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The Effect of Toll-like Receptor 2 Activation on the Non-opsonic Phagocytosis of Oral Bacteria and Concomitant Production of Reactive Oxygen Species by Human Neutrophils

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Chronic/cyclic neutropenia, leukocyte adhesion deficiency syndrome, Papillon-Lefèvre syndrome, and Chédiak-Higashi syndrome are associated with severe periodontitis, suggesting the importance of neutrophils in the maintenance of periodontal health. Various Toll-like receptor (TLR) ligands are known to stimulate neutrophil function, including FcR-mediated phagocytosis. In the present study, the effect of TLR2 activation on the non-opsonic phagocytosis of oral bacteria and concomitant production of reactive oxygen species (ROS) by human neutrophils was evaluated. Neutrophils isolated from peripheral blood were incubated with *Streptococcus sanguinis* or *Porphyromonas gingivalis* in the presence of various concentrations of Pam_3CSK_4, a synthetic TLR2 ligand, and analyzed for phagocytosis and ROS production by flow cytometry and chemiluminescence, respectively. Pam_3CSK_4 significantly increased the phagocytosis of both bacterial species in a dose-dependent manner. However, the enhancing effect was greater for *S. sanguinis* than for *P. gingivalis*. Pam_3CSK_4 alone induced ROS production in neutrophils and also increased concomitant ROS production induced by bacteria. Interestingly, incubation with *P. gingivalis* and Pam_3CSK_4 decreased the amounts of ROS, as compared to Pam_3CSK_4 alone, indicating the possibility that *P. gingivalis* survives within neutrophils. However, neutrophils efficiently killed phagocytosed bacteria of both species despite the absence of Pam_3CSK_4. Although *P. gingivalis* is poorly phagocytosed even by the TLR2-activated neutrophils, TLR2 activation of neutrophils may help to reduce the colonization of *P. gingivalis* by efficiently eliminating *S. sanguinis* , an early colonizer, in subgingival biofilm.

Key words: periodontitis, neutrophils, phagocytosis, reactive oxygen species (ROS), TLR2

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Introduction

Periodontitis is a common oral disease which affects globally more than half of the adult population [1-4]. Periodontitis is initiated by subgingival plaque biofilms. According to Socransky's classification, subgingival plaque bacteria are classified into six major complexes : red, orange, green, yellow, purple, and Actinomyces [4]. It is known that these bacteria colonize in a certain order: yellow, green, and purple complexes, and Actinomyces are early colonizers; the red complex is a late colonizer; and the orange complex acts

like a bridge between the early and late colonizers, called a bridging colonizer [4]. Therefore, the red complex bacteria are not detected in the absence of the orange complex bacteria or early colonizers. The red complex bacteria *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* are highly associated with periodontitis and distinctly more pathogenic than the others [5].

In the gingival sulcus, two major defense mechanisms regulate plaque bacteria and protect from the invasion of pathogens into gingival tissues. One is antimicrobial peptides, such as human cathelicidin LL-37, human beta defensin-1, and human beta defensin-2, that directly kill bacteria. The other is phagocytosis by macrophages and neutrophils [6]. Chronic/cyclic neutropenia, leukocyte adhesion deficiency syndrome, Papillon-Lefèvre syndrome and Chédiak-Higashi syndrome are associated with severe periodontitis, suggesting the importance of neutrophils in the maintenance of periodontal health [7-10].

Periodontal pathogens have diverse mechanisms to evade the host defense machinery in the gingival sulcus. First, there is interleukin (IL)-8 degradation. IL-8 is upregulated in epithelial cells by invading bacteria and serve as a key chemokine for neutrophils [11]. It has been reported that *P. gingivalis* does not block the mRNA expression of IL-8 but degrades IL-8 protein with its proteases [12,13]. Another mechanism is resistance to LL-37. LL-37 is produced by epithelial cells and neutrophils as a part of the chemical barrier of the epithelia at the gingival sulcus. According to several studies, the late colonizers are more resistant to LL-37 than other bacteria [14-17]. A third mechanism is survival within macrophages. *P. gingivalis* can survive within macrophages through crosstalk among several immune receptors, such as TLR2, CXC-chemokine receptor 4 (CXCR4), complement receptor 3 (CR3) and complement 5a receptor (C5aR) [18,19]. A fourth mechanism is resistance to phagocytosis by neutrophils that also occurs by red complex bacteria [17,20]. Therefore, the enhancement of neutrophil function could be a good target to prevent periodontitis. It is known that various Toll-like receptor (TLR) ligands stimulate neutrophil function, including FcR-mediated phagocytosis [21]. The aim of this study was to investigate whether the stimulation of TLR2 can enhance the non-opsonic phagocytosis of oral bacteria and subsequent killing in neutrophils.

Materials and Methods

Bacterial strains and growth conditions

All bacteria used in this study were obtained from the American Type Culture Collection (Bethesda, MD, USA). *P. gingivalis* ATCC 49417 was cultured in brain heart infusion broth (BHI; BD Biosciences, Franklin Lakes, NJ, USA) with 5 μg/ml Hemin (Sigma, St Louis, MO, USA) and 10 μg/ml Vitamin K (Sigma) under anaerobic condition (5% H_2, 10% CO_2 and 85% N_2). *Streptococcus sanguinis* 804 (NCTC 10904) was cultured in BHI broth under aerobic condition. Bacteria in the log phase were harvested and washed twice with phosphate-buffered saline (PBS; Gibco, NY, USA). Bacterial concentration was determined by flow cytometry as previously described [21].

Preparation of fixed bacteria samples for the phagocytosis assay and measurement of reactive oxygen species (ROS) production

To prepare fixed bacterial samples, bacteria were collected by centrifugation at 5,000 x g for 5 min at room temperature. The bacteria pellets were washed twice with PBS and then incubated with 3.7% paraformaldehyde for 20 min at room temperature. Finally, the samples were washed once and suspended in PBS. For phagocytosis assay, bacteria were stained with 5 μM 5[and-6]-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) at room temperature for 30 min. The bacteria were sonicated (5 cycles of 10 sec at the low power with sonicators, Fisher Scientific, Fair Lawn, USA) to dissociate aggregates and counted with a FACSCalibur (BD Bioscience, Franklin Lakes, NJ) to determine concentration.

Isolation of neutrophils

The use of human materials was approved by the Institutional Review Board at the SNU School of Dentistry (S-D2009005). Leftover human peripheral blood samples were provided by the Department of Diagnostic Medicine, Seoul National University Hospital. To minimize inter-subject variation, a mixture of 10 blood samples (1 ml each) was diluted in 1 x Dulbecco's phosphate-buffered saline (DPBS; Gibco, NY, USA) at 1:1, layered on a 10 ml FICOLL-PAQUE™ plus (Amersham BioSchiencem, Uppsala, Sweden), and centrifuged at 720 x g for 40 min. After centrifugation,

the pellet that contains the red blood cells (RBCs) and polymorphonuclear cells (PMNs) was obtained, and the PMNs were purified by lysing RBCs using hypotonic NaCl solutions. The purified cells were suspended in RPMI 1640 medium (HyClone, Logan, UT, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS; HyClone), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) and incubated on ice.

Phagocytosis assay

To examine the phagocytosing ability of neutrophils, purified neutrophils (1 x 10^5/assay) were incubated with CFSE-labeled bacteria at a cell to bacteria ratio of 1:25 in the presence of various concentrations of Pam_3CSK_4 (Invivogen, San Diego, CA, USA) for 60 min at 37℃. Incubated neutrophils were washed with DPBS. After quenching the fluorescence of the extracellular bacteria with 500 μl trypan blue (Gibco), the cells were analyzed with FACSCalibur. A negative control was prepared by adding the same amount of CFSE-labeled bacteria to neutrophils fixed with 3.7% formaldehyde. The phagocytosing ability of cells was solely analyzed for live cells that were gated based on the forward scatter and FL-3 fluorescence of trypan blue.

Enhanced chemiluminescence assay for ROS measurement

In 96-well plates, purified neutrophils (1x10^5/assay/100 μl) were incubated with unlabeled fixed bacteria at a cell to bacteria ratio of 1:25 in the presence of 0.5 mM luminol and various concentrations of Pam_3CSK_4 at 37℃ for 100 min. All the cells and reagents were resuspended in RPMI medium supplemented with 10% heat inactivated FBS and 2 mM L-glutamine. As a negative and positive control, neutrophils were stimulated with medium alone and 0.1 μM phorbol 12-myristate 13-acetate (Sigma), respectively. After plating, the plate was immediately placed in a microplate luminometer (BMGLabtech, Ortenberg, Germany), and light emission in relative light units was recorded every 10 min during the 100 min incubation at 37℃.

Antibiotic protection assay

To examine the intracellular killing of live *S. sanguinis* and *P. gingivalis* within neutrophils, purified neutrophils (1 x 10^5 cells/ 500 μl per FACS tube) were incubated with those bacteria at a cell to bacteria ratio of 1:25 in the presence of various concentrations of Pam_3CSK_4 at 37℃ for 30 min. Then, the extracellular bacteria were killed by incubating the neutrophils in antibiotic (gentamicin 30 μg/ml, amphotericin 15 μg/ml, and amoxicillin 5 μg/ml)-containing fresh medium for 30 min. The antibiotic-treated neutrophils were washed three times with cold DPBS and further cultured in fresh medium without antibiotics at 37℃ for 1, 2, or 4 h. The neutrophils were lysed with sterile distilled water for 10 min to liberate intracellular bacteria. The lysates were plated onto agar plates and incubated under the appropriate condition until colonies were observed by the naked eye.

Statistical analysis

All experiments were performed at least three times in triplicate. Statistical difference between the untreated control and the treated sample was analyzed with *t*-test. *P* values of < 0.05 were considered statistically significant.

Result

Pre-stimulation of neutrophils with Pam_3CSK_4 enhances the phagocytosis of *S. sanguinis* and concomitant ROS production.

To investigate the effect of TLR2 activation on the phagocytic function of neutrophils, neutrophils were pre-stimulated with Pam_3CSK_4, a synthetic TLR2 ligand, and incubated with CFSE-labeled *S. sanguinis*. Pre-stimulation with Pam_3CSK_4 increased not only the percentage of neutrophils that phagocytosed *S. sanguinis* but also the amount of *S. sanguinis* phagocytosed by each neutrophil (Fig. 1A Left and Right panels). Upon phagocytosis of the microbes, neutrophils produce ROS, one of the major weapons to kill phagocytosed microbes [23]. Therefore, the effect of pre-stimulation with Pam_3CSK_4 on ROS production was also evaluated. Non-stimulated neutrophils produced ROS upon exposure to *S. sanguinis* as expected. Pre-stimulation with Pam_3CSK_4 increased the ROS production by neutrophils more than twofold (Fig. 1B). These results suggest that Pam_3CSK_4 increases the phagocytosing ability and ROS production.

Simultaneous stimulation with Pam_3CSK_4 also enhances phagocytosing activity and concomitant ROS production in neutrophils.

Next, the effect of simultaneous stimulation with Pam_3

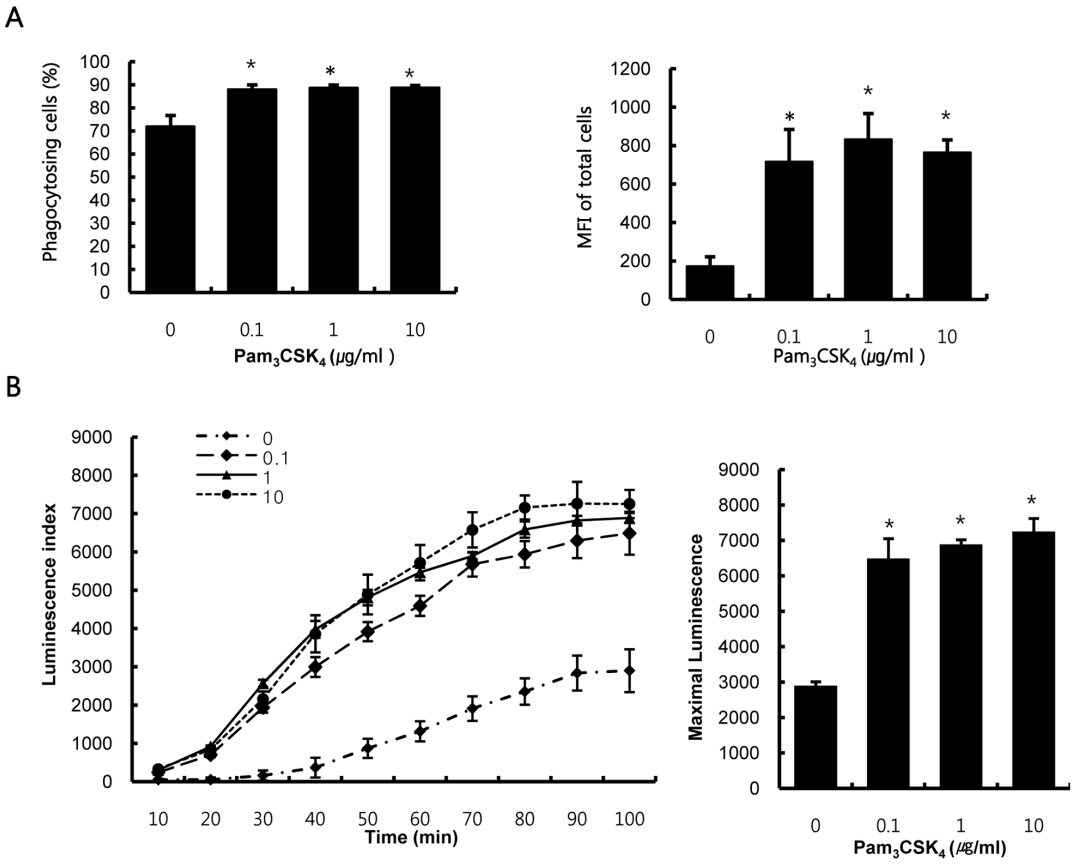

Fig. 1. Prestimulation of neutrophils with Pam_3CSK_4 enhances the phagocytosis of *S. sanguinis* and concomitant ROS production. (A) Neutrophils were pre-stimulated with various concentrations of Pam_3CSK_4 (μg/ml) at 37℃ for 60 min and then incubated with CFSE-labeled fixed *S. sanguinis* at 37℃ for 60 min. The cells were analyzed by flow cytometry after quenching the fluorescence of extracellular bacteria bound on the cell surface with trypan blue. Phagocytosis was expressed by percentage of phagocytosing cells and the mean fluorescence intensity (MFI) of total cells. (B) Neutrophils were pre-stimulated with various concentrations of Pam_3CSK_4 (μg/ml) at 37℃ for 60 min. Cells were then incubated with luminol and unlabeled fixed *S. sanguinis* at 37℃ for 100 min. ROS production was measured every 10 min and a column graph represents the maximal peak level during the measurement period. **P* < 0.05, compared with cells in the absence of Pam_3CSK_4.

CSK_4 on the phagocytosis of *S. sanguinis* was examined. Simultaneous stimulation presented a similar result with that of pre-stimulation, presenting the maximal effect by 1 μg/ml Pam_3CSK_4 (Fig. 2A Left panel). Simultaneous stimulation with Pam_3CSK_4 on the phagocytosis of *P. gingivalis* was also examined. The phagocytosis of *P. gingivalis* was increased by Pam_3CSK_4, also presenting the maximal effect by 1 μg/ml. However, the enhancing effect was greater for *S. sanguinis* than for *P. gingivalis* (Fig. 2A Right panel). Stimulation with Pam_3CSK_4 induced ROS production by itself and increased the bacteria-induced ROS production for both *S. sanguinis* and *P. gingivalis* in a dose-dependent manner (Fig. 2B Left panel). Different from the effect on phagocytosis, the maximal effect on ROS production was observed by 10 μg/ml. Interestingly, *P. gingivalis* attenuated the Pam_3CSK_4 (1 and 10 μg/ml)- induced ROS production (Fig. 2B Right panel).

Neutrophils efficiently kill phagocytosed bacteria even in the absence of a TLR2 stimulator

The increased ROS production induced by Pam_3CSK_4 may assist intracellular killing of phagocytosed bacteria in neutrophils. To determine the effect of Pam_3CSK_4 stimulation on the intracellular killing of bacteria, antibiotic protection assay was performed. Right after phagocytosis and killing of extracellular bacteria (0 h), the enhancement of phagocytosis by Pam_3CSK_4 was evident for both bacterial species. After 4 h of further culture, all *P. gingivalis* and 80% of *S. sanguinis* were killed even in the absence of Pam_3CSK_4 stimulation. The presence of 1 μg/ml Pam_3CSK_4 induced a fourfold

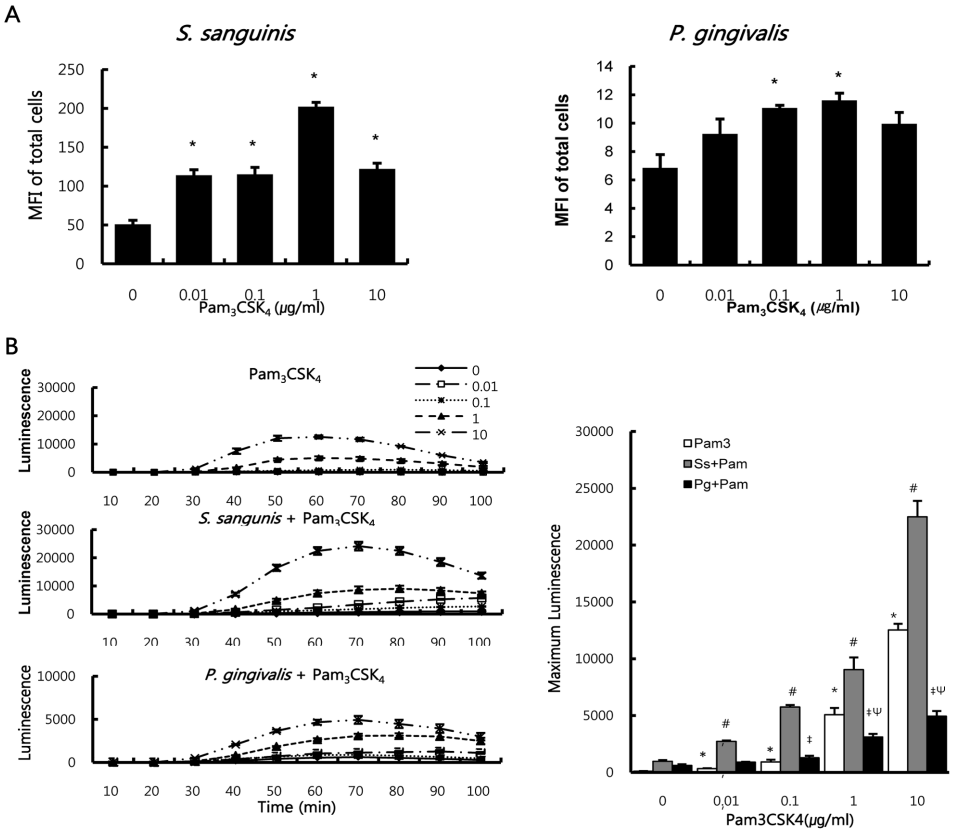

Fig. 2. Simultaneous stimulation of neutophils with Pam_3CSK_4 also enhances phagocytosing activity and concomitant ROS production in neutrophils. (A) Neutrophils were incubated with various concentrations of Pam_3CSK_4 (μg/ml) and CFSE-labeled fixed bacteria at 37℃ for 60 min and analyzed by flow cytometry after quenching the fluorescence of extracellular bacteria bound on the cell surface with trypan blue. Phagocytosis was expressed by MFI of total cells. *$P < 0.05$, compared with cells in the absence of Pam_3CSK_4. (B) Neutrophils were incubated with various concentrations of Pam_3CSK_4 (μg/ml) and unlabeled bacteria in the presence of luminol at 37℃ for 100 min. ROS production was measured every 10 min and a column graph represents the maximal peak level during the measurement. *, #, ‡, $P < 0.05$, compared with cells in the absence of Pam_3CSK_4 in each group. Ψ, $P < 0.05$, compared with cells with Pam_3CSK_4 alone.

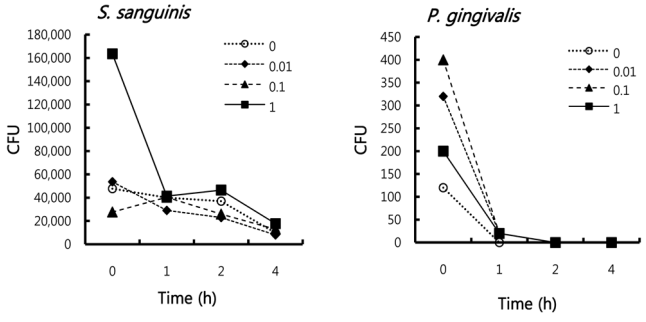

Fig. 3. Neutrophils efficiently kill phagocytosed bacteria even in the absence of a TLR2 stimulator. Neutrophils (1x 10^5/500 μl) were incubated with live bacteria (2.5 x 10^6) in the presence of various concentrations of Pam_3CSK_4 (μg/ml) at 37℃ for 30 min. The extracellular bacteria were killed by incubation with antibiotics for 30 min on ice, and the neutrophils were further cultured in fresh medium without antibiotics at 37℃ for indicated time. The intracellular bacteria were liberated by lysing the neutrophils and cultured on agar plates until colonies were observed by the naked eye. (CFU, colony forming unit)

increase in the phagocytosis of *S. sanguinis*, 89% of which was eliminated in 4 h (Fig. 3).

Discussion

Phagocytosis is the primary defense mechanism against bacteria. It involves several events including the recognition of pathogens through various cell surface receptors, receptor-mediated uptake, fusion of the phagosomes with lysosomes, generation of ROS, and killing of pathogens in the phagolysosomes [24]. A number of studies have shown that TLRs on the cell surface of neutrophils recognize various microbial structures such as lipopolysaccharides (TLR4), lipoproteins (TLR2), and bacterial DNA (TLR9), resulting in the production of cytokines and chemokines or the inhibition

of neutrophil apoptosis [25-27]. The present study showed that TLR2 activation enhances the phagocytosing ability and ROS production in neutrophils.

Stimulation of neutrophils with a TLR2 ligand increased phagocytosis of both *S. sanguinis* and *P. gingivalis*. Compared to *S. sanguinis*, *P. gingivalis* was resistant to phagocytosis by neutrophils even in the presence of Pam_3CSK_4. It is similar to the fact that *T. denticola* was resistant to phagocytosis by neutrophils compared to *S. sanguinis*, even after opsonization with specific antibodies [28]. Although the effect of Pam_3CSK_4 stimulation was not compared with the effect of opsonizing antibodies in parallel, 1 μg/ml Pam_3CSK_4 increased the phagocytosis of *S. sanguinis* about fourfold, which was comparable to the effect of specific opsonizing antibodies shown in the previous study [28].

Induction of ROS production by TLR2 activation in neutrophils has been already reported [25,29]. Therefore, it is not surprising that neutrophils exposed to bacteria produced increased amounts of ROS in the presence of Pam_3CSK_4. The increase in ROS production may be necessary to handle the increased amounts of phagocytosed bacteria. One interesting finding is attenuation of the Pam_3CSK_4-induced ROS production by *P. gingivalis*, which suggests the potential survival of *P. gingivalis* within neutrophils. However, the small number of phagocytosed *P. gingivalis* did not survive within neutrophils even in the absence of Pam_3CSK_4.

In summary, TLR2 activation increases phagocytosis of bacteria and ROS production by neutrophils. Although *P. gingivalis* is poorly phagocytosed even by the TLR2-activated neutrophils, TLR2 activation of neutrophils may help to reduce the colonization of *P. gingivalis* by efficiently eliminating *S. sanguinis*, an early colonizer, in subgingival biofilm.

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Conflict of interest

Authors declare no conflict of interest.

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