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Expression of Inflammasome Complex Following Various Oral Bacterial Infection in THP-1 Cells

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Interleukin-1b (IL-1β), a proinflammatory cytokine, regulates the innate immune responses against bacterial infection. Mature IL-1β is produced from pro-IL-1β by activated caspase-1, which in turn is activated by the inflammasome complex formation. In this study, we compared the inflammasome mRNA expression induced by *S. sanguinis, S. oralis, F. nucleatum* and *P. intermedia*. Among the tested bacteria, *S. sanguinis* induced the highest IL-1β secretion. *S. oralis, F. nucleatum* and *P. intermedia* induced very weak IL-1β secretion. *S. sanguinis* mostly induced the NLRP3 mRNA expressions. Although *F. nucleatum* did not induce high IL-1β secretion, it induced high expression levels of AIM2, NLRP2, and NLRP3. No specific inflammasomes were induced by *S. oralis* and *P.intermedia*. Studying the inflammasome complex activation induced by oral bacteria may thus enhance our understanding of the pathogenesis of oral diseases.

Key words: Oral bacteria, inflammasome, IL-1 beta, real-time PCR

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Introduction

Inflammation is a fundamental response of the innate immune system to noxious stimuli including bacterial infection. Interleukin-1β (IL-1β) plays a critical role in host defense against many pathogens by regulating innate immune and inflammatory responses [1]. Mature form of IL-1β is cleaved from pro-IL-1β by activated capsase-1 [2]. Inflammasomes are complexes that assemble into a platform for the activation of proinflammatory caspase-1. Inflammasomes include the Nod-like receptor (NLR) proteins NLR family pyrin domain containing 2 (NLRP2), NLR family pyrin domain-containing 3 (NLRP3), and NLR family CARD domain-containing 4 (NLRC4), as well as the DNA-sensing complex of absent in melanoma 2 (AIM2) [3].

Different inflammasomes are activated by various stimuli [4]. For example, The NLRP3 inflammasome isactivated by a large variety of stimuli, including microbial products and endogenous signals, such as urate crystal, silica, amyloid fibrils, and ATP[2]. NLRC4 becomes activated by cytosolic flagellin in cells infected with *Salmonella, Legionella*, and *Pseudomonas* [5]. AIM2 family members, which detects cytosolic DNA, can also activate caspase-1 [6].

Oral microflora consists of diverse microorganisms including bacteria, viruses, mycoplasma, fungi, and protozoa. There are more than 700 different bacterial species in the mouth [7]. Oral bacteria can induce inflammation either systemically or locally. Oral microflora can enter the

bloodstream and cause transient bacteremia in human after tooth brushing, tooth extraction, flossing or scalingand root planning [8]. Periodontitis, one of the most common diseases, is a chronic inflammatory disease of periodontium which is driven by pathogenic oral bacteria so called red complex including *Porphysomonas gingivalis, Tannerella forsythia*, and *Treponema denticola* [9]. Socransky *et al*. have described five microbial complexes in subgingival plaques. Among five complexes, orange complex and red complex are known to be involved in the initiation and progression of periodontal disease [10, 11].

Inour previous study, we have found that *Aggregatibacter actinomycetecomitans* infection induced AIM2 expression [12] and *P. gingivalis* infection stimulated AIM2 and NLRP3 expression in differentiated THP-1 cells [13]. The purpose of this study was to compare the inflammasome mRNA expression induced by various oral bacteria.

Materials and Methods

Cell culture and bacterial infection.

THP-1cells were seeded in 6-well plate and primed with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml). THP-1cells were infected with *Streptococcus oralis* (ATCC35037), *Streptococcus. sanguinis* (ATCC 10556), *Fusobacterium nucleatum* (KCTC 5549), or *Prevotella intermedia* (ATCC 25611) at multiplicity of infection 50 and 100 (MOI 50, 100). *S. oralis* and *S. sanguinis* were cultured in brain heart infusion broth (Difco, Detroit, MI, USA) at 37 ºC up to the late log phase of growth (optical density 1.0). *S. oralis* or *S. sanguinis*-infected cells were incubated for 45 min, washed 3 times, and incubated with serum free medium containing penicillin-streptomycin (Gibco, Carlsbad, USA) for 24 hr. *F. nucleatum,* or *P. intermedia* were cultured in Gifu Anaerobic medium (Nissui Seiyaju, Tokyo, Japan) at 37 ºC up to the late log phase of growth (optical density 1.0). Since, *F. nucleatum,* or *P. intermedia* are strict anaerobics, *F. nucleatum,* or *P. intermedia*-infected cells were incubated for 24 hours without washing.

Measurementof IL-1β secretion.

Quantities of IL-1β released to the culture medium after oral bacterial stimulation were analyzed using an enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's instructions (eBioscience, Waltham, MA, USA). A standard or sample solution was added to an ELISA well plate. After incubation for 2 hr at room temperature, anti-IL-1β antibody conjugated with biotin was added to the solution and incubated for 2 hr at room temperature. Streptavidin conjugated with horseradish peroxidase (HRP) was added and allowed to react for 30 minutes. Tetramethylbenzidine substrate solution was then added and allowed to react for 30 minutes. Level of cytokine expression was assessed by an ELISA reader at 450 nm. Each densitometric value was obtained from three independent experiments and expressed as mean ± SD.

Real-time RT-PCR.

Total RNA was isolated with the RNeasy kit (Qiagen, Hilden, Germay) in accordance with the manufacturer's instructions, and cDNA was synthesized with a reverse transcription system(Bioneer Co, Daejeon, South Korea). Real time q-PCR was conducted by an ABI 7500 Real Time PCR System (Applied Biosystems, Waltham, MA, USA) using Taqman Universal PCR Master Mix (Applied Biosystems, Waltham, MA, USA). The level of mRNA expression was normalized with that of GAPDH gene expression values. PCR analysis were used with predeveloped TaqMan assay primers and probes (GAPDH: Hs99999905, AIM2: Hs00915710, NLRP2: Hs01546932, NLRP3: Hs00918082, NLRC4: Hs00892666).

Statistics

Statistically significant differences between samples were analyzed with an unpaired, one-tailed Student's t test. The data are shown as the mean ± SD. A p value of <0.05 was considered statically significant.

Results

IL-1β secretion in THP-1 cells varies among oral bacteria infection.

To determine how much oral bacteria induce inflammation, we examined IL-1β secretion. We differentiated cells of a human acute monocytic leukemia cell line (THP-1) to macrophage-like cells by treatment with PMA. The release of IL-1β after infection with various oral bacteria was detected in culture supernatants by ELISA (Fig. 1). Among

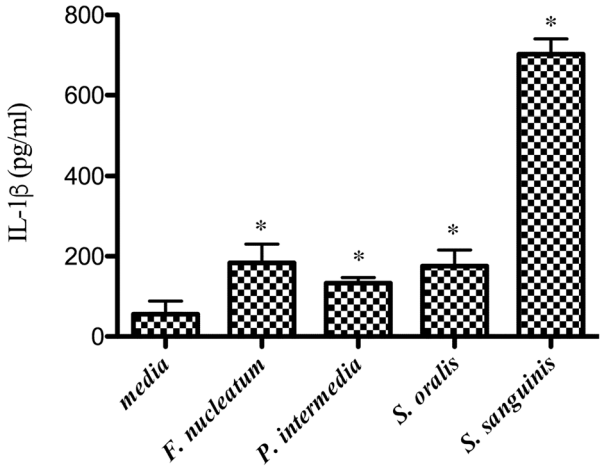

Figure 1. Production of IL-1β after treatment with various oral bacteria. PMA differentiated THP-1 cells were infected with *S. oralis, S. sanguinis, F. nucleatum*, or *P. intermedia* at MOI 100. *S. oralis* and *S. sanguinis* were infected for 45 min, washed with PBS 3 times, and incubated with medium containing penicillin-streptomycin for 24 hr. The levels of IL-1 β production in culture supernatants were measured by ELISA. Values are expressed as mean ± SD obtained from three independent experiments. *p<0.05 vs media.

the tested bacteria, *S. sanguinis* showed highest IL-1β secretion while *F. nucleatum, P. intermedia* and *S. oralis* showed weak IL-1β secretion.

Characterization of inflammasome component mRNA expression following oral bacteria infection.

To characterize specific inflammasome component induced by each oral bacterial, mRNA was extracted after the bacterial infection. *S. sanguinis*, which showed the highest IL-1β secretion, specifically induced NLRP3 expression after 24 hr infection (Fig. 3). *F. nucleatum* induced AIM2, NLRP2, NLRP3 expression at 6 hr (Fig. 2) and NLRP2 and NLRP3 at 24hr after infection (Fig. 3). *P. intermedia* and *S. oralis* rather showed decreased expression of inflammasome complex following the bacterial infection (Fig. 2, Fig. 3).

Discussion

There are a variety of bacterial species in the mouth. Oral

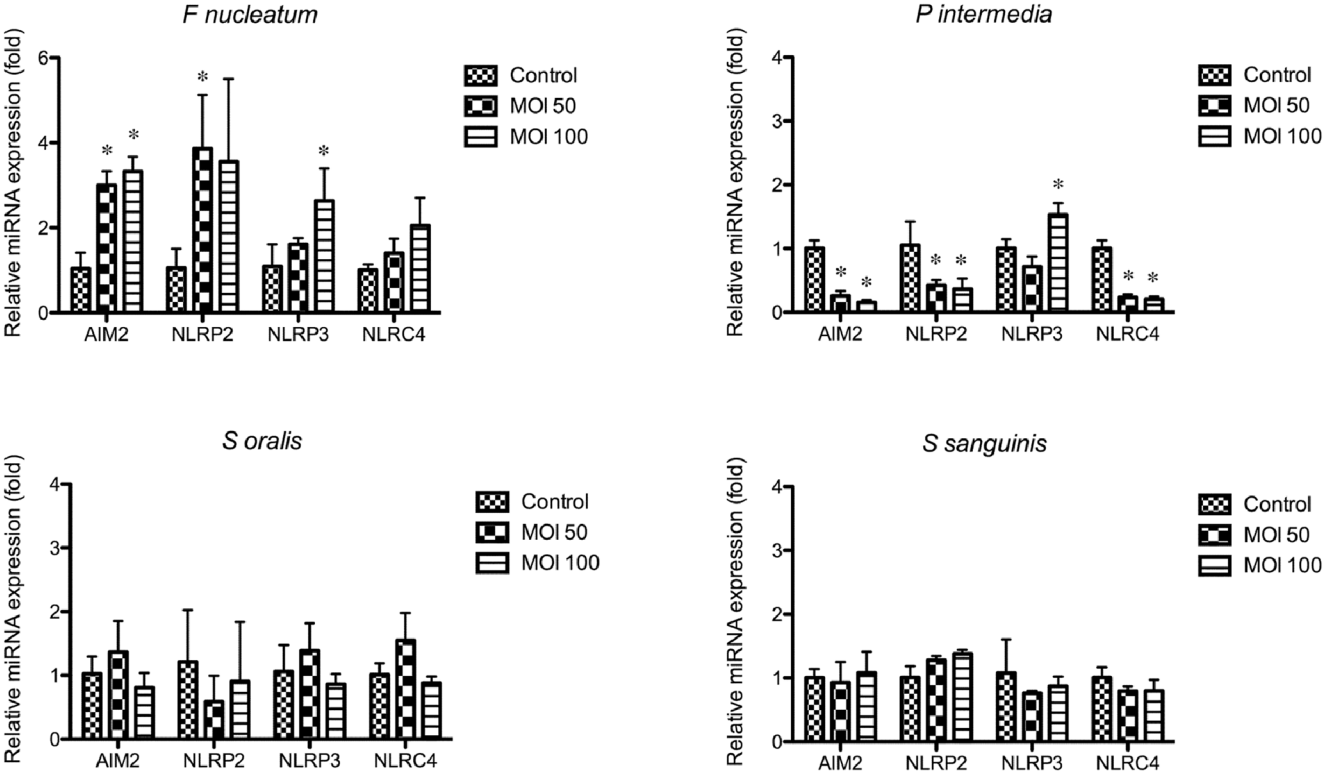

Figure 2. Inflammasome gene expression study. PMA differentiated THP-1 cells were infected with *S. oralis, S. sanguinis, F. nucleatum,* or *P. intermedia* at MOI 50 or 100. *S. oralis* and *S. sanguinis* were infected for 45 min, washed with PBS 3 times, and incubated with medium containing penicillin-streptomycin for 6 hr. The mRNA expressions of each inflammasome genes were determined by real-time RT-PCR. Values are expressed as mean ± SD obtained from three independent experiments. * p<0.05 vs control.

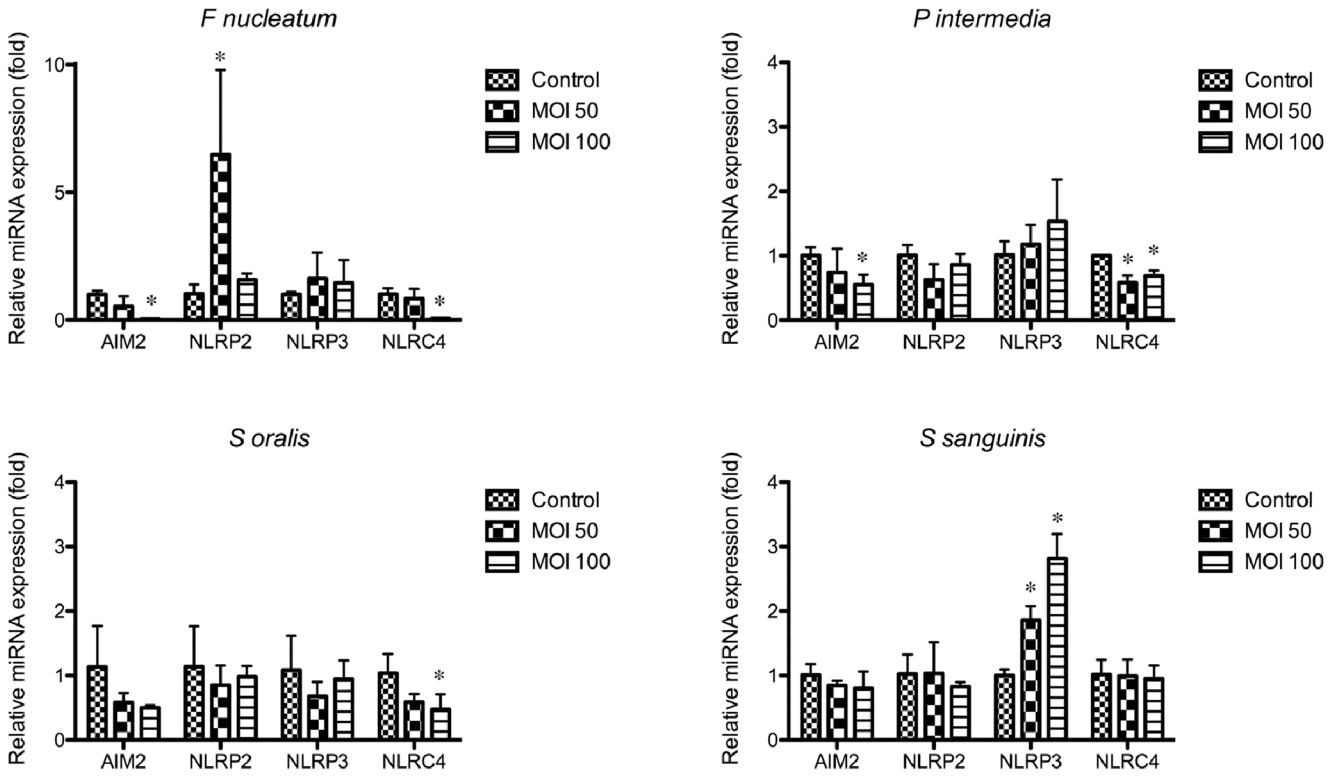

Figure 3. Inflammasome gene expression study. PMA differentiated THP-1 cells were infected with *S. oralis, S. sanguinis, F. nucleatum,* or *P. intermedia* at MOI 50 or 100. *S. oralis* and *S. sanguinis* were infected for 45 min, washed with PBS 3 times, and incubated with medium containing penicillin-streptomycin for 24 hr. The mRNA expressions of each inflammasome gene were determined by real-time RT-PCR. Values are expressed as mean ± SD obtained from three independent experiments. * $p<0.05$ vs control.

bacteria can induce inflammation either systemically or locally. Oral microflora can enter the bloodstream and cause transient bacteremia. Periodontitisis an infection-driven chronic inflammatory disease of periodontium. In our previous study, we have reported that *A. actinomycetemcomitans* and *P. gingivalis* activate inflammasome and induce IL-1β secretion in THP-1 cells [12, 13]. In this study, we infected various oral bacteria to THP-1 cells to determine the production of IL-1β secretion and the inflammasome component induced by the bacterial infection. Differentiated THP-1 cells, which reflect systemic inflammatory response against oral bacteria entering blood stream as well as local inflammation and show higher inflammatory response than monocytes, were treated with oral bacteria for 6 hr and 24 hr to determine early and late inflammasome responses [13].

Among the tested bacteria, *S. sanguinis* induced the highest IL-1β secretion. *S. sanguinis* is an gram-positive bacterium that has been well recognized as a key player in colonization of the human oral cavity [14]. The viridans streptococci are the most common cause of native-valve infective endocarditis, and *S. sanguinis* is one of most commonly implicated with this disease [15]. *S. sanguinis* also serves as a tether for the attachment of other oral microorganisms that colonize the tooth surface and contributes to the development of caries and periodontal disease [16]. When *S. sanguinis* were infected to PMA differentiated THP-1 cells, *S. sanguinis* induced NLRP3 expression. The NLRP3 inflammasome is one of the well studied inflammasome complex that is essential for processing and secretion of IL-1β via activation of caspase-1 [17]. Diverse types of NLRP3 inflammasome activators, such as uric acid, asbestos, silica, and extracellular ATP, could induce caspase-1 activation and IL-1β secretion [18]. Besides, various bacterial pathogens including *P. gingivalis* have been reported to activate NLRP3 [13]. Thus, *S. sanguinis* may also activate NLRP3 to induce IL-1β secretion in THP-1 cells.

F. nucleatum is considered to be a key oral bacterium in

recruiting periodontal pathogens into subgingival dental plaque. The majority of research has focused on the role of *Fusobacterium* spp. in gingivitis and periodontitis, where it is the most frequently isolated species from dental plaque. *F. nucleatum*is believed to play an important role in bridging periodontal pathogens insubgingival dental plaque [10]. Although, *F. nucleatum* did not induce strong production of IL-1β in THP-1 cells by itself, *F. nucleatum* strongly induced the expression of AIM2, NLRP2 and NLRP3 at 6 hr and NLRP2 expression at 24hr after infection. AIM2 controls inflammasome activation, IL-1β secretion, and cell death in response to bacterial dsDNA [6, 19]. NLRP2 enhances caspase-1 activation and IL-1β secretion but shows inhibitory influence on NF-kB activation [20]. Thus, induction of AIM2, NLRP3 and NLRP2 could prime the host cells more susceptible to produce IL-1β when periodontal pathogens including *P. gingivalis* are recruited by *F. nucleatum*.

P. intermedia is frequently found in subgingival plaque from patients with periodontal diseases and is considered one of the periodontal pathogens [21]. *P. intermedia* has also been associated with other oral infections, including endodontic infections [22], and acute necrotizing ulcerative gingivitis [23]. Lipopolysaccharide from *P. intermedia* has been widely used to stimulate macrophages to induce IL-1β production [24, 25]. However, using live *P. intermedia* did not strongly induce inflammasome mRNA expression or IL-1 β secretion in this study. Since, *P. intermedia* is a member of orange complex, it is possible that *P. intermedia* have to interact with other bacteria in orange complex to mediate inflammatory response.

S. oralis is an alpha-hemolytic *Streptococcus* and one of the dominant commensal bacteria of human oral cavity. *S. oralis* belongs to the mitis group which also includes the commonoral species *Streptococcus mitis* and *S. sanguinis*. However, *S. oralis* did not induce inflammasome mRNA expression or high amount of IL-1β secretion which was different from those induced by *S. sanguinis*. Okahashi *et al.* have reported that H_2O_2 produced by *S. oralis* induce cell death on THP-1 human macrophage cell line. In addition, they have shown that the cytotoxic effect was independent of inflammatory responses, because H_2O_2 was not a potent stimulator of tumor necrosis factor-α production in macrophages [26]. Thus, cytotoxic effect of *S. oralis* may have inhibited inducing the inflammatory responses.

In summary, each of oral bacteria such as *S. sanguinis, F. nucleatum*, *P. intermedia,* or *S. oralis* stimulated various amounts of IL-1β production and different inflammasome mRNA expression, which can be involved in IL-1β secretion. This study may help our understanding of oral inflammatory diseases.

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Conflict of interest

The authors declare that they have no conflicting interest.

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