이학석사 학위논문

Enterococcus faecalis induces caspase-1 activation and IL-1β production in macrophages

*Enterococcus faecalis*에 의해 대식세포에서 유도되는 caspase-1 활성화와 IL-1β 생성

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치의생명과학과 면역 및 분자미생물 전공

양 하 힘

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Abstract

Enterococcus faecalis induces caspase-1 activation and IL-1β production in macrophages

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Objectives

Enterococcus faecalis is a Gram-positive bacterium and causes various diseases using its virulence factors. Inflammasome is a component of the innate immune system. Recent studies of inflammasome activation have focused on the pathogenesis of diverse inflammatory and autoimmune diseases. Inflammasome activation results in caspase-1 activation, which is required for the

processing of pro-interleukin-1 beta (pro-IL-1β) to its secreted form (IL-1β) as well as pyroptosis, a form of pro-inflammatory cell death. The purpose of this study was to investigate whether endodontic infection associated with *E. faecalis* induces inflammasome activation.

Methods

THP-1 macrophages were treated with live *E. faecalis*. Caspase-1 activation, pro-IL-1β expression and IL-1β secretion were detected by immunoblotting, real-time reverse-transcription polymerase chain reaction (real-time RT-PCR), and enzyme-linked immunosorbent assay (ELISA), respectively. Pyroptosis was measured by lactate dehydrogenase (LDH) release and propidium iodide (PI) staining. Secreted IL-1β and pyroptosis were detected in the presence of caspase-1 inhibitors. Adenosine triphosphate (ATP) release was measured by an ATP bioluminescence assay kit. *E. faecalis*-induced inflammasome activation was measured by immunoblotting in the presence of the ATP receptor antagonist, oxATP. To determine whether the Nod-like receptor family protein 3 (NLRP3) inflammasome was associated with *E. faecalis*-induced caspase-1 activation and IL-1β production, knockdown of NLRP3 was conducted using siRNA. To verify whether *E. faecalis*

internalization is a prerequisite for inflammasome activation, CFSE-labelled *E. faecalis* was detected by flow cytometry and confocal laser scanning microscopy in the presence or absence of cytochalasin D. Caspase-1 activation, pro-IL-1β expression and IL-1β production were detected by immunoblotting in the presence of cytochalasin D. To determine which signaling pathway contributes to the *E. faecalis*-induced pro-IL-1β expression, NF-κB pathway activation and MAP kinase pathway activation were measured by immunoblotting in the presence or absence of signaling inhibitors.

Results

E. faecalis induced caspase-1 activation and pro-IL-1β expression in macrophages, which resulted in IL-1β secretion. *E. faecalis* significantly induced ATP release, a mechanism of NLRP3 inflammasome activation, and oxATP treatment inhibited *E. faecalis*-induced caspase-1 activation. *E. faecalis* significantly increased LDH release and PI uptake, which are characteristics of pyroptosis. *E. faecalis*-induced caspase-1 activation and IL-1β secretion were decreased by the knockdown of NLRP3. *E. faecalis* was internalized into THP-1 macrophages, but blocking *E. faecalis* internalization by treatment with cytochalasin D did not affect *E.*

faecalis-induced caspase-1 activation, pro-IL-1β expression and IL-1β production. The *E. faecalis*-induced pro-IL-1β expression was mediated via NF-κB and MAP kinase activation. These results suggest that *E. faecalis* may contribute to the progression of pulpal inflammation by stimulating excessive secretion of IL-1β and cell death.

Keywords : caspase-1, *Enterococcus faecalis*, inflammasome, interleukin-1 beta, pyroptosis

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CONTENTS

1. Introduction

Enterococcus faecalis is a facultatively anaerobic Gram-positive bacterium. *E. faecalis* is a commensal bacterium that inhabits the gastrointestinal tract and oral cavity (1, 2). However, its ability to resist multiple antibiotics can result in nosocomial infections (3-5). It has been previously reported that *E. faecalis* can translocate into epithelial cell monolayers and circulate into the bloodstream and spleen, and can also survive in macrophages by manipulating the apoptotic signaling pathway (6-8). In addition, some strains of *E. faecalis* can survive within macrophages for more prolonged periods than other strains (9). Therefore, it is widely regarded as an opportunistic pathogen (10). *E. faecalis* is a prominent microorganism found in biofilms, which protect bacteria against antibiotics and phagocytosis (11, 12). In particular, *E. faecalis* is involved in biofilm-mediated infections caused by indwelling medical devices, orthopedic implants, and catheters (13-15). *E. faecalis* expresses various genes involved in biofilm formation and quorum sensing (16-18). *E. faecalis* is found in secondary and persistent endodontic infections rather than primary endodontic infections and is one of the most abundantly found species in root canal-treated teeth when examined by culture-dependent and culture-independent molecular methods (19-24). This bacterium is highly resistant to various irrigants and

medicaments that are used for endodontic treatment, including sodium hypochlorite and calcium hydroxide (25-27).

Recent studies on inflammasome activation via bacterial infection have centered on chronic inflammatory and autoimmune diseases (28). An inflammasome is a multimeric protein complex composed of a cytoplasmic sensor molecule, the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) and pro-caspase-1 (29). Several inflammasome complexes have been discovered, and the uncontrolled activation of inflammasomes is associated with many inflammatory diseases (30-35). Inflammasome activation results in the maturation of pro-caspase-1, which is required for the maturation of pro-interleukin-1 beta (IL-1β) and pyroptosis (36). IL-1β is produced in a pro-form (31 kDa) inside cells and is processed enzymatically to a mature form (17 kDa). This process is indispensable for its secretion and functional role. Pro-caspase-1 in inflammasome complexes is processed to the active form of caspase-1, which comprises tetramers composed of p10 and p20 subunits. IL-1β is a key inflammatory cytokine involved in host defense against pathogens (37). However, undue secretion of IL-1β heightens inflammation, finally leading to tissue impairment and inflammatory diseases (38-40). Active caspase-1 is also involved in pro-inflammatory cell death which is characterized by cellular

swelling, nuclear condensation, plasma membrane rupture, and the release of pro-inflammatory cellular contents (41). Although various bacteria have been shown to activate caspase-1 in different inflammasome complexes (41, 42), the ability of *E. faecalis* to activate caspase-1 has not yet been examined.

As the analyses of microorganisms from inflammatory root canals with inflammation have shown a high proportion of *E. faecalis* (22, 43), it was hypothesized that *E. faecalis* activates caspase-1, leading to IL-1β processing and pyroptosis in macrophages as part of the innate immune response. To elucidate this hypothesis, the inflammatory effects of live *E. faecalis* on macrophages were evaluated.

2. Materials and Methods

2.1. Chemicals and antibodies

Chemicals and antibodies were purchased from the list below. Phorbol 12-myristate 13-acetate (PMA), trichloroacetic acid solution (TCA), oxidized ATP (oxATP), cytochalasin D, N-*p*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), propidium iodide (PI), and phalloidin-tetramethylrhodamine B Isothiocyanate (Rhodamine-phalloidin) were from Sigma (St. Louis, MO, USA). Pam3CSK4 was from Invivogen (San Diego, CA, USA). Ac-YVAD-CHO, SB 203580, SP 600125 and PD 98059 were from Calbiochem (San Diego, CA, USA). Z-YVAD-FMK and the LDH-cytotoxicity assay kit were from BioVision (Palo Alto, CA, USA). Anti-human caspase-1 antibody (recognizing pro-caspase-1 p45, p50 and caspase-1 p20), phospho-SAPK/JNK (Thr183/Tyr185) MAPK antibody, phospho-p44/42 MAPK (Thr202/Tyr204) antibody, phospho-p38 MAP kinase (Thr180/Tyr182) antibody and IκB-α antibody were from Cell Signaling Technology (Beverly, MA, USA). Anti-human IL-1β antibody (recognizing pro-IL-1β p31 and IL-1β p17) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-NLRP3/NALP3 mAb (Cryo-2) were from Adipogen (San Diego, CA, USA). Anti-human actin antibody and brain-heart infusion (BHI) were from BD Biosciences (San Jose, CA, USA). Horseradish peroxidase-conjugated anti-rabbit IgG antibody,

horseradish peroxidase-conjugated anti-goat IgG antibody, horseradish peroxidase-conjugated anti-mouse IgG antibody and the human IL-1β ELISA assay kit were from R&D Systems (Minneapolis, MN, USA). The ATP bioluminescence assay kit and complete protease inhibitor cocktail were from Roche (Mannheim, Germany). Penicillin-streptomycin, carboxylfluorescein diacetate succinimidyl ester (5(6)-CFSE), Hoechst 33342, Stealth siRNA for NLRP3, the stealth siRNA negative control duplex and Lipofectamine RNAiMAX were from Invitrogen (Carlsbad, CA, USA). Easy-BLUE total extraction kit was from iNtRON Biotechnology (Sungnam, Korea). Aqueous/dry mounting medium was from Biomeda (Foster City, CA, USA). Triton X-100 was from MERCK (Darmstadt, Germany)..

2.2. Bacterial strain and growth condition

E. faecalis (American Type Culture Collection [ATCC] 29212) was cultured in an anaerobic atmosphere chamber (10% CO_2, 5% H_2, and 85% N_2) using BHI broth (BD bioscience).

2.3. Cell culture and treatment

To investigate the pro-inflammatory cytokines, *E. faecalis*

was cultured everyday, inoculated to an initial optical density (OD) value at 600 nm of 0.05 in fresh BHI medium, and cultured overnight. At early stationary phase, *E. faecalis* was harvested by centrifugation at 930 x g for 20 min and washed with 1X phosphate buffered saline (PBS). *E. faecalis* was resuspended in RPMI 1640 medium (Hyclone, Logan, UT, USA), and the number of *E. faecalis* was counted using a hemocytometer. THP-1 cells (ATCC TIB-202), a human monocytic cell line, were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin (Invitrogen). The cells were seeded in 6-well plates (2×10^6 cells/well) and differentiated into macrophage-like cells by treatment with 0.5 μM PMA for 3 hours. PMA-differentiated THP-1 macrophages were washed with serum-free medium without antibiotics and infected with *E. faecalis* at a multiplicity of infection (MOI, the ratio of the bacterial number to the number of host cells) of 1, 5, 20, 50 and 100 for 1, 3 and 6 hours, respectively, at 37℃ in the presence of 5% CO_2.

In some experiments, THP-1 macrophages were pre-treated with caspase-1 inhibitors Z-YVAD-FMK, Ac-YVAD-CHO, cytochalasin D, or oxATP for 30 min or 1 hour before stimulation with *E. faecalis*. As a positive control for caspase-1 activation and IL-1β secretion, a synthetic bacterial lipopeptide called Pam3CSK4 was used.

2.4. Immunoblotting

Caspase-1 and IL-1β in the culture supernatants and pro-caspase-1, pro-IL-1β, and β-actin in the cell lysates were detected by immunoblotting. Cell lysates and supernatants of THP-1 macrophages treated with *E. faecalis* or Pam3CSK4 were prepared as follows. After centrifugation of the cells at 600 x g for 10 min, the supernatants and cell pellets were collected individually. The supernatants were mixed with 10% TCA at 4℃ for 30 min and centrifuged at 16,000 x g for 10 min. After removing the TCA, the remaining protein precipitates were mixed well with 15 $\mu\ell$ of 0.1 N NaOH. The cell pellets were lysed using RIPA buffer, which is composed of 10 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM ethylenediamine tetraacetic acid, 5 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride and a complete protease inhibitor cocktail. The cell lysates and the precipitated supernatants were mixed with 5X sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), subjected to SDS-PAGE, and transferred to methanol activated-polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk for 1 hour and were then introduced to rabbit anti-human caspase-1 antibody in 5% bovine serum albumin at 4℃ for 2 days. After washing with PBS-0.1% Tween 20 (AMRESCO, Solon, OH, USA), the membranes were reacted to horseradish

peroxidase-conjugated anti-rabbit IgG goat antibody in 5% skim milk for 1 hour at room temperature. After washing with PBS-0.1% Tween 20, the target proteins were detected with standard enhanced chemiluminescence reagents (Neuronex, Pohang, Korea). To detect pro-IL-1β, IL-1β, NLRP3, and β-actin, the membranes were stripped with a stripping buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 0.8% β-mercaptoethanol) and reacted to the corresponding antibodies.

2.5. Real-time RT-PCR

The total RNA of THP-1 cells treated with *E. faecalis* was isolated with an easy-BLUE total extraction kit and complementary DNA was synthesized from 1 ㎍ of the extracted RNA with M-MLV reverse transcriptase (Promega, Madison, WI, USA) and dNTP mixture (TakaRa, Otsu, Shiga, Japan). For real-time RT-PCR, cDNA (2 ㎕) was mixed with 10 ㎕ of SYBR (Applied Biosystems, Warrington, UK) and primer pairs (10 pmol each) in a 20 ㎕ reaction volume. After incubation at 95℃ for 10 min, a polymerase chain reaction was performed for 40 cycles comprised of a denaturation condition at 95℃ for 15 seconds and an annealing and extension condition at 60℃ for 1 min. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference

gene to normalize expression of IL-1β and NLRP3. The sequences of primers used were as follows: 5' -GCC AAT CTT CAT TGC TCA AGT GTC-3' and 5' -TTG CTG TAG TGG TGG TCG GA-3' for IL-1β; 5' -GAA ACC TTT CTT CCA TGG CTC A-3' and 5' -GGG ATT CGA AAC ACG TGC ATT AT-3' for NLRP3; 5' -GTC GCC AGC CGA GCC-3' and 5' -TGA AGG GGT CAT TGA TGG CA-3' for GAPDH.

2.6. ELISA

The culture supernatants of *E. faecalis*-infected THP-1 macrophages were subjected to ELISA to determine the concentration of the secreted IL-1β using ELISA assay kits according to the manufacturer's protocol.

2.7. Measurement of ATP release

PMA-differentiated THP-1 cells were treated with *E. faecalis* (MOI of 1, 5, 20, and 50) or 0.5 μg/mL Pam3CSK4 for 1.5 hours. The level of extracellular adenosine triphosphate (ATP) was determined using an ATP bioluminescence assay kit according to the manufacturer's protocol. The culture supernatants of *E. faecalis* or Pam3CSK4-treated THP-1 macrophages were mixed

with 0.3% TCA and were kept at 4℃ for 30 min. After adding 4 volumes of 250 mM Tris-acetate (pH 7.75), a luciferase reagent was added to the mixture, and luminescence was measured by the Glo-Max 96 Microplate Luminometer (Promega).

2.8. RNA interference assay

The pre-designed Stealth select RNAi siRNA (NLRP3) was purchased from Invitrogen. The gene ID is 114548 and the gene accession number is NM-001079821 (Homo sapiens NLR family, pyrin domain containing 3 (NLRP3). The pre-designed NLRP3 siRNA sequences were as follows: 5' -ACC GCG GUG UAC GUC UUC UUC CUU U-3' and 5' -AAA GGA AGA AGA CGU ACA CCG CGG U-3' for siNLRP3 duplex. NLRP3 siRNA and siRNA negative controls were resuspended to a concentration of 20 μM, and Lipofectamin RNAiMAX diluted in 250 $\mu\ell$ of serum-free, antibiotic-free RPMI was mixed with NLRP3 siRNA or a negative control to a final concentration of 30, 50, and 100 nM. PMA-differentiated THP-1 macrophages (2 x 10^6 cells/well in 6-well plates) in RPMI medium containing 10% FBS were transfected by the RNAiMAX/siRNA mixture for 36 hours and 48 hours at 37℃ (5% CO_2 atmosphere). After transfection, THP-1 cells were washed with serum-free RPMI medium and infected with *E. faecalis* at an

MOI of 50 for 6 hours. The knockdown of the NLRP3 gene and protein was determined by real-time RT-PCR and immunoblotting, respectively.

2.9. Fluorescence labelling of bacteria and cells

In order to determine the binding and internalization ability of *E. faecalis* into THP-1 macrophages, *E. faecalis* and THP-1 cells were labelled with fluorescence molecules.

To label *E. faecalis,* overnight-grown *E. faecalis* was harvested in 1X PBS and diluted to OD at a 600 nm reading of 1.0, and *E. faecalis* was stained with 50 μM CFSE for 1 hour at room temperature. The bacteria were then washed twice with 1X PBS and resuspended with 1 ml of serum-free RPMI medium without antibiotics.

To stain THP-1 macrophages, the cells in 24-well plates (2 x 10^5 cells/well) were fixed with 4% paraformaldehyde at RT for 15 min and permeabilized with 0.1% Triton X-100 at RT for 1 min. The cells were washed with chilled PBS twice and reacted to 5 μg/ml of rhodamine-phalloidin for 15 min and 10 μM of Hoechst for 30 min at room temperature.

2.10. Bacterial binding and internalization assay

To examine whether *E. faecalis* binds and is internalized in THP-1 macrophages, THP-1 cells (2 X 10^5 cells/well in 24-well plates) were differentiated into macrophage-like cells with 0.1 μM PMA overnight. After differentiation, the cells were washed with serum-free RPMI medium and treated with CFSE-labelled *E. faecalis* or serum-free RPMI medium without antibiotics at an MOI of 50 or 100 for 1, 3, and 6 hours. THP-1 cells were washed with pre-warmed DPBS and treated with 4% paraformaldehyde for fixation. After washing with chilled PBS, the cells were permeabilized by 0.1% Triton X-100. Rhodamine-phalloidin (5 ㎍/ml) was used for actin staining, and 10 ㎛ Hoechst was used for nucleus staining for 15 min or 30 min, respectively. The cells were mounted with aqueous/dry mounting medium to avoid direct light overnight. Images were acquired by confocal laser scanning microscopy (LSM 700, Carl Zeiss, Jena, Germany).

For flow cytometric analysis, PMA-differentiated THP-1 cells were infected with CFSE-labelled *E. faecalis* at an MOI of 50 for 6 hours. In some experiments, THP-1 cells were pre-treated with cytochalasin D for 1 hour before *E. faecalis* infection, The cells were detached using 1X trypsin-EDTA, and 1ml of 10% FBS in RPMI was used for neutralization of trypsin. The cells were collected in FACS tubes and resuspended with 300 ㎕ of chilled

DPBS after centrifugation at 400 x g for 5 min. The fluorescence of bacteria was measured by a FACScalibur (BD bioscience), and 0.4% trypan blue was used for quenching extracellular *E. faecalis*.

2.11. Cell death assay

Cytoplasmic lactate dehydrogenase (LDH) release and PI uptake through the plasma membrane pore are characteristics of pyroptosis, which are mediated by active caspase-1. PMA-differentiated THP-1 cells (1 x 10^5 cells/well in 96-well plates) were stimulated with *E. faecalis* or Pam3CSK4 for 6 hours. Released LDH was normalized to the maximum LDH release obtained by treatment with the cell lysis solution included in the LDH cytotoxicity assay kit.

To quantify PI-positive cells, the cells were double stained with 30 μM PI and 10 μM Hoechst 33342 for 30 min. The cells were fixed with 4% formaldehyde for 15 min and washed with PBS and chilled distilled water. The cells were mounted with aqueous/dry mounting medium. Images of PI-positive cells were acquired by confocal laser scanning microscopy (LSM 700), and the number of PI-positive cells were counted using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.12. NF-κB and MAP kinase activation assay

THP-1 cells (5 x 10^6 cells/dish) were differentiated into macrophage-like cells with 0.5 μM PMA in 60Φ dishes for 3 hours. After washing with serum-free RPMI medium, THP-1 macrophages were incubated with 5 ml of serum-free medium for 24 hours at 37℃ in the presence of 5% CO_2. After harvesting overnight-cultures of *E. faecalis*, the number of *E. faecalis* was determined by measuring the OD value using a microtitter plate. THP-1 macrophages were infected with *E. faecalis* at an MOI of 50 or stimulated with 0.5 μg/ml of Pam3CSK4 for 1 hour or for 0 to 2 hours. IκB-α, p-p38, p-ERK, and p-JNK were detected in the cell extracts by immunoblotting.

To determine whether NF-κB and MAP kinase activation is involved in pro-IL-1β expression in *E. faecalis*-infected THP-1 cells, PMA-differentiated THP-1 cells (2 x 10^6 cells/well in 6-well plates) were pre-treated with TPCK (10 μM), SB 203580 (30 μM), SP 600125 (30 μM), and PD 98059 (50 μM) for 30 min before infection to inhibit NF-κB, p38, JNK, and ERK pathways, respectively.

2.13. Statistical analysis

Statistical difference between the untreated control and the treated samples was analyzed using Student's *t*-test. A *p* value of <0.05 was considered statistically significant.

3. Results

3.1. *E. faecalis* Activates Caspase-1 in THP-1 macrophages

E. faecalis-induced caspase-1 activation and IL-1β secretion of THP-1 macrophages were measured by immunoblotting of culture supernatants, while pro-caspase-1 and pro-IL-1β expression were measured by immunoblotting of cell lysates. Activation of caspase-1 was determined by the appearance of cleaved caspase-1 product (p20) in culture supernatants. *E. faecalis* induced caspase-1 activation and IL-1β secretion in a dose-dependent manner (Fig. 1A). Increased IL-1β secretion was also dose-dependently detected by ELISA (Fig. 1B). Because the IL-1β secretion in macrophages is proceeded by pro-IL-1β expression, *E. faecalis* was expected to induce pro-IL-1β production. Dose-dependent pro-IL-1β expression was detected in cell lysates infected with *E. faecalis* (Fig. 1A). In addition, pro-IL-1β expression at the RNA level was confirmed by real-time RT-PCR (Fig. 1C). Pam3CSK4 was used as a positive control for pro-IL-1β expression via the Toll-like receptor (TLR) 2 and caspase-1 activation via the NLRP7 inflammasome (44).

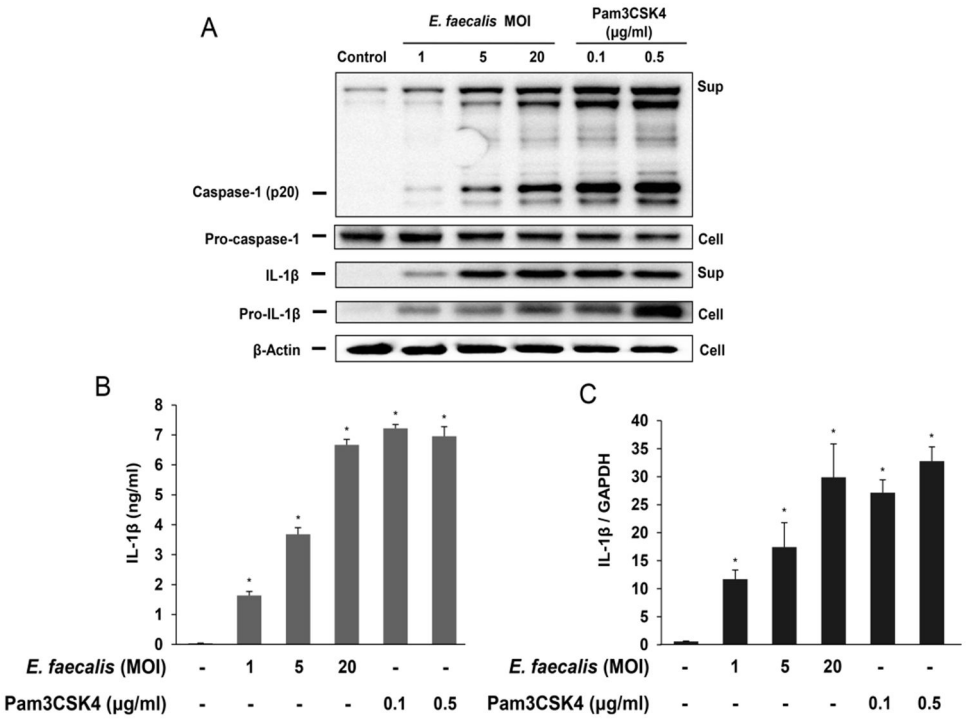

Figure 1. *E. faecalis* induces caspase-1 activation and IL-1β secretion. THP-1 macrophages were infected with live *E. faecalis* (MOI of 1, 5, and 20) or Pam3CSK4 (0.1 and 0.5 μg/mL) for 6 hours. (A) Caspase-1 and IL-1β were secreted into the culture supernatants (Sup) and pro-caspase-1, pro-IL-1β, and β-actin in the cell lysates (Cell) were detected by immunoblotting. (B) Secreted IL-1β was measured by ELISA. (C) mRNA expression of pro-IL-1β was measured by real-time RT-PCR. $^{*}p < 0.05$ compared with the non-treated control.

To confirm that *E. faecalis*-induced IL-1β secretion was caused by active caspase-1, THP-1 cells were pre-treated with the caspase-1 inhibitors (Z-YVAD-FMK and Ac-YVAD-CHO) before infection with *E. faecalis*. These inhibitors significantly inhibited *E. faecalis*-induced IL-1β secretion (Fig. 2). These results suggest that *E. faecalis* induces IL-1β secretion in macrophages by the simultaneous induction of pro-IL-1β expression and processing of pro-IL-1β to IL-1β by caspase-1 activation.

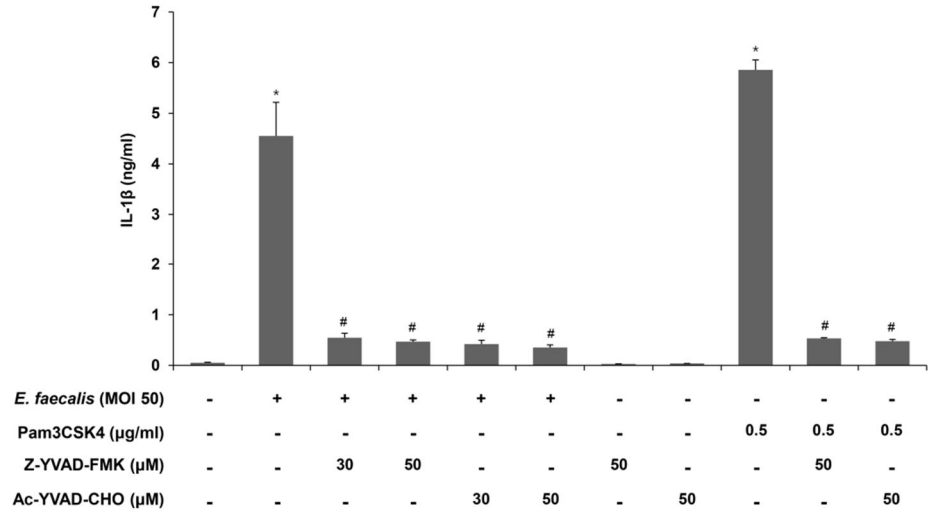

Figure 2. *E. faecalis*-induced IL-1β secretion is dependent on caspase-1. THP-1 macrophages were pre-treated with caspase-1 inhibitors (Z-YVAD-FMK and Ac-YVAD-CHO) for 30 min before infection with *E. faecalis* for 6 hours. Secreted IL-1β was detected by ELISA. $^{*}p < 0.05$ compared with the non-treated control and $^{\#}p < 0.05$ compared with *E. faecalis*- or Pam3CSK4-treated cells.

3.2. *E. faecalis* Induces Pyroptosis

Because pyroptosis is active caspase-1-dependent inflammatory cell death, LDH release and PI uptake were analyzed to determine whether pyroptosis was induced by *E. faecalis* infection. *E. faecalis* induced LDH release in a dose-dependent manner in THP-1 macrophages (Fig. 3A). In addition, *E. faecalis*-stimulated cells were stained with PI, whereas non-stimulated cells were not (Fig. 3B). The number of PI-positive cells was significantly higher in the *E. faecalis*-infected cells than the uninfected cells (Fig. 3C). Caspase-1 inhibitor Ac-YVAD-CHO inhibited *E. faecalis*-induced PI uptake (Fig. 3B and C). These results indicates that *E. faecalis* induces pyroptosis.

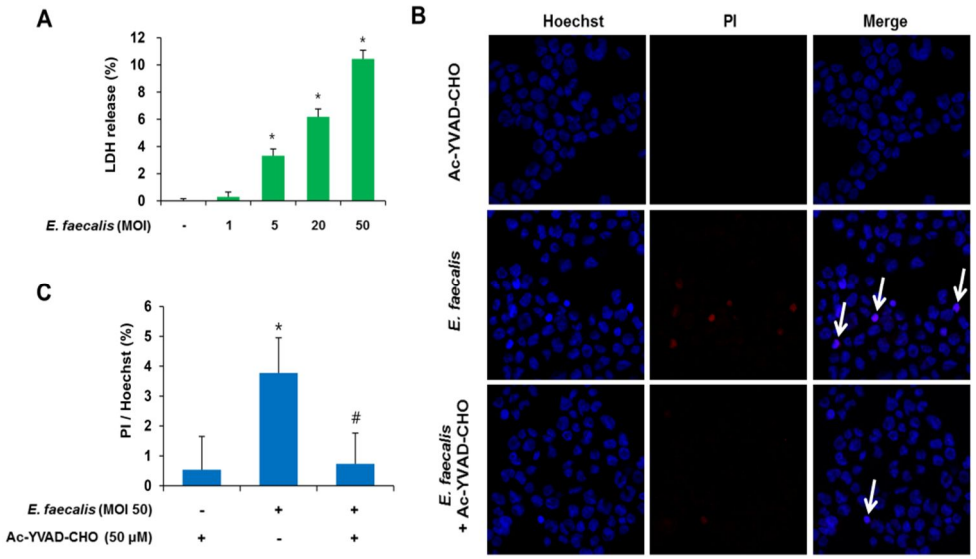

Figure 3. *E. faecalis* induces LDH release and PI uptake. THP-1 macrophages were infected with *E. faecalis* for 6 hours. (A) LDH levels in culture supernatants were measured using an LDH cytotoxicity assay kit. (B) Cells were stained with PI and Hoechst 33342. Images were acquired with a confocal laser scanning microscope. (C) The number of PI-positive cells out of a total 1000 cells was counted in 10 randomly chosen fields using Image J software. Data are shown as mean ± standard deviation of the percentage of PI-positive cells. $^{*}p < 0.05$ compared with the non-treated control and $^{\#}p < 0.05$ compared with *E. faecalis*-stimulated cells

3.3. *E. faecalis* Induces ATP Release, Resulting in Caspase-1 Activation

A high concentration of extracellular ATP is a well-known caspase-1 activator via the NLRP3 inflammasome by binding to the P2X7 receptor and causing the release of intracellular potassium through the pannexin-1 channel (45). Stressed or infected host cells can release ATP as a danger signal (46). *E. faecalis* induced ATP release in a dose-dependent manner (Fig. 4A), and a P2X7 receptor antagonist oxATP inhibited *E. faecalis*-induced caspase-1 activation and IL-1β secretion (Fig. 4B).

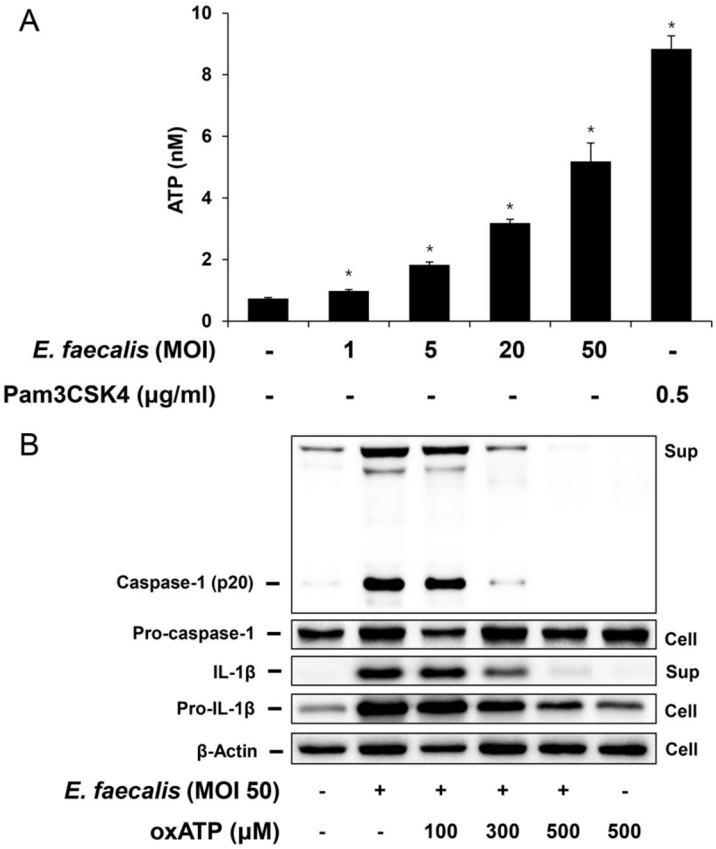

Figure 4. Caspase-1 activation by *E. faecalis* is associated with ATP release. (A) THP-1 macrophages were infected with *E. faecalis* for 1.5 hours, and extracellular ATP levels in the culture supernatants were determined using an ATP bioluminescence assay kit. $^{*}p < 0.05$ compared with the non-treated control (B) THP-1 macrophages were pre-treated with a P2X7 receptor antagonist oxATP for 30 min prior to infection with *E. faecalis* at an MOI of 50 for 6 hours. Caspase-1 and IL-1β secreted into the culture supernatants (Sup) and pro-caspase-1, pro-IL-1β, and β-actin in the cell lysates (Cell) were detected by immunoblotting.

3.4. NLRP3 Knockdown Decreases Caspase-1 Activation and IL-1β Secretion Induced by *E. faecalis*

Caspase-1 activation is induced by several inflammasome components such as NLRP3, NLRP7, and AIM2 (29). As ATP release is known to be associated with the NLRP3 inflammasome activation, knock own of the NLRP3 gene was conducted by using siRNA technology. Transfection of THP-1 macrophages with NLRP3 siRNA resulted in a decrease of the expression of NLRP3 mRNA and protein as compared to the control siRNA-transfected cells (Fig. 5A and 5B). *E. faecalis*-induced caspase-1 activation, IL-1β secretion and NLRP3 protein are decreased by NLRP3 knockdown (Fig. 5B). However, knockdown of NLRP3 did not completely decrease caspase-1 activation (Fig. 5C).

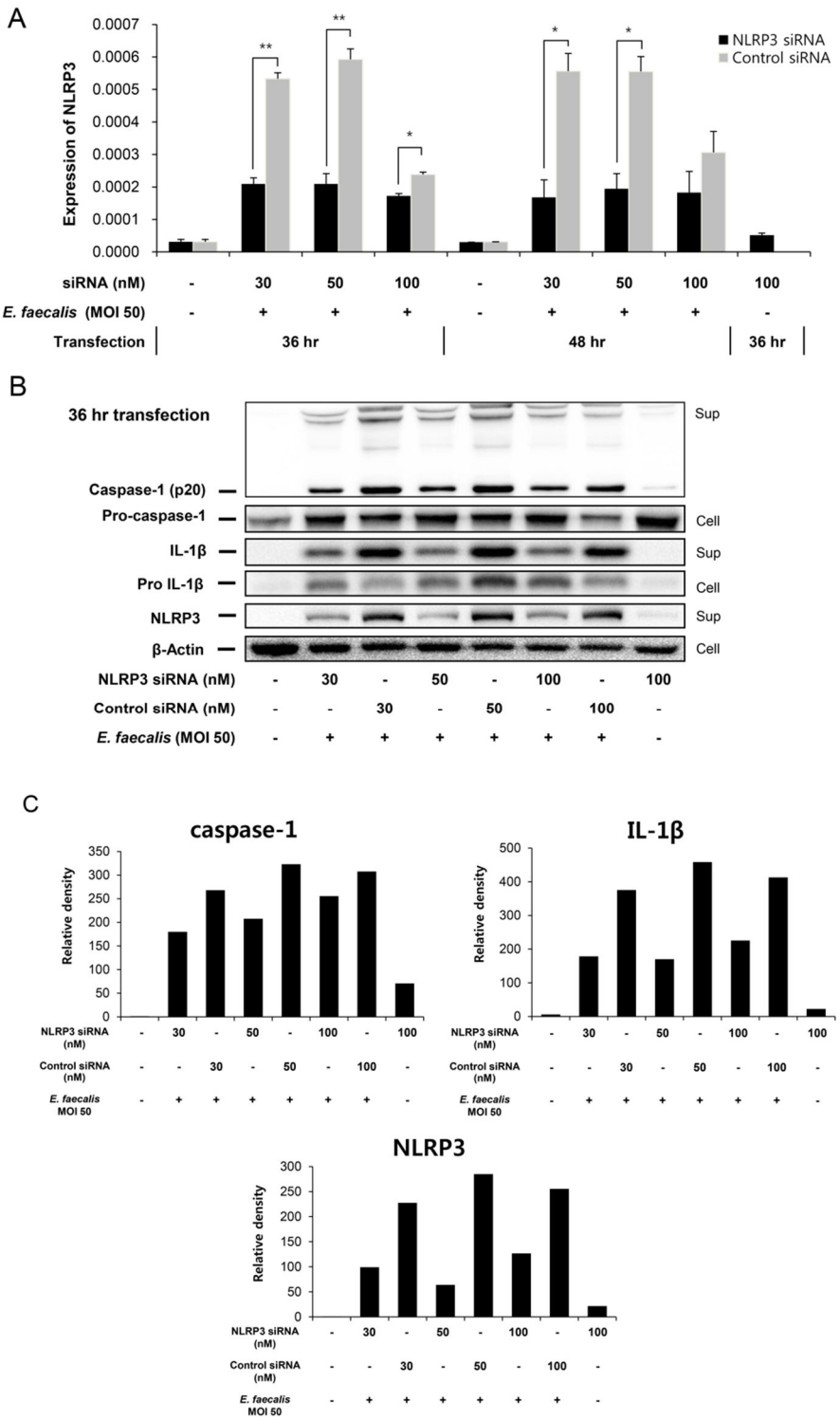
A
Expression of NLRP3
0.0007
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
NLRP3 siRNA
Control siRNA
siRNA (nM) - 30 50 100 - 30 50 100 100
E. faecalis (MOI 50) - + + + - + + + -
Transfection 36 hr 48 hr 36 hr
B
36 hr transfection
Caspase-1 (p20)
Pro-caspase-1
IL-1β
Pro IL-1β
NLRP3
β-Actin
Sup
Cell
Sup
Cell
Sup
Cell
NLRP3 siRNA (nM) - 30 - 50 - 100 - 100
Control siRNA (nM) - - 30 - 50 - 100 -
E. faecalis (MOI 50) - + + + + + + -
C
caspase-1
IL-1β
NLRP3
Relative density
NLRP3 siRNA (nM)
Control siRNA (nM)
E. faecalis MOI 50

Figure 5. NLRP3 knockdown reduces active form of caspase-1 and IL-1β. THP-1 macrophages were transfected with NLRP3 siRNA or control siRNA for 36 hours. The cells were then infected with *E. faecalis* for 6 hours. To confirm RNA interference and inflammasome activation, (A) half of the cells were used to analyze the mRNA expression of NLRP3 by real-time RT-PCR, and (B) the other half was used for the detection of NLRP3, caspase-1 and IL-1β secretion into the culture supernatants, and pro-caspase-1, pro-IL-1β and β-actin in the cell lysates by immunoblotting. (C) Relative density of each protein compared to β-actin in Fig. 5B was analyzed by Image J software. $^{*}P < 0.05$ compared with the non-treated control and $^{**}p < 0.005$ compared with the non-treated control

3.5. *E. faecalis* Adheres to and is Internalized into THP-1 cells

THP-1 macrophages have several receptors that exist both on the surface and inside of cells that recognize foreign substances and bacteria (47). Inflammasomes are activated by soluble molecules, bacterial adhesion, or invasion (48). To determine whether *E. faecalis* adheres to and internalizes into THP-1 cells, the cells were infected with CFSE-labelled *E. faecalis* and were subsequently stained with rhodamine-phalloidin to visualize F-actin and with Hoechst dye to counterstain the nucleus. *E. faecalis* bound to THP-1 cells, and increased incubation time resulted in increased internalization of the bacteria (Fig. 6).

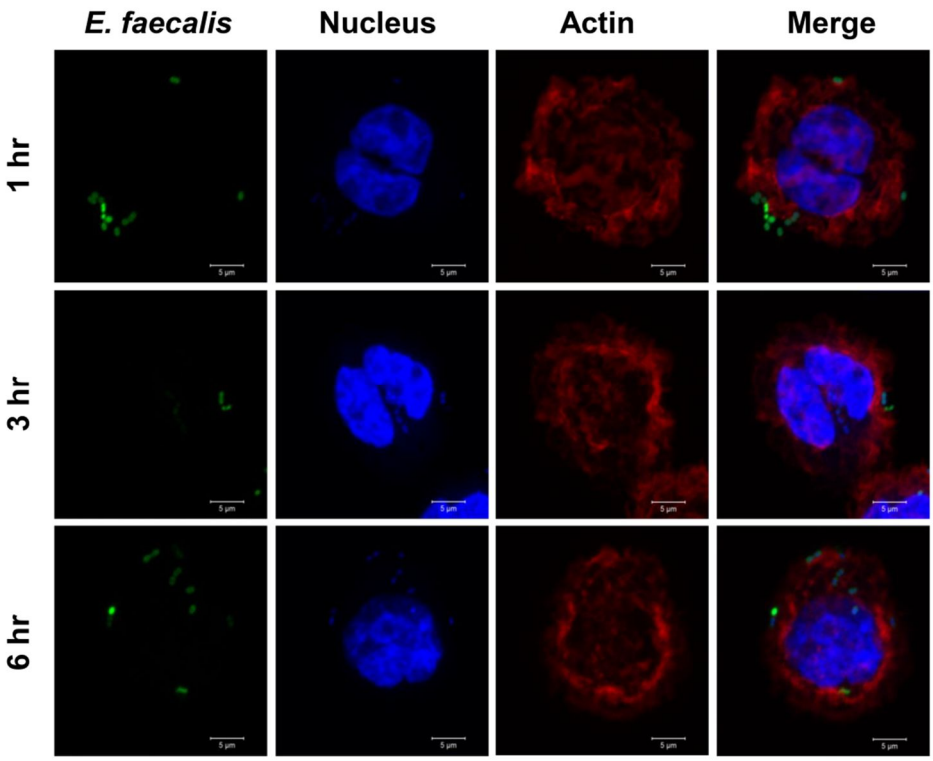

Figure 6. Adhesion and internalization of *E. faecalis* is time-dependently observed. THP-1 cells were inoculated with CFSE-labeled *E. faecalis* at an MOI of 100 for 1, 3, or 6 hours. THP-1 cells were stained with Hoechst (10 μM) and rhodamine-phalloidin (5 μg/ml). The cells were observed using a confocal laser scanning microscope. Original magnification x 2000.

To block the bacterial internalization, further experiments were performed by using cytochalasin D which inhibits actin polymerization and induces actin depolymerization. *E. faecalis* internalization was observed in the non-treated THP-1 cells (Fig. 7). On the other hand, the pre-treated THP-1 cells with cytochalasin D resulted in the inhibition of CFSE-labeled *E. faecalis* internalization when detected by confocal laser scanning microscopy (Fig. 7)

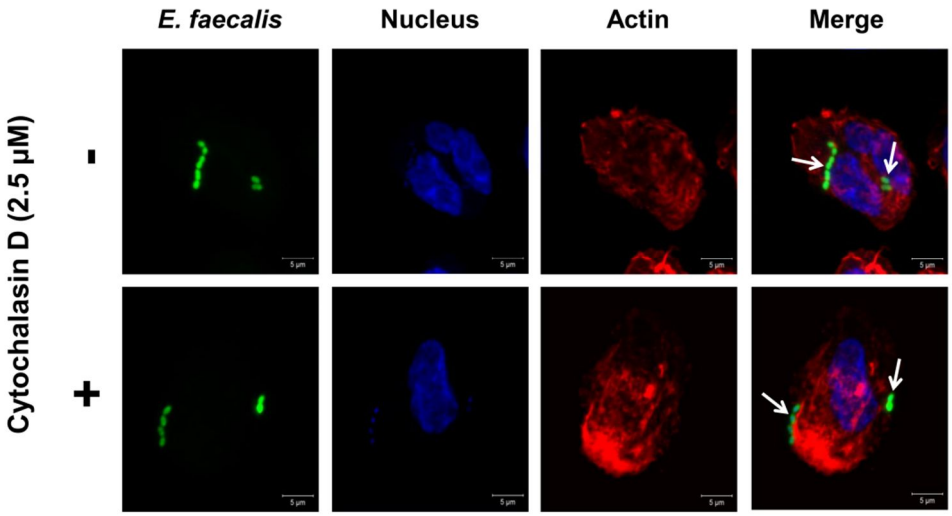

Figure 7. Cytochalasin D inhibits internalization of *E. faecalis*. THP-1 cells were pre-treated with cytochalasin D (2.5 μM) or RPMI 1640 medium before infection of CFSE-labeled *E. faecalis* MOI 50 infection for 6 hours. *E. faecalis* was stained by CFSE (10 μM) and THP-1 cells were stained with Hoechst (10 μM) and rhodamine-phalloidin (5 μg/ml). The cells were observed using a confocal laser scanning microscope. The arrow in the upper panel indicates the internalized *E. faecalis* and the arrow in the lower panel points the adhered *E. faecalis*. Original magnification x 2000

To quantify *E. faecalis*-internalized THP-1 cells, the *E. faecalis*-treated THP-1 cells were analyzed by flow cytometry. The percentage of *E. faecalis*-adhered and internalized cells decreased in a dose-dependent manner due to cytochalasin D (Fig. 8A). CFSE fluorescence of extracellular *E. faecalis* was quenched by trypan blue. The percentage of *E. faecalis*-internalized cells also significantly decreased due to cytochalasin D (Fig. 8B),

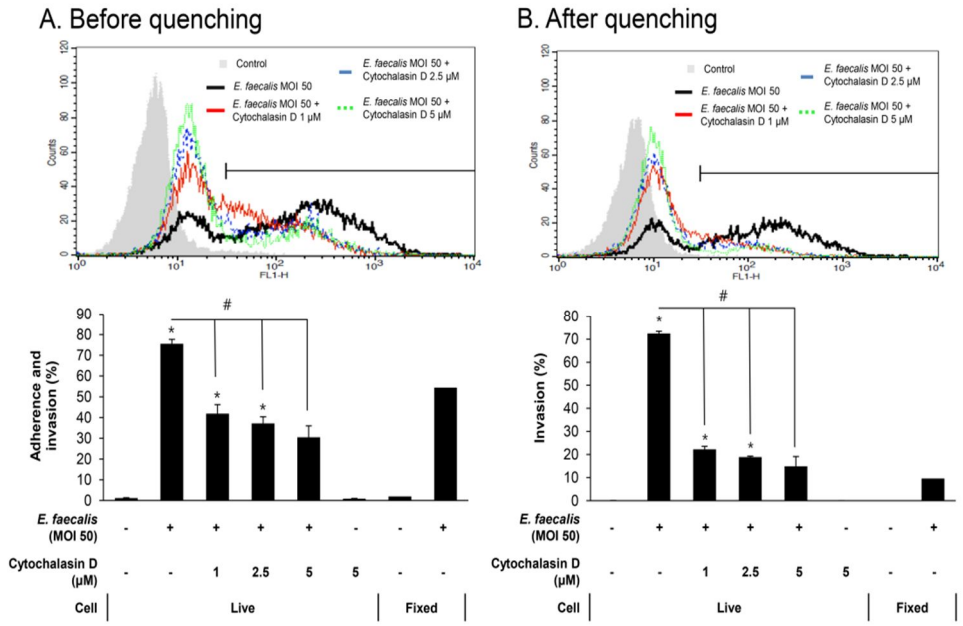

Figure 8. Adhesion and internalization of *E. faecalis* is blocked by cytochalasin D. THP-1 cells were pre-treated with cytochalasin D or RPMI 1640 medium for 1 hour before infection with CFSE-labeled live *E. faecalis* for 6 hours. (A) Adhesion of *E. faecalis* was analyzed by flow cytometry. (B) Internalization of *E. faecalis* was analyzed by flow cytometry after the CFSE fluorescence of non-internalized *E. faecalis* was quenched by 0.4% trypan blue. Fixed THP-1 cells that were infected with CFSE-labeled *E. faecalis* were used as a negative control. * $p < 0.05$ compared to control; $^{\#}$ $p < 0.05$ compared to *E. faecalis*-infected group.

3.6. Inflammasome Activation Is not Dependent on Internalization of *E. faecalis*

To determine whether the internalization ability of *E. faecalis* affects inflammasome activation, THP-1 cells were treated with cytochalasin D prior to *E. faecalis* infection for 1, 3, and 6 hours. The supernatants and cell lysates were collected separately and subjected to immunoblotting. Although *E. faecalis* internalization was inhibited by cytochalasin D (Fig. 7 and 8), caspase-1 activation, pro-IL-1β expression, and IL-1β secretion were not inhibited by cytochalasin D (Fig. 9). These results indicate that *E. faecalis* internalization is not required for *E. faecalis*-induced inflammasome activation.

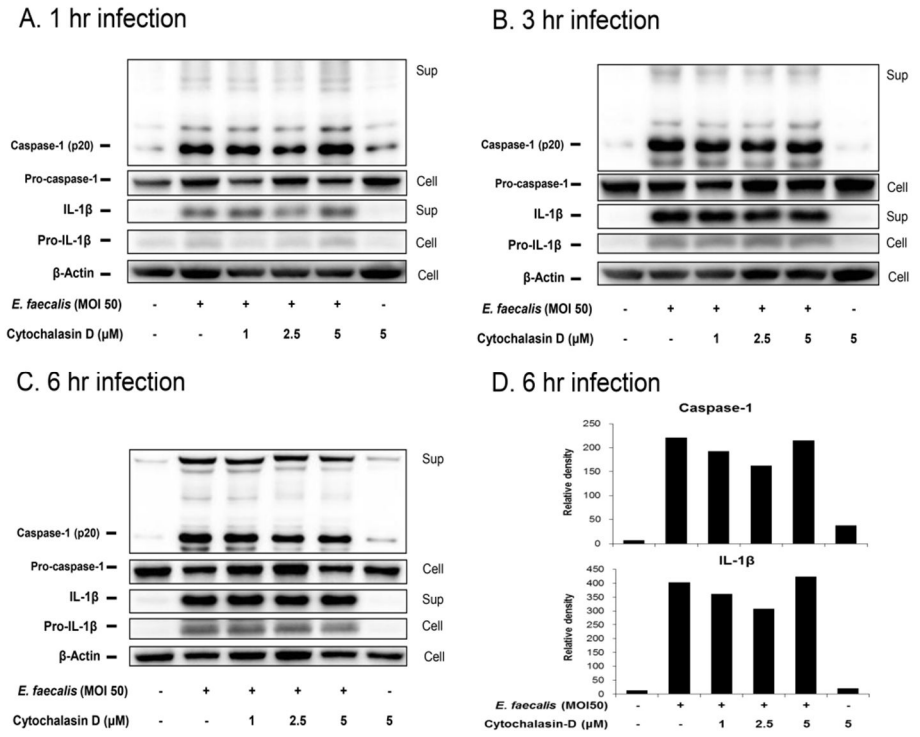

Figure 9. Inflammasome activation is not inhibited by blocking the *E. faecalis* internalization. THP-1 cells were pretreated with cytochalasin D for 1 hour before infection with *E. faecalis* at an MOI of 50 for 1, 3, and 6 hours. (A-C) Caspase-1 and IL-1β in the culture supernatants (sup) and pro-caspase-1, pro-IL-1β, and β-actin in the cell lysates (cell) were detected via immunoblotting. (D) Relative density of each protein compared to β-actin was analyzed by Image J software.

3.7. *E. faecalis* Activates NF-κB and MAP kinase Pathway in THP-1 cells

To determine the signaling pathways that are involved in pro-IL-1β expression by *E. faecalis*, THP-1 macrophages were stimulated with live *E. faecalis* or Pam3CSK4 for the indicated amount of time. After each cell lysate was obtained separately, IκB-α degradation and MAP kinases phosphorylation were determined by immunoblotting using specific antibodies. *E. faecalis* induced phosphorylation of p38, ERK, and JNK as well as degradation of IκB-α in THP-1 macrophages, in a time-dependent manner (Fig. 10A). The induction of pro-IL-1β expression in *E. faecalis*-infected THP-1 cells was inhibited by pretreatment of NF-κB (TPCK) and MAP kinase signaling inhibitors, including p38 (SB 203580), JNK (SP 600125), and ERK (PD 98059) (Fig. 10B). These results indicate that *E. faecalis* induces pro-IL-1β expression through the NF-κB and MAP kinase signaling pathways in THP-1 macrophages.

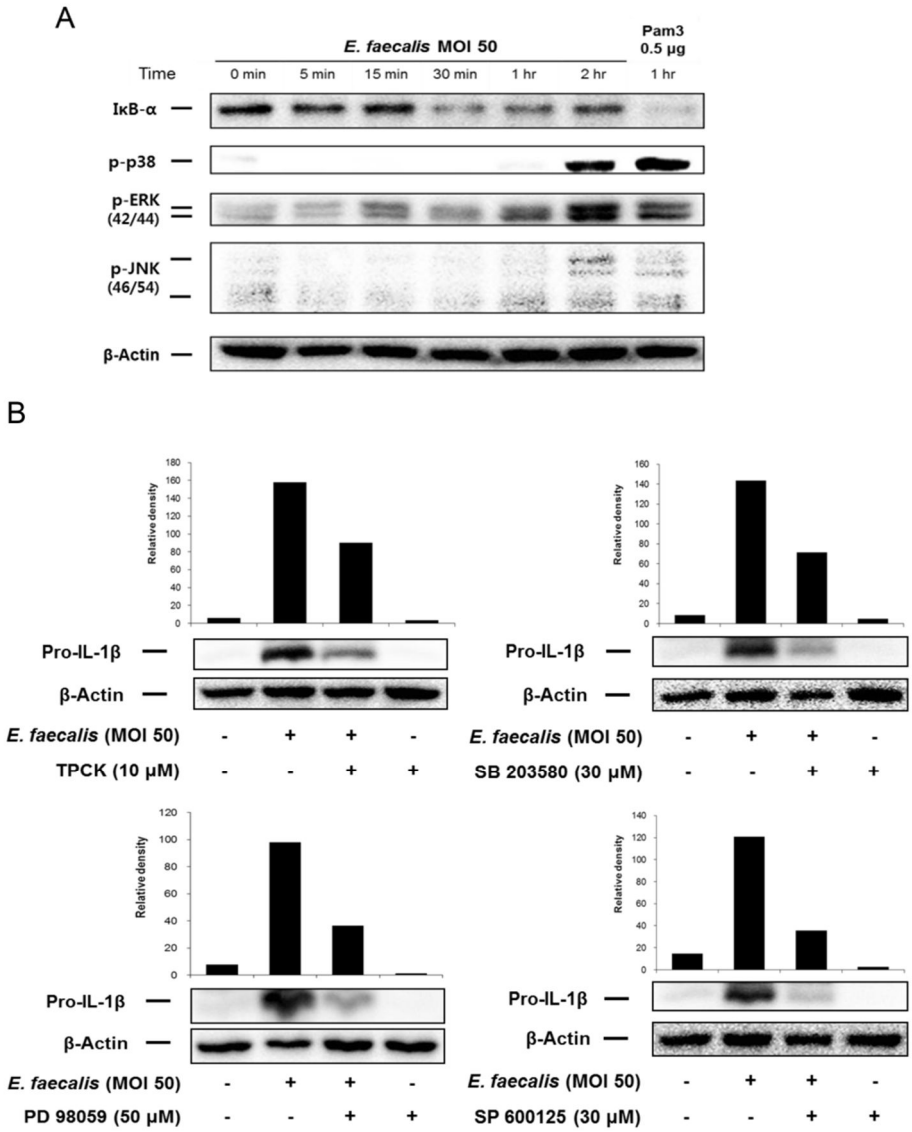

Figure 10. NF-κB and MAP kinase signaling pathways are involved in *E. faecalis*-induced pro-IL-1β expression in THP-1 cells. (A) THP-1 cells were infected with live *E. faecalis* for the indicated time. *E. faecalis*-infected THP-1 cell lysates were analyzed by immunoblotting for IκB-α degradation and phosphorylation of p38,

ERK, and JNK. As a positive control, a synthetic bacterial lipopeptide called Pam3CSK4 was used (49-51). (B) THP-1 cells were pre-treated with signaling inhibitors for 30 min before being infected with *E. faecalis* for 6 hours. Pro-IL-1β and β-actin in the cell lysates were detected by immunoblotting and relative density was analyzed by Image J software.

4. Discussion

Inflammatory responses in the host protect cells from microbial attack. However, if the inflammatory response exceeds the protective response, it may be destructive to surrounding tissues and cause inflammatory diseases. Endodontic infection is an inflammatory disease ranging from being asymptomatic to life-threatening (52). It is caused by polymicrobial infection-induced inflammation. Endodontic infection is classified into three types. In the primary intraradicular infection, both Gram-negative and Gram-positive bacteria are detected as initiators of early invasion and colonization. However, in the secondary and persistent intraradicular infection, fewer bacteria are detected than in primary intraradicular infection, and Gram-positive facultative and more anaerobic bacteria are predominantly detected than Gram-negative and aerobic bacteria (23). As *E. faecalis* is resistant to antimicrobial agents and can survive in harsh environments using several virulence factors, it is abundantly found in secondary and persistent intraradicular infection (23, 53).

Pulp cells are composed of fibroblasts, undifferentiated mesenchymal cells, odontoblasts, and other cell types including macrophages, lymphocytes, and dendritic cells (54, 55). All these cells participate in the inflammatory host defense response. Chronic inflammation persists in diseased pulp because of mechanical insults

or bacterial invasion. Chronic pulp inflammation is characterized by the presence of high or increased numbers of macrophages, lymphocytes, and plasma cells (55).

This study demonstrated that *E. faecalis* induces caspase-1 activation, pro-IL-1β expression, and secretion of active IL-1β. IL-1β plays a critical role in host defense against bacterial infection. IL-1β is tightly controlled by separate regulation of pro-IL-1β expression and its processing to a mature form (45). THP-1 cells, which can be differentiated into macrophages by PMA, have been widely used to study the separate regulation of IL-1β expression and secretion (45, 56). PMA-differentiated THP-1 macrophages were used to investigate the ability of *E. faecalis* to induce both caspase-1 activation and IL-1β production. Simultaneous stimulation of these two steps can lead to overproduction of IL-1β and cause exaggerated inflammation and proinflammatory cell death, a process known as pyroptosis. IL-1β secretion and pyroptosis by *E. faecalis* were inhibited by caspase-1 inhibitors. These results indicate that *E. faecalis* induced-IL-1β secretion and pyroptosis depend on *E faecalis* induced-caspase-1 activation.

Increased IL-1β production in inflamed pulp tissue has been detected by immunohistochemical staining and ELISA, and increased IL-1β production has been detected in cultured pulp

fibroblasts treated with *Escherichia coli* lipopolysaccharide (57). *Porphyromonas endodontalis* lipopolysaccharide increases IL-1β mRNA expression and IL-1β secretion from human dental pulp cells without changing the activity of caspase-1 (58). Stimulation with interferon gamma or cytosolic double-strand DNA increases IL-1β production in human dental pulp cells via the AIM2 inflammasome in a time and concentration-dependent manner (59, 60). *E. faecalis* (ATCC 19433) at an MOI of 300 significantly increases IL-1β mRNA expression in RAW264.7 cells, a mouse macrophage cell line (61). Lipoteichoic acid of *E. faecalis* increases IL-1β release in peripheral blood mononuclear cells (62). According to the present study, *E. faecalis*-infected THP-1 macrophages released ATP, which functions as a signal inducer for NLRP3 inflammasome activation. *E. faecalis*-induced caspase-1 activation and IL-1β production was inhibited by the P2X7 receptor antagonist oxATP or NLPR3 knockdown. However, NLRP3 siRNA did not completely inhibit caspase-1 activation. This result may imply other inflammasomes can also be involved in *E. faecalis*-induced caspase-1 activation. The role of inflammasomes other than NLRP3 in caspase-1 activation by *E. faecalis* remains undiscovered.

Caspase-1 activation and IL-1β production are induced by recognition of pathogen-associated molecular patterns or damage-

associated molecular patterns. Some bacteria and bacterial products induce inflammasome activation in the host cytoplasm. Conversely, others activate caspase-1 and IL-1β production via membrane receptors (63-65). Although the internalization abilities of *E. faecalis* have been already reported by other research groups (66), how *E. faecalis* initiates caspase-1 activation and IL-1β secretion has not yet been studied. *E. faecalis* internalized in THP-1 macrophages in a time-dependent manner, and these events were effectively inhibited by the pretreatment of an actin polymerization inhibitor, cytochalasin D. Although *E. faecalis* internalization was inhibited by cytochalasin D, *E. faecalis*-induced caspase-1 activation and IL-1β production were not affected.

Activation of NF-κB signaling pathway and MAP kinase pathway results in increased expression of pro-IL-1β (67, 68). *E. faecalis* infection induced IκB-α degradation and phosphorylation of p38, ERK, and JNK for activation of NF-κB and MAP kinase signaling pathways in THP-1 macrophages.

In summary, *E. faecalis* simultaneously induced pro-IL-1β expression and caspase-1 activation in THP-1 macrophages, resulting in IL-1β secretion. The bacterium also induced pyroptosis, an inflammatory cell death, which was dependent on caspase-1 activation. ATP release was involved in *E. faecalis*-induced caspase-1 activation via the NLRP3 inflammasome.

Activation of NF-κB and MAP kinase signaling pathways was associated with pro-IL-1β expression from *E. faecalis* infection. *E. faecalis* internalization was not essential for inflammasome activation and pro-IL-1β expression in THP-1 macrophages.

5. Conclusion

Finding that caspase-1 is activated by *E. faecalis* improves the understanding of excessive pulp inflammation, which is essential to provide effective treatment strategies. The identification of bacterial components at the molecular level and discovery of mechanisms involved in activation of the inflammasome will provide insights into the pathogenesis of infectious diseases caused by multidrug-resistant *E. faecalis.*

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국문초록

*Enterococcus faecalis*에 의해 대식세포에서 유도되는 caspase-1 활성화와 IL-1β 생성

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1. 목 적

*Enrerococcus faecalis*는 그람 양성균으로 다양한 독성인자를 이용해 여러 질환을 일으키는 것으로 알려져 있다. 인플라마좀은 선천면역계의 구성요소 중 하나이다.

인플라마좀 활성에 대한 최근의 연구는 다양한 염증성 질환과 자가면역 질환의 발병에 초점이 맞춰져 있다. 분비형 IL-1β와 pyroptosis는 선천면역을 구성하는 세포내 단백질 복합체인 인플라마좀에 의해 활성화되는 caspase-1에 의해 활성화된다. 이 연구의 목적은 근관내 감염과 관련되어 있는 *E. faecalis*가 인플라마좀 활성을 유도하는지 확인하는 데 목적이 있다.

2. 방 법

THP-1 대식세포에 살아있는 *E. faecalis*를 처리하였다. Caspase-1 활성, pro-IL-1β 발현, 그리고 IL-1β 분비를 면역 블로팅, 실시간 역전사효소 중합연쇄반응과 효소결합면역분석법을 수행하여 각각 확인하였다. Pyroptosis를 젖산탈수소효소 분비와 프로피디움 요오드화물 염색을 통해 측정하였으며, 분비된 IL-1β와 pytoptosis를 caspase-1 억제제를 투여한 상태에서 측정하였다. ATP 분비 여부는 ATP 생물발광 기법을 통해 확인하였다. ATP 수용기의 길항제인 oxATP를 처리한 상태에서 면역 블로팅을 통해 *E. faecalis*가 유도하는 인플라마좀 활성화를 확인하였다. NLRP3 인플라마좀이 caspase-1 활성과 IL-1β 생산에 연관되어 있는지 확인하기 위해 NLRP3 siRNA를 수행하였다. *E. faecalis*의 침투가 인플라마좀 활성에 필요한지 확인하기 위해 cytochalasin D를 처리한 상태에서 유세포분석기와 공초점 레이저 현미경을 사용해 분석하였으며, 면역 블로팅을 통해 caspase-1 활성, pro-IL-1β 발현과 IL-1β 생산을 확인하였다. *E. faeclais*가 유도하는 pro-IL-1β 발현에 기여하는 신호 전달경로를 확인하기 위해, NF-κB와 MAPK 신호전달 경로를 면역 블로팅으로 확인하였다.

3. 결 과

*E. faecalis*로 대식세포를 자극했을 때, caspase-1 활성과 pro-IL-1β 발현을 유도함으로써 IL-1β 분비를 일으킨다. *E. faecalis*는 NLRP3 인플라마좀 활성화를 일으키는 ATP의 분비를 증가시키고, oxATP에 의해 caspase-1 활성이 억제된다. *E. faecalis*는 pyroptosis의 특징인 젖산탈수소효소 분비와 프로피티움 요요드화물 흡수를 증가시킨다. *E. faecalis*가 유도하는 caspase-1 활성과 IL-1β 분비는 NLRP3 결핍에 의해 감소되었다. *E. faecalis*는 대식세포에 침투할 수 있으나, cytochalasin D에 의해 억제된 *E. faecalis*의 침투는 caspase-1 활성, pro-IL-1β 발현과 IL-1β 생산에 영향을 주지 않았다. *E. faecalis*가 유도하는 pro-IL-1β의 발현은 NF-κB와 MAPK 활성을 통해 진행되었다. 이 결과를 통해 *E. faecalis*는 과도한 IL-1β의 분비와 세포사멸을 통해 근관내 감염에 기여할 수 있을 것으로 여겨진다.

주요어 : caspase-1, *Enterococcus faecalis*, 인플라마좀, interleukin-1 beta, pyroptosis

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