured at a wavelength of 650 nm with a spectrophotometer against the standard medium during the bacterial growth. The OD results were calculated as the means of at least three measurements.

Cytokine analysis

To determine the concentration of cytokines released into the culture media after *A. actinomycetemcomitans* infection, the supernatant was analyzed using an ELISA kit (Biolegend, San Diego, CA, USA) according to manufacturer's instructions. The plates were read using an ELISA reader (Tecan, Männedorf, Switzerland) at 450/570 nm.

Statistics

Statistically significant differences between samples were analyzed with the SPSS 13.0 statistical software program (SPSS Inc., IL, USA). The data were shown as the mean ± SD. A p value of < 0.05 was considered statically significant.

Results

1. Effect of UDCA on the viability of THP−1−derived macrophages and the growth of *A. actinomycetemcomitans*

To examine the effect of UDCA on THP-1 cells and *A.*

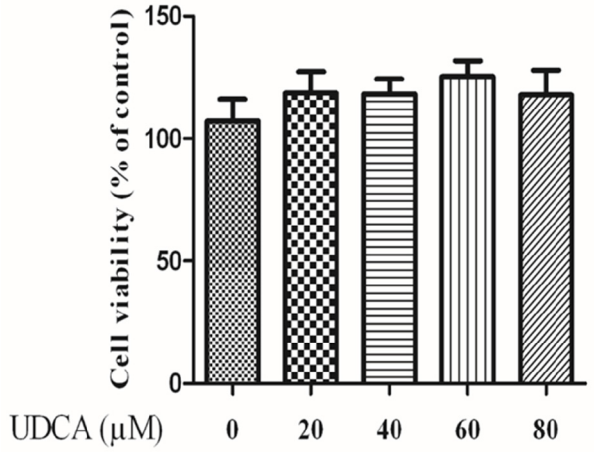

Fig 1. Cytotoxic effect of UDCA on the THP-1-derived macrophages. THP-1-derived macrophages were treated with various concentrations of UDCA for 24 h, and the cytotoxicity was measured by MTT assay. Data are presented as mean ± SD (n=6).

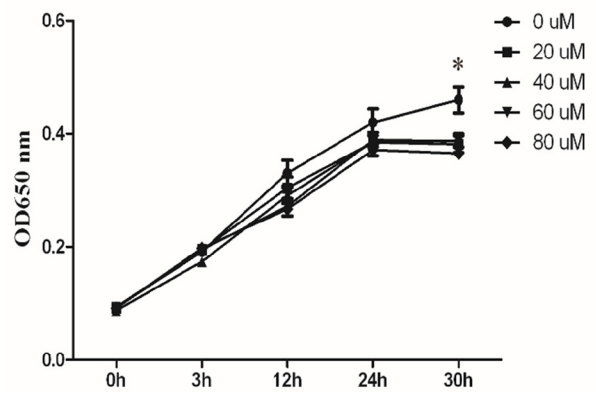

Fig 2. Effect of UDCA against *A. actinomycetemcomitans* growth. *A. actinomycetemcomitans* was grown in TSB broth containing 1% yeast extract with or without UDCA (20, 40, 60 and 80 μM). The OD for growth was measured at various time points with a spectrophotometer at a wavelength at 650 nm. The data represent mean values ± SD (n=6). *p<0.05 vs 0 mM.

actinomycetemcomitans growth, UDCA was added to THP-1-derived macrophages and *A. actinomycetemcomitans*, respectively. THP-1 cells treated with UDCA up to 80 mM did not show any cytotoxicity (Fig. 1). *A. actinomycetemcomitans* showed similar growth pattern between UDCA-treated group and non-treated control group at early growth phase. At late logarithmic phase, 80 μM of UDCA showed some degree of inhibition (10%, $p < 0.05$). After 30 h of incubation, UDCA-treated group significantly decreased bacterial growth suggesting delayed effect of UDCA on *A. actinomycetemcomitans* growth (Fig. 2). UDCA below 80 μM did not show any toxic effect on both THP-1 cells and *A. actinomycetemcomitans* growth for 24 h. Thus, this concentration was used in subsequent experiments.

2. Effect of UDCA on the production of pro-inflammatory cytokines in *A. actinomycetemcomitans*-infected THP-1-derived macrophages

We investigated the effects of UDCA on proinflammatory cytokines production induced by *A. actinomycetemcomitans* infection. *A. actinomycetemcomitans* infection (MOI 50) significantly increased the production of both IL-1β and TNF-α. The production of IL-1β and TNF-α was significantly inhibited by UDCA in a dose-dependent manner (Fig. 3). The

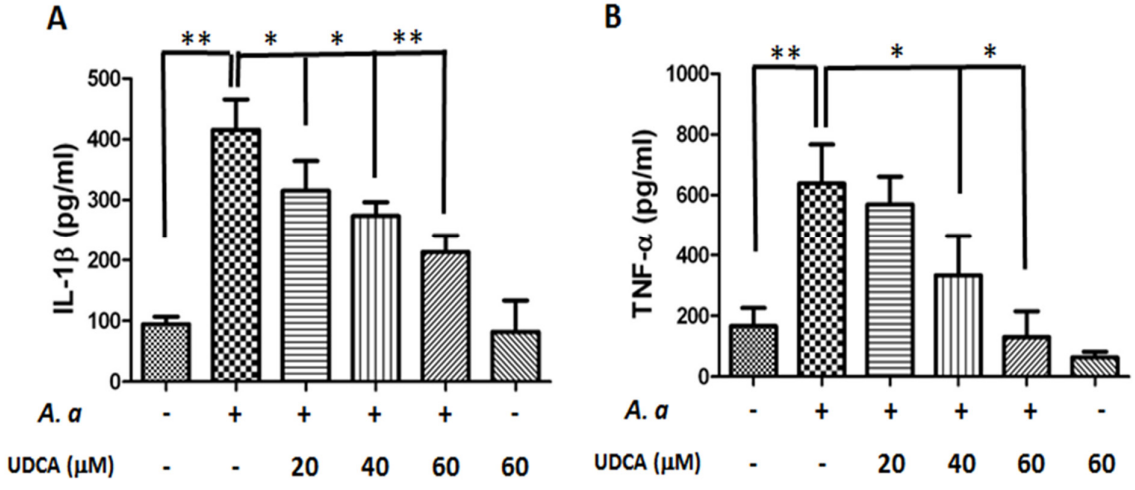

Fig 3. UDCA inhibited the release of pro-inflammatory cytokines induced by *A. actinomycetemcomitans* infection. THP-1-derived macrophages were pretreated with UDCA (20, 40 and 60 μM) for 30 minutes and then were infected with *A. actinomycetemcomitans* (MOI of 50) for 24 h. Cell culture supernatant was collected to determine the concentration of the cytokines. IL-1β (A) and TNF-α (B) production was determined by ELISA. The data represent mean ± SD values (n=4). * p<0.05, ** p<0.01.

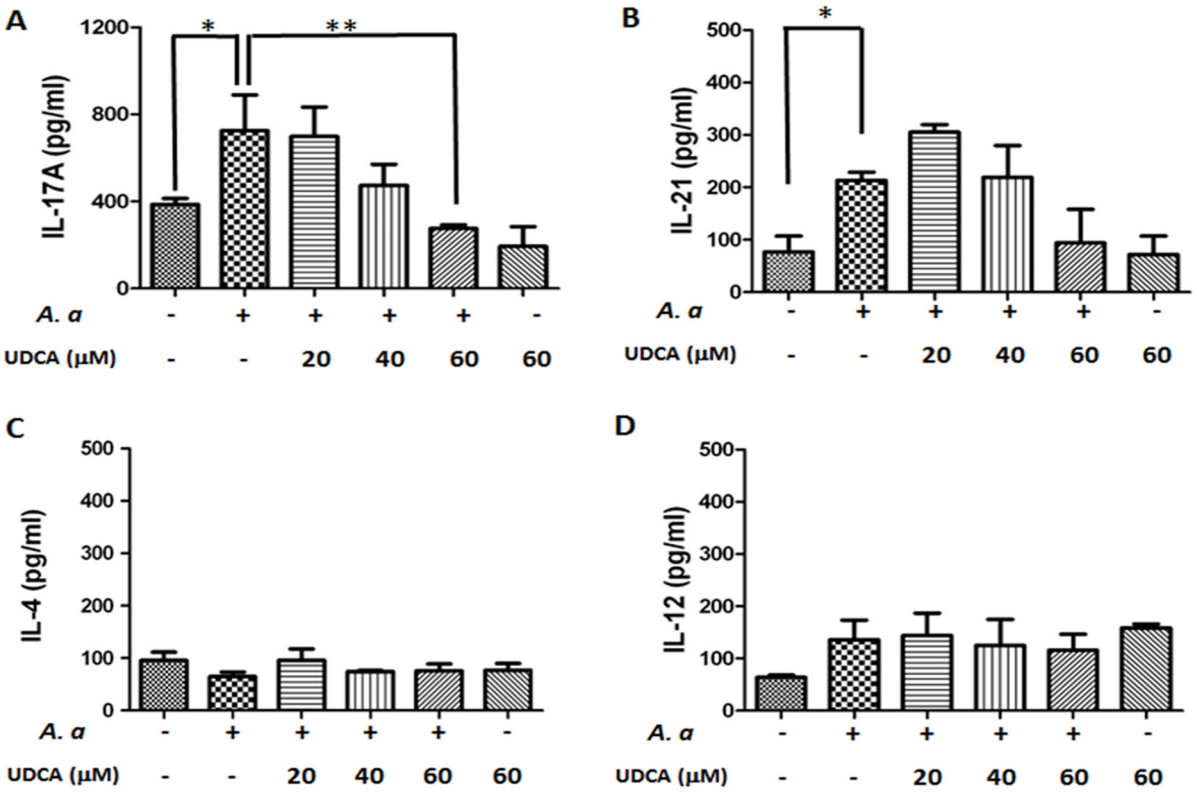

Fig 4. UDCA inhibited the release of adaptive immunity-related cytokines induced by *A. actinomycetemcomitans* infection. THP-1-derived macrophages were pre-treated with UDCA (20, 40 and 60 μM) for 30 minutes and then were infected with *A. actinomycetemcomitans* (MOI of 50) for 24 h. Cell culture supernatant was collected to determine the concentration of cytokines. IL-17A (A), IL-21 (B), IL-4 (C) and IL-12 (D) production was determined by ELISA. The data represent mean ± SD values (n=4). * $p<0.05$, ** $p<0.01$.

results indicate that UDCA can be effective in inhibiting the production of proinflammatory cytokines induced by *A. actinomycetemcomitans* infection in THP-1-derived macrophages.

3. Effect of UDCA on the production of adaptive immune response-related cytokines in *A. actinomycetemcomitans*-infected THP-1-derived macrophages.

To determine if UDCA also has the effect on adaptive immune response adaptive immunity-related cytokines including IL-17A, IL-21, lL-4 and IL-12 production was analyzed. *A. actinomycetemcomitans* infection (MOI 50) significantly increased the production of both IL-17A and IL-21. The production of IL-17A and IL-21 was significantly inhibited by UDCA at 60 μM (Fig. 4). lL-4 and IL-12 were not secreted from THP-1 cells following *A. actinomycetemcomitans* infection (Fig. 5). These results suggest that UDCA is effective in inhibiting the production of Th17-related cytokines in *A. actinomycetemcomitans*-infected THP-1-derived macrophages.

Discussion

Periodontitis is characterized by inflammatory disease in tooth surrounding tisuse and overgrowth of periodontal pathogenic bacteria including *A. actinomycetemcomitans*, *P. gingivalis*, *Treponema denticola*, and *Tannerella forsythia* [11]. *A. actinomycetemcomitans* is the major etiologic agent for aggressive periodontitis [12]. Upon *A. actinomycetemcomitans* infection, dense invasion and excessive inflammatory response of inflammatory cells including monocytes and macrophages occurs at the affected gingiva [13]. Although cytokines produced by bacterial infection play protective roles in the elimination of the pathogen, the overproduction of proinflammatory cytokines is directly related to periodontal breakdown and alveolar bone resorption [14]. Therefore, the tight regulation of cytokine secretion is required for the treatment of periodontitis. It is important anti-inflammatory strategy to reduce the production of pro-inflammatory cytokine

and the susceptibility of inflammatory cells to stimuli.

In this study, the effects of UDCA on *A. actinomycetemcomitans* infected THP-1-derived macrophages were examined. UDCA significantly inhibited the production of IL-1β and TNF-α induced by *A. actinomycetemcomitans* infection. Secretion of pro-inflammatory cytokines including IL-1β and TNF-α is one of the most important initial responses used by the host to resist periodontal microorganisms [15]. These cytokines activate osteoclast and eventually cause bone resorption [16]. They also affect the activation or suppression of inflammation. As is well known, IL-1β and TNF-α are closely related to the progression of periodontitis [7]. Therefore, on the hypothesis of UDCA may work as anti-inflammatory agent, we investigated if UDCA modulated the expression of inflammatory cytokines in *A. actinomycetemcomitans*-infected THP-1-derived macrophages.

Macrophages play a critical role bridging between innate and adaptive immunity during inflammatory responses. It has been reported that a strong innate response leads to a Th1 and Th17 response under the influence of IL-12, IL-21 and IL-17A while a weak innate response leads to a Th2 response under the influence of IL-4 cytokine [17]. IL-17A is the major cytokine involved in mediating Th17 response. IL-17A induces the expression of receptor activator of nuclear factor k B ligand (RANKL) on osteoblasts and stimulates the differentiation and activation of osteoclasts, which can influence bone resorption mediated by these cells [18]. Many studies have demonstrated the presence of IL-17A in periodontal tissues, crevicular gingival fluid, saliva, and plasma of patients with periodontal disease [19]. Along with IL-17A, Th17 cells also produce IL-21, IL-22 and IL-26. Dutzan *et al.* showed an increase in IL-21 levels in chronic periodontitis, a positive correlation of IL-21 with probing depth, clinical attachment levels, IL-1β, IL-6, and Th17 cytokines such as IL-17A and IL-23 [20]. We have also determined IL-12 and IL-4 for Th1 and Th2 response, but *A. actinomycetemcomitans* did not stimulate macrophages to induce their production. This experiments indicated that UDCA significantly suppressed the IL-17A and IL-21 production induced by *A. actinomycetemcomitans* infection.

In summary, this study showed that UDCA could modulate both innate inflammatory response and Th17-mediated inflammatory response. Park *et. al* reported the inhibitory effects of UDCA on periodontal disease progression using clinical, microbiological, and histometrical parameters [21]. Sombetzki *et al.* reported that UDCA attenuated the inflammatory response in livers of infectedmice[22]. Martinez-Moya *et al.* reported anti-inflammatory effect of UDCA in experimental colitis. In addition to modulating inflammatory responses, UDCA has strong growth inhibitory effects on HSC-3 cells, human oral squamous carcinoma cells, but had almost no effect on HOK normal oral cells [23]. Thus, UDCA may have strong ant-inflammatory effect against various infectious diseases but has minimal effect on normal cells, which potentiates its clinical use.

In conclusion, our study demonstrated that UDCA suppressed the production of IL-1β, TNF-α, and IL-17A in THP-1-derived macrophages infected with *A. actinomycetemcomitans*. Our experimental results suggest that UDCA may serve as a potential therapeutic agent for preventing or treating inflammation-associated oral disease such as aggressive periodontitis.

Acknowledgements

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Conflict of interest

The author's declare that there is no conflict of interest that would prejudice the impartiality of this work.

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