이학박사 학위논문

Role of *Porphyromonas gingivalis* Gingipains in Caspase-1 Activation and Phagocytosis of *Tannerella forsythia*

Caspase-1 활성화와 *Tannerella forsythia*의 포식작용에서 *Porphyromonas gingivalis* Gingipain의 역할

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정영정

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by

Young-Jung Jung

Under the supervision of

Professor Bong-Kyu Choi, Ph. D.

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지도교수 최 봉 규

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ABSTRACT

Role of *Porphyromonas gingivalis* Gingipains in Caspase-1 Activation and Phagocytosis of *Tannerella forsythia*

Young-Jung Jung
Program in Immunology and Molecular Microbiology in Dentistry
Department of Dental Science
The Graduate School
Seoul National University

Objectives

In the pathogenesis of periodontitis, *Porphyromonas gingivalis* plays a role as a keystone pathogen that dysregulates host immune responses and results in dysbiosis in oral microbial communities. Arg-gingipains (RgpA and RgpB) and Lys-gingipain (Kgp) are essential to the virulence of *P. gingivalis*. Therefore, gingipains are often considered as therapeutic targets. The aim of this study was to elucidate the roles of gingipains in caspase-1 activation in *P. gingivalis*-infected macrophages and to investigate the roles of gingipains in the modulation by *P. gingivalis* of phagocytosis of *Tannerella forsythia* by macrophages.

Methods

Macrophages differentiated from THP-1 cells with phorbol-12-myristate-13-acetate (PMA) and peripheral blood mononuclear cells (PBMC)-derived macrophages

were infected with *P. gingivalis* or its gingipain mutants for 6 hours to determine whether *P. gingivalis* activates caspase-1 and whether gingipain mutation affects the caspase-1 activation. Caspase-1, interleukin (IL)-1β, and lactate dehydrogenase (LDH) in the culture supernatants were analyzed with immunoblot, ELISA, and LDH cytotoxicity assay. To examine the role of gingipain protease activity, macrophages were infected with *P. gingivalis* in the presence or absence of leupeptin, a cysteine protease inhibitor, or *P. gingivalis* preincubated with gingipain-specific inhibitors, KYT-1 and KYT-36. Degradation of proteins that were released from cells upon caspase-1 activation was analyzed after incubation of *P. gingivalis* in the culture supernatants of macrophages that had been stimulated with heat-killed *P. gingivalis*. Intracellular caspase-1 activity and ATP release were measured after infection with *P. gingivalis* and its gingipain mutants, or *P. gingivalis* preincubated with KYT-1 or KYT-36. To assess the effect of processing of surface proteins by gingipains on caspase-1 activation, cells were infected with the gingipain-null mutant that had been cultured overnight with gingipain-containing culture supernatants of *P. gingivalis*.

To determine whether *P. gingivalis* coinfection has an effect on *T. forsythia* phagocytosis, PMA-differentiated THP-1 cells and PBMC-derived macrophages were infected with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled *T. forsythia* in the presence or absence of *P. gingivalis* or its gingipain mutants for 1 hour. Phagocytosis of *T. forsythia* was analyzed by flow cytometry and confocal microscopy. Coaggregation between the two bacterial species was assessed, and the effect of amino acids that inhibit the coaggregation on *T. forsythia* phagocytosis was examined. The role of gingipain protease activity was determined using KYT-1 and KYT-36. Intracellular persistence/survival of *T. forsythia* was

analyzed by flow cytometry, confocal microscopy, and 16S rRNA-based viability assay after antibiotic protection.

Results

Infection with *P. gingivalis* at low multiplicity of infections (MOIs), but not at high MOIs, resulted in low levels of IL-1β and LDH without detectable active caspase-1 in the culture supernatants. The proteins released from caspase-1-activated cells were rapidly degraded by gingipains. However, *P. gingivalis* expressing gingipains induced higher intracellular caspase-1 activity in the infected cells than the gingipain-null mutant. The increased intracellular caspase-1 activity was associated with ATP release from the infected cells. In addition, growing the gingipain-null mutant in the culture supernatants containing gingipains enhanced caspase-1 activation by the mutant. In contrast, inhibition of the protease activity of Kgp or Rgp increased the caspase-1-activating potential of wild-type *P. gingivalis*, indicating an inhibitory effect of the protease activities of Kgp and Rgp.

Phagocytosis of *T. forsythia* was significantly enhanced by coinfection with *P. gingivalis* in an MOI- and gingipain-dependent manner. Mutation of either Kgp or Rgp in the coinfecting *P. gingivalis* resulted in attenuated enhancement of *T. forsythia* phagocytosis. Inhibition of coaggregation between the two bacterial species reduced phagocytosis of *T. forsythia* in the mixed infection, and the coaggregation was dependent on gingipains. Inhibition of gingipain protease activities in coinfecting *P. gingivalis* abated the coaggregation and the enhancement of *T. forsythia* phagocytosis. However, direct effect of protease activities of gingipains on *T. forsythia* seemed to be minimal. Although most of the phagocytosed *T. forsythia* were cleared in infected cells, more *T. forsythia*

remained in cells coinfected with gingipain-expressing *P. gingivalis* than in cells coinfected with the gingipain-null mutant or infected only with *T. forsythia* at 24 and 48 hours post-infection.

Conclusion

Considering that gingipains are major promising therapeutic targets, it is of great importance to fully characterize the roles of gingipains in the pathogenicity of *P. gingivalis*. This study demonstrated that gingipains play contradictory roles in caspase-1 activation by *P. gingivalis* monoinfection, and that gingipains are essential in the augmentation of *T. forsythia* phagocytosis in the mixed infection. This study provides clues to the role of gingipains in the mechanism by which *P. gingivalis* dysregulates host immune responses.

Keywords: *Porphyromonas gingivalis*, Gingipains, Proteases, Caspase-1, *Tannerella forsythia*, Phagocytosis

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Contents

I. Introduction

1. Periodontitis

1.1. Periodontitis: definition, prevalence, and outcomes

Periodontitis is a chronic inflammatory disease caused by complex interactions between bacteria in subgingival biofilms and host immune responses. Its consequence is the destruction of the tooth-supporting tissues, which are comprised of the periodontal ligament, gingival, cementum, and alveolar bone. Periodontitis is one of major causes of tooth loss in adults. Periodontitis, along with dental caries, is the most important oral health burden worldwide. It is one of the most common chronic inflammatory diseases in adult populations, with severe periodontitis found in 5-15% of the global adult population [1]. Children and adolescents can also be affected by several forms of periodontitis including aggressive periodontitis, chronic periodontitis, and periodontitis as a manifestation of systemic diseases [2]. For example, early-onset aggressive periodontitis has been reported to affect 2% of young individuals and causes premature tooth loss [1]. Despite improvement in oral hygiene practices and advances in our understanding of periodontitis pathogenesis over the last few decades, the prevalence of severe periodontitis has not decreased [3,4].

Numerous epidemiological studies have shown the association of periodontal diseases with multiple systemic conditions [5]. On one hand, along with behavioral factors, such as smoking, alcohol consumption, some systemic conditions such as diabetes mellitus, obesity, and metabolic syndrome are risk factors for periodontal diseases [6]. On the other hand, periodontitis is associated with an increased risk for several systemic diseases including cardiovascular diseases, diabetes,

rheumatoid arthritis, and adverse pregnancy outcomes [7]. To explain the association, several hypotheses have been suggested: the common susceptibility model (genetic susceptibility), systemic inflammation model (increased level of circulating cytokines and inflammatory mediators), direct infection model (direct infection of distant tissues like blood vessels by bacterial dissemination), and cross-reactivity/molecular mimicry model (cross-reactivity of T cells and antibodies specific for bacterial antigens with self-antigens) [8].

1.2. Pathogenesis of periodontitis

The most important etiologic factor in the pathogenesis of periodontitis is polymicrobial biofilms in the subgingival crevice although complex interactions of other etiological factors, including systemic health status, genetic factors, and behavioral factors, are involved [6]. The classical study by Socransky et al. [9] categorized numerous bacterial species in subgingival plaque samples into 5 major microbial complexes (red, orange, yellow, green, and purple) of bacteria based on their association with periodontitis. The so-called "red-complex" consortium comprising *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* have been considered as the disease-causing agents of periodontitis. Recent studies using 16S rRNA gene sequencing have suggested that some novel microorganisms such as *Filifactor alocis* and *Synergistetes* species are also associated with periodontitis [10-12].

A recent model for periodontitis pathogenesis suggests that polymicrobial synergy and dysbiosis (PSD), but not individual pathogens, causes periodontal diseases [13,14]. In this model, transition from periodontal health to disease is caused by a shift from symbiosis to dysbiosis in the subgingival microbiota.

Dysbiotic microbiota overactivate the inflammatory responses, resulting in tissue destruction, which further facilitates dysbiosis by providing nutrients and environmental niches to pathogens [15]. The initial shift to dysbiosis is driven by colonization of keystone pathogens and their immune subversion [16]. Keystone pathogens, even at low-abundance, can increase the quantity of the normal microbiota and change the microbial composition in the oral cavity. These changes in turn support and stabilize dysbiosis and cause inflammation. A representative keystone pathogen is *P. gingivalis* [14].

2. *Porphyromonas gingivalis*

2.1. General characteristics of *P. gingivalis*

P. gingivalis is a Gram-negative, asaccharolytic, obligatory anaerobe. Its characteristic black-pigmented colonies on blood agar plates are caused by accumulation of hemin (iron protoporphyrin IX, derived from erythrocytic hemoglobin) on the bacterial surface and within bacterial cells [17]. *P. gingivalis* is detected frequently in subgingival biofilms of periodontitis patients (up to 85% compared to 23% in those of healthy individuals) [18]. Its number is positively correlated with pocket depth, a representative clinical indicator of periodontitis [19]. *P. gingivalis* has been implicated in the development and progression of systemic diseases [20]. For example, its association with cardiovascular disease has been supported by its detection in human atherosclerotic plaques [21] and *in vivo* animal studies [22]. *P. gingivalis* has also been implicated in rheumatoid arthritis via citrullination of host proteins by its peptidylarginine deiminase [23].

2.2. Virulence factors of *P. gingivalis*

P. gingivalis exhibits both immune stimulatory and evasive properties [24], which contribute to the pathogenesis of periodontitis and its associated systemic diseases [16]. Virulence of *P. gingivalis* varies depending on the strains [25-27], and its high pathogenic potential has mainly been ascribed to its ability to evade the host immune system. Key virulence factors (Table 1) of *P. gingivalis* include lipopolysaccharide (LPS), fimbriae, serine phosphatase (SerB), nucleotide diphosphate kinase (NDK), and multiple proteases [28,29]. *P. gingivalis* expresses modified forms of LPS, which suppress Toll-like receptor (TLR)-4 signaling [30] and non-canonical inflammasome activation [22]. Its major fimbriae (FimA) inhibit the TLR2-dependent antimicrobial pathway through the interaction with CXC-chemokine receptor 4 (CXCR4) [31]. *P. gingivalis* utilizes its minor fimbriae (Mfa1) to bind DC-SIGN for entry to and survival within dendritic cells (DC), and subversion of DC functions [32]. SerB dephosphorylates Ser536 residue of p65 subunit of NF-κB, resulting in inhibition of its nuclear translocation and interleukin-8 (IL-8) production in gingivalis epithelial cells [33]. *P. gingivalis* hydrolyzes ATP using its NDK, which inhibits ATP-induced apoptosis of epithelial cells and contributes to its intracellular persistence [34,35].

Table 1. Virulence factors of *P. gingivalis* involved in immune subversion

Virulence factor	Mechanism	References
Lipid A 1- and 4'-phosphatases, lipid A deacylase	Lipid A modifications	[22,30,36]
SerB	Dephosphorylation of NF-kB p65	[33]
Peptidylarginine deiminase	Citrullination of host proteins	[23]
NDK	Hydrolysis of extracellular ATP	[34,35]
FimA fimbriae Mfa1 fimbriae Hemagglutinins	Binding to host cells and tissue : Invasion Dysregulation of host defense responses	[31,32,37]
Gingipains (RgpA, RgpB, Kgp)	Binding to host cells Degradation of host defense proteins (cytokines, chemokines, antibacterial peptides) Complement inactivation Cleavage of cell surface receptors	[38-48]

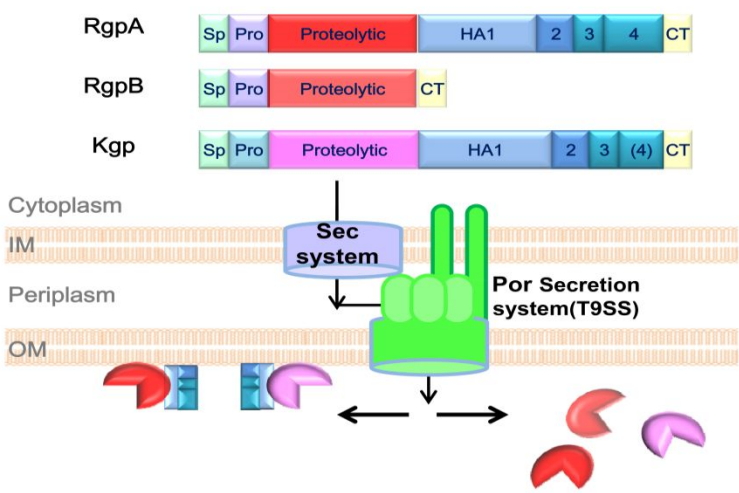
Adapted from Zenobia and Hajishengallis [28]

2.3. Gingipains

2.3.1. Characteristics of gingipains

Because *P. gingivalis* is asaccharolytic and dependent on peptides and proteins as nutrient sources, it produces various endopeptidases and exopeptidases [29]. Among its multiple proteolytic enzymes, cysteine proteases referred to as gingipains [Arg-X-specific Arg gingipains (RgpA and RgpB) and Lys-X-specific Lys gingipain (Kgp)] are responsible for most of the proteolytic activity (at least 85%) of *P. gingivalis* [49]. RgpA and Kgp are multi-domain proteins and contain hemagglutinin/adhesin domains as well as catalytic domains (Fig. 1A) [50,51]. Gingipains can be produced as outer membrane-associated forms or secreted forms via the Por secretion system (Fig. 1A) [52-54]. Gingipains perform diverse functions both in bacteria and during the infection process. They are indispensible not only for nutrient uptake and growth [55] but also for the maturation of other *P. gingivalis* virulence factors such as major and minor fimbriae, periodontain and gingipain themselves (Fig. 1B) [29,56].

A



B

Bacterial proteins processed by Kgp and Rgp		
Pro-Rgp	→	Rgp
HArep	→	HbR
Pro-PrtP	→	PrtP
Pro-PrtT	→	PrtT
Pro-Kgp	→	Kgp
Pro-periodontain	→	Periodontain
Bacterial proteins processed by Rgp		
Pro-fimbrilin	→	Fimbrilin (major fimbriae)
75 kDa OMP precursor	→	75 kDa OMP (minor fimbriae)
Pro-Tpr	→	Tpr
Bacterial protein processed by Kgp		
Pro-Prolyl tripeptidyl-peptidase	→	Ptp

Figure 1. Schematic representation of structures, secretion of gingipains and their role in processing of bacterial proteins [29,51,52,54]**.** IM, inner membrane; OM, outer membrane; SP, signal peptide; Pro, prodomain; HA, hemagglutinin/ adhesion domain; CT, C-terminal domain; HArep, hemagglutinin/adhesin repeats in gingipains, HagA, and TonB-associated adhesin domain; HbR, hemoglobin-binding receptor; OMP, outer membrane protein.

2.3.2. Functions of gingipains in the pathogenesis of periodontitis

Gingipains are essential to the pathogenic potential of *P. gingivalis* [50,55,57]. They are involved in most stages during the establishment of *P. gingivalis* infection, including adhesion, invasion, and survival [58,59]. Gingipains contribute to the bacterial adhesion to host cells through processing other bacterial surface proteins like major and minor fimbriae, as well as the adhesin domains of RgpA and Kgp [38,56]. The pathogenicity of *P. gingivalis* is further facilitated by the disruptive and evasive proteolytic effects of gingipains on host innate immune responses, via dysregulation of cytokine/chemokine networks, complement systems, intracellular signaling, and cell proliferation/death [39-41]. Gingipains degrades complement factors such as C3 [43] as well as proinflammatory cytokines like IL-6 and IL-8 [44], while C5a generated by Arg-gingipains can bind C5aR and inhibits nitric oxide production in macrophages [45]. Gingipains also have several subversive effects on the adaptive immune system. The proteases inactivate some effector cytokines like IL-4 as well as immunoglobulins [42,46,47] and attenuate T-cell proliferation by inhibiting IL-2 accumulation [48]. Thus, gingipains are regarded as promising therapeutic targets for the prevention and treatment of periodontitis and associated systemic diseases.

3. *Tannerella forsythia*

3.1. General characteristics of *T. forsythia*

T. forsythia, another member of the “red complex” consortium, is a Gram-negative, rod-shaped, asaccharolytic, obligatory anaerobe. It is also implicated in an increased risk for systemic inflammation like cardiovascular disease [21,60,61].

Although *T. forsythia* is relatively less characterized than *P. gingivalis* because of its fastidious growth requirements, recent studies have identified several virulence factors that *T. forsythia* utilizes to modulate host immune responses.

3.2. Virulence factors of *T. forsythia*

Representative virulence factors include BspA, surface-layer (S-layer)-associated glycoproteins, lipoproteins, glycosidases, and proteases [29,62]. A cell surface-associated and secreted protein BspA, which binds to TLR-2, mediates binding and entry of *T. forsythia* into oral epithelial cells and leads to IL-8 production [63,64]. It also binds to fibronectin and fibrinogen in the extracellular matrix [65]. *In vivo* studies have shown that BspA is responsible for *T. forsythia*-induced alveolar bone loss and can facilitate atherosclerosis in mice [60,64,66]. The cell surface S-layer contributes to serum resistance of *T. forsythia* [67] as well as adhesion and invasion into oral epithelial cells [68]. It delays inflammatory responses in macrophages [69], and glycosylation of S-layer suppresses the expression of cytokines related to T helper 17 responses in macrophages and DCs [70]. Karilysin, a metalloprotease, has been demonstrated to inhibit all the three complement pathways and to increase soluble TNF-α concentration by cleaving the membrane-bound form of TNF-α [71,72]. *T. forsythia* expresses surface lipoproteins that can induce expression of proinflammatory cytokines. It also expresses several glycosidases that may jeopardize the functional integrity of the periodontium and promote the progression of periodontal inflammation [62].

4. Mixed infection with *P. gingivalis* and *T. forsythia*

4.1. Interspecies bacterial interaction

Previous studies that used traditional culture methods and those that used recent culture-independent methods have identified more than 700 species of bacteria in human oral cavity [73]. With substantial diversity among people, approximately 100-200 species are found in the oral cavity of an individual [73]. Among microbial habitats in the oral cavity, such as gingival sulcus, teeth, tongue, and cheeks as well as carious lesions and endodontic infections, over 400 species of bacteria have been detected in subgingival biofilm [74,75]. Polymicrobial communities are developed through interspecies interactions and adaptation within the surrounding microenvironments. Interspecies bacterial interactions in a polymicrobial community involve communication via direct contact as well as soluble mediators [76]. Outcomes of interbacterial interactions are different from the simple sum of activites observed with each bacterial species [77]. The interactions include neutralism (no effect on each other), competition (for growth-limiting nutrients and physical resources), commensalism (one benefits from the other, but not vice versa), mutualism (synergy in growth or survival), and ammensalism (indirect negative effect, such as production of an antagonist that inhibits the growth of the other). Furthermore, interference or promotion of growth-dependent signaling, or neutralization of virulence factors of one species by another species is also possible [78].

4.2. Synergistic pathogenicity of *P. gingivalis* and *T. forsythia*

P. gingivalis is frequently isolated together with *T. forsythia* as complexes within the microbial communities of active periodontitis lesions [9,10]. In addition,

mixed infection of *T. forsythia* and *P. gingivalis* has been shown to cause synergistic abscess formation and additive alveolar bone loss in murine models [79,80]. This synergism between the two bacterial species can be attributed to several factors. *T. forsythia* stimulates the growth of *P. gingivalis* [81], and *P. gingivalis* promotes the growth of *T. forsythia* in multi-species biofilms [82], indicating their symbiotic relationship. Karilysin of *T. forsythia* and Arg-gingipains of *P. gingivalis* synergistically inactivate complement pathways [72]. In addition, *P. gingivalis* enhances invasion of *T. forsythia* into oral epithelial cells [63]. Although some studies have failed to find synergistic inflammatory responses to mixed infection with *P. gingivalis* and *T. forsythia* [83,84], this combination of bacteria synergistically induces IL-6 production in murine macrophages [85].

5. Macrophages and phagocytosis

5.1. Role of macrophages in periodontitis

Macrophages were first identified as phagocytic cells by Metchnikoff. Along with monocytes and DCs, they belong to the mononuclear phagocytic system, which is derived from committed hematopoietic stem cells in bone marrow [86]. Macrophages are found in all body tissues with great diversity in their phenotypes and functions. They respond to environmental challenges as well as to physiological changes through their diverse receptors including cytokine receptors, pattern recognition receptors, and phagocytic receptors [87]. Macrophages play pivotal roles in host defense, inflammation, and tissue homeostasis. They function as sentinel cells by moving throughout tissues and sensing non-self or damaged tissues, which initiates phagocytosis and clearance by macrophages [88]. Classically activated macrophages (M1) mediate inflammatory responses through

production of pro-inflammatory mediators, such as reactive oxygen and nitrogen species, proinflammatory cytokines, and chemokines. However, depending on the activation conditions, they can switch to anti-inflammatory phenotypes (M2, alternatively activated macrophages) and participate in tissue repair/remodeling and maintenance of homeostasis. Macrophages are also important players in the development of adaptive immunity by activating or suppressing T cells through their role as antigen presenting cells [89].

In a healthy periodontium, it is essential to maintain a controlled immune-inflammatory state in response to normal symbiotic microbiota. In periodontitis, overt inflammation leads to periodontal tissue destruction. Macrophages play complicated roles in the pathogenesis of periodontitis. First of all, macrophages, together with neutrophils, are responsible for phagocytosis of invading microbes and foreign substances, which is essential to limit propagation of infection and inflammation. Because the oral cavity and gingival tissues can be an entry to the inside of the body, clearance of invading pathogens in gingival tissues is important to prevent persistent infection and systemic dissemination of an invading pathogen. They are also involved in resolution of inflammation and maintenance of tissue homeostasis in gingival tissues [90].

Macrophages, on the other hand, can inadvertently contribute to the progression of periodontitis through inflammation and tissue destruction. Infiltration of macrophages in gingival tissues increases in active periodontitis lesions compared to inactive sites or healthy tissues [91,92], and their infiltration increases with the progression of periodontitis [93]. Macrophages are major immune cells that produce inflammatory cytokines such as IL-1 and TNF-α as well as chemokines including MIP-1α and IL-8, which lead to further recruitment and

activation of inflammatory cells [90]. It has been reported that depletion of macrophages reduces alveolar bone loss in a mouse periodontitis model [92], indicating that excessive macrophage infiltration can be detrimental to periodontal health. Macrophages are precursor cells that can be differentiated into osteoclasts in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL). In addition, proinflammatory cytokines such as IL-1 and IL-6 from macrophages can promote osteoclastogenesis [94]. Macrophages contribute to tissue destruction by degrading extracellular matrix and providing attachment sites for osteoclasts by degrading the demineralized organic bone matrix [95].

5.2. Phagocytosis in macrophages

Phagocytosis is one of three major strategies to internalize extracellular substances. Large particles (> 0.5 μm) such as bacteria are engulfed by cells via phagocytosis whereas pinocytosis is a process for internalizing solutes and fluid. Receptor-mediated endocytosis is mainly involved in uptake of macromolecules, small particles, and virus [96]. The mechanism of phagocytosis is remarkably complex. A diverse array of receptors and signaling pathways are involved, and the mechanism shows substantial heterogeneity depending on the features of particles and receptors [97].

The initial step of phagocytosis is particle recognition. Direct recognition of microbes is mediated by receptors that identify ligands on the microbial surfaces, such as mannose receptor (CD206), dectins, scavenger receptors (SR-A, MARCO, and CD36), CD14, and integrins (CD11b/CD18) [96,98]. Alternatively, phagocytes recognize particles that are opsonized with mannose binding proteins, surfactant

protein A, immunoglobulins, or complement (C3b, C4b, and iC3b) via C1q receptor, SPR210, Fc receptors, or complement receptors, respectively [96,98]. Particles with multiple ligands, like bacteria, are recognized by several receptors that can synergize or antagonize each other. Furthermore, non-phagocytic receptors influence activity of phagocytic receptors. For example, activation of TLR-2, 3, 4, and 9 promotes phagocytosis in macrophages via upregulation of scavenger receptors, Fc receptors, and complement receptors [99,100]. In addition, binding of *P. gingivalis* fimbriae to TLR2 transactivates complement receptor CR3 via inside-out signaling [101].

Particle recognition initiates the process for particle internalization: actin rearrangement, membrane extension around the particles, and membrane fusion to form phagosomes. This process involves multiple signaling pathways including protein kinase C, phosphoinositide 3-kinase, phospholipase C, Rho family of GTPase, and motor proteins such as myosin [102]. Phagosomes then fuse with acidic lysosomes to form mature phagolysomes, where microbes are killed and particles are digested by reactive oxygen and nitrogen species, antimicrobial proteins, and hydrolytic enzymes [103].

Phagocytosis is a major defense strategy against microorganisms that breach the epithelial barrier and is connected to inflammatory responses. Recognition of the specific nature of pathogens is facilitated after phagolysomal fusion via pattern recognition receptors located in the lysosomal compartment. This enables macrophages to exhibit immune responses tailored to specific pathogens [97]. For instance, differential signal transduction pathways are activated by engagement of different combination of TLRs, resulting in different inflammatory responses. After phagocytic killing of microbes, MHC class II molecules are loaded with peptides

derived from microbes. The MHC/peptide complexes are then presented to cognate T cells in the presence of cytokines that macrophages have produced. This antigen presentation process guides adaptive immunity [89]. Engulfment of apoptotic bodies, called efferocytosis, contributes to resolution of inflammation through induction of anti-inflammatory cytokines, such as TGF-β and IL-10 [104-106]. Efferocytosis also facilitates clearance of some microbes such as *Mycobacterium tuberculosis* and *Streptococcus pneumonia* [107,108]. However, some pathogens have developed strategies to survive within macrophages through escaping phagosomes (*Listeria monocytogenes* and *Shigella flexneri*), inhibiting phagosomal maturation (*Mycobacterium spp*. and *Salmonella spp*.), survival in phagolysosome (*Legionella pneumophila*, *Leishmania spp. (amastigotes)*, and *Coxiella burnetii*), or inhibiting cell death (*Toxoplasma gondii*) [109]. In these circumstances, macrophages provide intracellular niches, where the pathogens are protected from antibodies or antimicrobial effectors, and can promote dissemination of the pathogens [110].

6. Inflammasome

6.1. Inflammasome and caspase-1 activation

Caspase-1, along with caspase-4 and caspase-5, belongs to the inflammatory caspase subfamily, which plays a central role in the regulation of inflammation and host defenses against microbial infection [111]. Like other caspases, caspase-1 is produced as a zymogen, pro-caspase-1, consisting of a caspase recruitment domain (CARD domain), a large subunit (p20), and a small subunit (p10) (Fig. 2) [112]. Caspase-1 becomes proteolytically active by forming two heterodimers of p20 and p10 after it is recruited to intracellular multiprotein complexes, called the

inflammasomes [113].

Inflammasome assembly results from the activation of intracellular innate immune sensors that include several members of the NOD-like receptor (NLR) family (NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, NAIP1/2/5/6, and NLRC4) and PYHIN family (AIM2, IFI16, and PYRIN), by specific pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Fig. 2A and B, Table 2) [114,115]. In most cases, the adaptor ASC (apoptosis-associated speck-like protein with a caspase-recruitment domain) is needed, and ASC proteins assemble to form large protein aggregates or specks [116]. Noncanonical activation of caspase-1 involves caspase-11 or human caspase-4 binding/activation by cytosolic LPS, which leads to assembly of NLRP3 inflammasome and activation of caspase-1 (Fig. 2C) [117,118]. In addition, caspase-8-dependent activation of caspase-1 by *Yersinia pestis* and its virulence factor YopJalso has been reported, and the activation is independent of inflammasome components (Fig. 2D) [119,120].

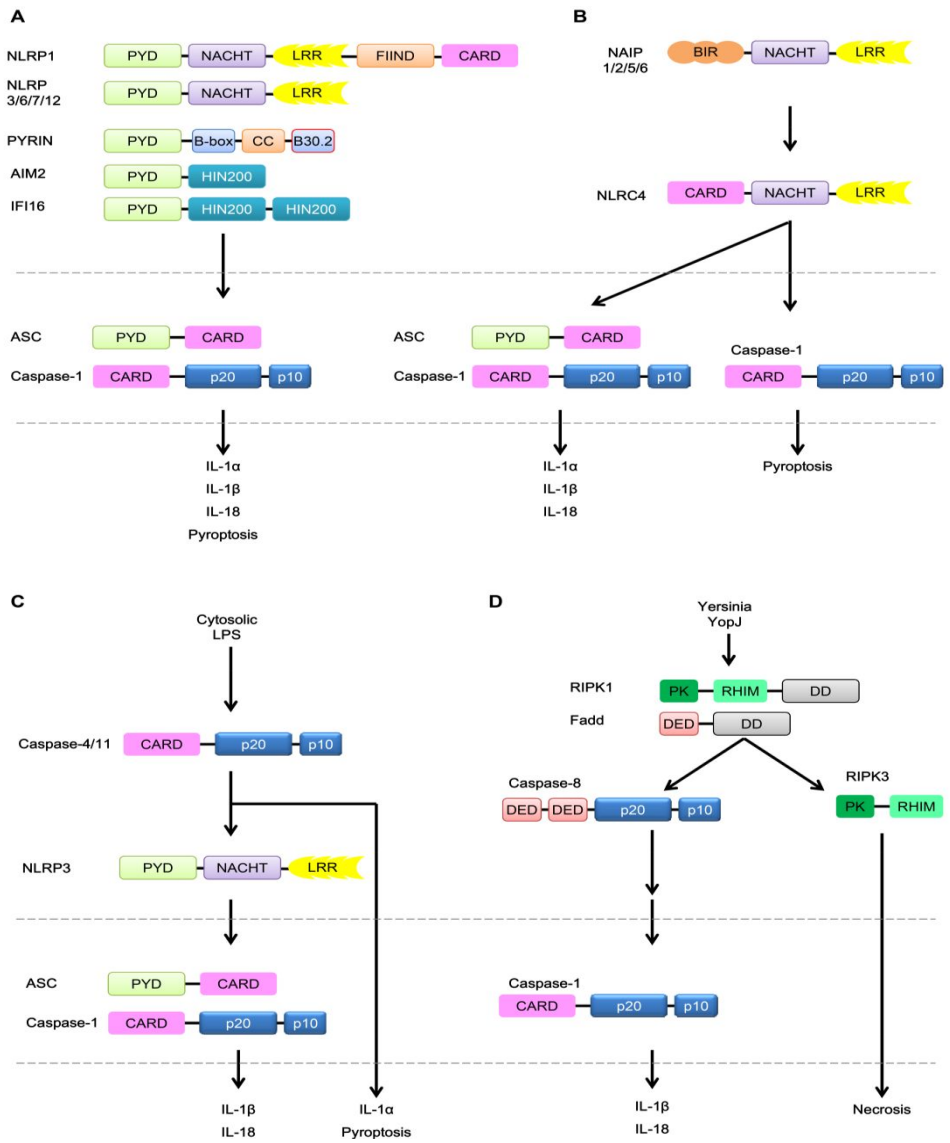


Figure 2. Canonical and non-canonical activation of caspase-1 [115,118]**.** BIR, baculovirus inhibitor of apoptosis domain; CARD, caspase recruitment domain; DD, death domain; DED, death effector domain; HIN200, hematopoietic interferon-inducible nuclear proteins with a 200 amino acid repeat; LRR, leucine rich repeat; NACHT, NAIP, CIITA, HETE and TP1 domain; PK, protein kinase; PYD, pyrin domain; RHIM, Receptor-interacting protein (RIP) homotypic interaction domain.

Table 2. Inflammasome activation by bacterial infection [115,121,122]

Inflammasome		Signaling mechanism	Ligands/stimulants
NLRP1		Proteolytic cleavage	*Anthrax* lethal toxin
NLRP3		K+ efflux, lysosomal destabilization, ROS	Pore forming toxins: *V. cholera* (Hyl), EHEC, *S. aureus* (hemolysin), *L. monocytogenes* (LLO), *S. pneumoniae* (pneumolysin) Extracellular ATP (via P_2X_7 receptor) Complement *T. denticola* (Td92), *F. nucleatum*, *P. gingivalis*
		Caspase-4/11	Cytosolic LPS: *Burkholderia*, *Haemophilus*, *Proteus*, *Vibrio*
NLRP6			Commensal microbiota
NLRP7			Acyl lipopeptide: *L. monocytogenes*, *S. aureus*, Mycoplasma
NLRP12			*Y. pestis*
NAIP1	NLRC4		T3SS needle proteins: *Chromobacterium violaceum* (Cprl), *Salmonella* Typhimurium (Prgl), EHEC (E. prl), *B. thailandensis* (BsaL), *S. flexneri* (Mxil), *P. aeruginosa* (PscF)
NAIP2			T3SS rod proteins: *Salmonella* Typhimurium (PrgJ), *B. thailandensis* (BsaK)
NAIP5/6			Flagellins: *Legionella pneumophila* (FlaA), *Salmonella* Typhimurium (FliC), *Y. pestis* (FliC2), *Photorhabdus luminescence* (Fla), *P. aeruginosa* (FliC)
AIM2			Cytosolic DNA: *Francisella tularensis*, *F. novocida,* *L. monocytogenes*, *P. gingivalis* *L. pneumophila* Δ*sdhA*
IFI16			Non-bacterial cytosolic DNA: Kasposi's sarcoma-associated herpes virus
PYRIN		Rho modification	*Clostridium botulinum* (C3), *C. difficile* (TcdB), *Histophilus somni* (lbpA), *V. parahemolyticus* (VopS)
?		RIPK1, RIPK3, Fadd, caspase-8	*Y. enterocolitica*, *Y. pestis* (YopJ)

6.2. Consequences of caspase-1 activation

Major events after caspase-1 activation include (1) processing of biologically inactive pro-IL-1β (31 kDa) and pro-IL-18 (24 kDa) to the bioactive IL-1β (17 kDa) and IL-18 (18 kDa), (2) secretion of IL-1β, IL-18, and IL-1α (both pro-form and mature form), and (3) pyroptosis.

IL-1β is a the major proinflammatory cytokine, and its activity is tightly controlled at multiple levels because of its pleiotropic functions in innate and adaptive immunity, metabolism, and bone homeostasis as well as inflammation [123]. Its transcription and translation is induced by the binding of PAMPs or DAMPs to pattern recognition receptors (PRRs). Then, the maturation of the pro-form into the bioactive IL-1β and its secretion is executed by active caspase-1 [124].

Unlike IL-1β, pro-IL-18 is constitutively expressed in many cell types including monocytes and keratinocytes [125,126]. IL-18, in synergy with IL-12, induces IFN-γ, skewing helper T cell polarization toward Th1 cells [127]. Alternatively, stimulation of γδ T cells and CD4 T cells with IL-18 in the presence of IL-23 increases IL-17 production [128]. Pro-IL-1α is also constitutively expressed in nonhematopoietic cells while the expression is inducible in mononuclear phagocytes [129]. Although cleavage of pro-IL-1α to its mature protein is executed by calpain [130], both pro-IL-1α and IL-1α can be secreted upon caspase-1 activation or cell death, and they bind to IL-1 receptor 1 (IL-1R1) [131].

An additional consequence of caspase-1 is proinflammatory cell death, known as pyroptosis. Pyroptosis serves as an efficient bacterial clearance mechanism by preventing the intracellular replication of pathogens, releasing them

from infected cells and exposing them to killing by neutrophils [132]. Proinflammatory cytoplasmic contents released from dying cells and endogenous danger signals (such as HGMB1) secreted from caspase-1-activated cells alert and recruit other immune cells [133,134]. Therefore, caspase-1 plays an important role in eliminating the intracellular niche of pathogens in vivo.

6.3. Inflammasome activation by *P. gingivalis*

A few previous studies have shown that *P. gingivalis* activates caspase-1 or inflammasomes [121,135,136]. Taxman et al. [135] reported that *P. gingivalis* induces expression and secretion of IL-1β in an ASC- and caspase-1-dependent manner in human monocytes. It also has been reported that *P. gingivalis* and its LPS induce NLRP3- and ASC-dependent cell death in macrophages, which was not dependent on caspase-1 [136]. In addition, Park et al. [121] have shown that *P. gingivalis* infection of macrophages activates AIM2 and NLRP3 inflammasomes, leading to caspase-1 activation and pyroptosis and that ATP release, potassium efflux, and cathepsin B (a lysosomal enzyme) are involved in the activation of NLRP3 inflammasomes.

Some previous studies, however, have shown that *P. gingivalis* fails to activate capase-1 activation [22,122,137]. A previous study reported that in macrophages, *P. gingivalis* does not activate caspase-1 and that it also blocks activation of NLRP3 inflammasomes by other inducers like *Fusobacterium nucleatum*, monosodium urate, and alum, through inhibiting their endocytosis [122]. Another study showed that *P. gingivalis* inhibits ATP-mediated NLRP3 inflammasome and caspase-1 activation in gingival epithelial cells by hydrolyzing ATP using its NDK [137]. In addition, *P. gingivalis* infection does not induce non-canonical caspase-1 activation

by cytosolic LPS because of its modified, immune-evasive LPS [22].

7. Aims of this study

Recent studies on inflammasome and caspase-1 activation by *P. gingivalis* have shown inconsistent results [22,121,122,135-137], and the role of gingipains in caspase-1 activation by *P. gingivalis* infection has not yet been investigated. Because *P. gingivalis* and *T. forsythia* are frequently isolated together in periodontitis lesions [9,10], *P. gingivalis* may have an effect on phagocytosis and clearance of *T. forsythia*. The aims of this study were as follows:

1) To elucidate the role of gingipains in caspase-1 activation by *P. gingivalis* in macrophages

2) To investigate whether *P. gingivalis*, in particular via its gingipains, influences phagocytosis of *T. forsythia* by macrophages

II. Materials and Methods

1. Bacterial strains and growth conditions

P. gingivalis (ATCC 33277, FDC 381, ATCC 49417, W50) and *F. nucleatum* ATCC 25586 were grown in brain heart infusion (BHI) broth supplemented with hemin (5 μg/mL) and vitamin K (1 μg/mL) under anaerobic conditions (10% CO_2, 10% H_2, and 80% N_2) at 37°C for 1 day. *T. forsythia* ATCC 43037 was grown in new oral spirochete broth (ATCC medium 1494) supplemented with vitamin K (0.02 μg/ml) and N-acetylmuramic acid (0.01 μg/ml) under the anaerobic condition at 37°C for 2 days.

Gingipain mutants of *P. gingivalis* ATCC 33277 [KDP129 (*kgp*$^-$), KDP133 (*rgpA*$^-$ *rgpB*$^-$), and KDP136 (*kgp*$^-$ *rgpA*$^-$ *rgpB*$^-$)] were kindly provided by Dr Koji Nakayama of Nagasaki University, Nagasaki, Japan. The mutant strains were maintained in enriched BHI broth (37 g of BHI, 5 g of yeast extract, and 1 g of cysteine per liter) supplemented with hemin, vitamin K, and the following antibiotics as described previously [55]: chloramphenicol (20 μg/ml), erythromycin (10 μg/ml), and tetracycline (0.7 μg/ml).

Where indicated, overnight cultures of *P. gingivalis* ATCC 33277 were washed with phosphate buffered saline (PBS) and preincubated in PBS containing 5 mM leupeptin (Sigma, St. Louis, MO, USA), 10 μM KYT-1 (Carbobenzoxy-Lys-Arg-CO-Lys-N-$(CH_3)_2$, Peptide Institute, Osaka, Japan) or KYT-36 (Carbobenzoxy-Glu(NHN(CH_3)Ph)-Lys-CO-NHCH$_2$Ph, Peptide Institute) under the anaerobic condition at 37°C for 1 hour. Then, the bacteria were washed with PBS and used for infection.

2. Treatment of *P. gingivalis* gingipain-null mutant with membrane vesicle-depleted culture supernatants

For the experiments to examine the effect of processing by gingipains of surface proteins in *P. gingivalis* KDP136, membrane vesicle-depleted culture supernatants (VDS) of *P. gingivalis* ATCC 33277 and its gingipain mutant strains were prepared as described previously [58]. Culture supernatants of *P. gingivalis* after overnight culture were collected by centrifugation at 10,000 x g for 20 minutes at 4°C, followed by filtration through 0.2-μm-pore-size filter (Minisart, Sartorius Stedim Biotech GmbH, Goettingen, Germany). The culture supernatants were depleted of membrane vesicles by ultracentrifugation at 100,000 x g for 50 minutes at 4°C, and the obtained VDS were kept frozen at -80°C until use.

The gingipain-null mutant KDP136 was cultured overnight in fresh culture medium containing 30% VDS of wild-type *P. gingivalis* or its gingipain mutants in the presence or absence of 10 μM KYT-1 or KYT-36. Because VDS of *P. gingivalis* contain several bacterial components as well as gingipains, gingipain-specific inhibitors KYT-1 and KYT-36 were used to determine whether the effect of VDS was attributable to gingipains. The overnight cultures of KDP136 were washed with PBS and used for infection.

3. Cell cultures

THP-1 cells (ATCC TIB-202), a human monocytic cell line, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Paisley, UK), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL) in a humidified 5% CO_2 atmosphere at 37°C.

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Paque (GE healthcare, Uppsala, Sweden) density gradient centrifugation from whole blood donated by healthy volunteers with the approval of Institutional Review Board of Seoul National University (IRB No. S-D20130004). The cells were then cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin for 6 days. After adherent cells were detached using trypsin/EDTA (Gibco BRL), the cells were seeded into 6-well plates (2 x 10^6 cells/well) and cultivated for 24 hours before infection to investigate caspase-1 activation.

To investigate phagocytosis in primary macrophages, $CD14^+$ monocytes were isolated from PBMCs by positive selection using anti-human CD14 magnet particles with the BD IMag™ cell separation magnet (BD Biosciences, San Jose, CA, USA). To differentiate them into macrophages, the cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in the presence of 10 μg/ml macrophage colony-stimulating factor (Peprotech, Princeton, NJ, USA) for 6 days. On the day before the phagocytosis assay, adherent cells were detached using trypsin/EDTA, seeded into 24-well plates (5 x 10^5 cells/well), and further cultivated for 24 hours.

4. Infection for caspase-1 activation

THP-1 cells (2 x 10^6 cells/well in six-well plates) were differentiated into macrophage-like cells with 0.5 μM PMA for 3 hours. The differentiated cells or PBMC-derived macrophages were stimulated with the indicated MOI of live *P. gingivalis*, heat-killed *P. gingivalis* (95°C, 60 minutes), live *F. nucleatum*, or 0.5 μg/ml Pam3CSK4 (InvivoGen, San Diego, CA, USA) for 6 hours unless otherwise

stated. For inhibition of *P. gingivalis* proteases during infection, wild-type *P. gingivalis* was preincubated in 0.2 or 5 mM leupeptin (Sigma, St. Louis, MO, USA)-containing PBS for 30 minutes, and THP-1 cells were infected with *P. gingivalis* in the presence of leupeptin at the indicated concentration. In experiments to examine the effects of caspase-1 inhibition, the cells were pre-treated with the caspase-1 inhibitor Z-YVAD-FMK (Biovision, Palo Alto, CA, USA) or Ac-YVAD-CHO (Calbiochem, San Diego, CA, USA) at the indicated concentration for 30 minutes before stimulation with live or heat-killed *P. gingivalis* for 6 hours. Where indicated, THP-1 cells were preincubated with 300 μM oxATP (Sigma), a P_2X_7 receptor antagonist, for 30 minutes and then infected with *P. gingivalis* for 6 hours.

5. Immunoblotting

After treatment of macrophages with *P. gingivalis* or other stimuli, the culture supernatants of the cells were collected, and the proteins in the culture supernatants were precipitated with 10% trichloroacetic acid (TCA, Sigma). After centrifugation at 16,000 x g for 10 minutes at 4°C, the protein pellets were neutralized using 0.1 M NaOH. The cells were washed once with ice-cold DPBS and lysed with RIPA buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM EDTA, 5 μM Na_3VO_4, 1 mM PMSF, and 1 x protease inhibitor cocktail (Roche, Manheim, Germany)] on ice with intermittent vortexing. The lysates were clarified by centrifugation at 16,000 x g for 45 minutes at 4°C. The protein concentration of the lysates was determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). The TCA-precipitated supernatants and the cell lysates were mixed with sample buffer (supplemented with dithiothreitol) and heated at 95°C for 5

minutes for protein denaturation. The proteins were separated by SDS-PAGE (12%) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Blots were incubated for 1 hour with blocking solution (Tris-buffered saline [TBS] containing 0.1% Tween 20 and 5% bovine serum albumin or nonfat dried milk) and were probed with the following primary antibodies: rabbit polyclonal anti-caspase-1 from Cell Signaling Technology (Beverly, MA, USA), goat polyclonal anti-IL-1β from Santa Cruz Biotechnology (Santa Cruz, CA, USA), mouse monoclonal anti-actin from BD Biosciences, rabbit polyclonal anti-HMGB1 from Millipore, and mouse monoclonal anti-NLRP3 and rabbit polyclonal anti-ASC from AdipoGen (Liestal, Switzerland). The binding of the primary antibodies was visualized using horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, anti-goat IgG, and anti-mouse IgG secondary antibodies (R&D systems, Minneapolis, MN, USA) and ECL Western blotting substrate (SUPEX, Dyne-Bio, Sungnam, Korea). β-Actin was used as a loading control.

6. ELISA

The concentration of IL-1β and IL-8 in the culture supernatants was measured using the Quantikine sandwich ELISA Kit (R&D Systems) according to the manufacturer's instructions.

7. Real-time reverse-transcription PCR

Total RNA from THP-1 cells was isolated with an easy-BLUE^{TM} total extraction kit (iNtRON Biotechnology, Sungnam, Korea), and cDNA was synthesized using an M-MLV Reverse Transcriptase kit (Promega, Madison, WI, USA) and oligo dT primer (Cosmo GENETECH, Seoul, Korea). For real-time RT-

PCR, cDNA was mixed with primer pairs (200 nM each) and 10 μl of 2X SYBR Premix Ex Taq^{TM} (Takara Bio Inc., Otsu, Shiga, Japan) in a 20 μl reaction volume. Following initial denaturation at 95°C for 5 minutes, cDNA was amplified for 40 cycles of denaturation (95°C, 15 sec), annealing (60°C, 15 sec), and extension (72°C, 33 sec) in an ABI PRISM 7500 Fast Real-Time PCR System (Applied biosystems, Foster City, CA, USA). Melting curve analysis of the PCR products was performed to ensure specificity of PCR amplification. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping control. The gene expression level was normalized against GAPDH and calculated by $2^{-\Delta\Delta CT}$ method. The sequences of the forward and reverse primers (Cosmo GENETECH) used for the real-time PCR were 5'-AGCTGTACCCAGAGAGTCC-3' and 5'-ACCAAATGTGGCCGTGGTTT-3', respectively for IL-1β, and 5'-GTGGTGGACCTGACCTGC-3' and 5'-TGAGCTTGACAAAGTGGTCG-3', respectively for GAPDH.

8. Measurement of cell death

8.1. LDH cytotoxicity assay

An increased lactate dehydrogenase (LDH) level in culture supernatant is a widely used marker for cell death because this cytoplasmic enzyme is rapidly released into extracellular space upon damage of the plasma membrane. THP-1 cells (1 x 10^5 cells/100 μl in a 96-well plate) were differentiated and stimulated with live *P. gingivalis*, live *F. nucleatum*, or heat-killed *P. gingivalis* at the indicated MOI for 6 hours. The LDH levels in the culture supernatants of the THP-1 cells in triplicate wells were measured using an LDH cytotoxicity assay kit (Biovision) according to the manufacturer's instructions. Relative amount of LDH (%) was

calculated as [(LDH_{Sample} – $LDH_{Control}$) / ($LDH_{Maximum}$ – $LDH_{Control}$) x 100]. For maximal LDH release (100%), cells were lysed with the lysis solution included in the kit.

8.2. Propidium iodide exclusion assay

Uptake of propidium iodide (PI), a membrane impermeant dye, was used as a marker for pore formation in the THP-1 cells, which is a sign of pyroptosis. To quantitate PI uptake, THP-1 cells were stained with 20 μM PI (Sigma) at 37°C for 30 minute after the cells were infected with the indicated MOI of live *P. gingivalis* for 6 hours. The cells were then analyzed using flow cytometry (FACSCalibur, BD Biosciences) and Cell Quest Pro (BD Biosciences). The percentage of PI-positive cells was calculated.

9. Protein degradation assay

To assess whether *P. gingivalis* proteolytically degrades proteins that are released from caspase-1-activated cells, the culture supernatants of THP-1 cells that had been stimulated for 6 hours with heat-killed *P. gingivalis* equivalent to an MOI of 100 were used as substrates for the bacterial proteases. The culture supernatants, after filtration (0.2-μm-pore-size filter), were incubated with live *P. gingivalis* (1 x 10^9 bacteria/ml or 2 x 10^8 bacteria/ml equivalent to an MOI of 500 or 100, respectively) for 1, 3, or 6 hours at 37°C. The culture supernatants were then analyzed by immunoblot analysis or LDH cytotoxicity assay.

10. Intracellular caspase-1 activity assay

After THP-1 cells were infected with *P. gingivalis* for 30 minutes or 2 hours,

the cells were detached using trypsin/EDTA. Active caspase-1 within the cells was stained using FAM-YVAD–FMK (FLICA™ Caspase-1 Assay Kit, Immunochemistry Technologies, Bloomington, MN, USA) at 37°C for 1 hour according to the manufacturer's instructions. After washing with the wash buffer (included in the kit), the cells were fixed with the fixative (included in the kit). Intracellular active caspase-1 was then measured by flow cytometry (FACSCalibur), and the caspase-1 activity index was calculated as [(% positive cells x mean fluorescence intensity) / 100], which was adapted from a formula previously described for phagocytosis [101].

11. Determination of extracellular ATP concentrations

After THP-1 cells were stimulated with the indicated MOI of live *P. gingivalis* for 50 minutes or 2 hours, the ATP concentration of the culture supernatants was determined using an ATP bioluminescence assay kit (Roche) according to the manufacturer's instructions. ATP-degrading enzymes in culture supernatants were inactivated by incubating the culture supernatants with 0.3% TCA at 4°C for 30 minutes. After mixing the supernatants with 4 volumes of 250 mM Tris acetate (pH 7.75), the ATP assay reagents was added to the mixture as previously described [138]. The ATP levels in the culture supernatants were calculated after measuring the luminescence using GloMax® 96 Microplate Luminometer (Promega).

12. Phagocytosis of *T. forsythia*

THP-1 (5×10^5 cells/well) were seeded into 24-well plates and differentiated into macrophage-like cells with 0.1 μM PMA overnight. Differentiated THP-1 cells and PBMC-derived macrophages were infected with carboxyfluorescein diacetate

succinimidyl ester (CFSE, Molecular Probes, Eugene, OR, USA)-labelled *T. forsythia* in the presence or absence of unlabelled *P. gingivalis* at the indicated MOI for 1 hour. To examine the effect of gingipains of coinfecting *P. gingivalis* on phagocytosis of *T. forsythia,* wild-type and gingipain mutant strains of *P. gingivalis* were used. In experiments to examine phagocytosis of *P. gingivalis*, the cells were infected with CFSE-labelled *P. gingivalis* in the presence or absence of unlabelled *T. forsythia* at the indicated MOI for 1 hour.

In experiments to examine the role of gingipain protease activities, overnight cultures of wild-type *P. gingivalis* were preincubated in PBS containing 10 μM KYT-1 or KYT-36 under anaerobic conditions at 37°C for 1 hour. After washing with PBS, *P. gingivalis* were used for mixed infection with CFSE-labelled *T. forsythia*.

In experiments examining the effect of gingipains on *T. forsythia*, CFSE-labelled *T. forsythia* was preincubated in fresh culture medium containing 30% VDS of wild-type *P. gingivalis* or gingipain mutant strains in the presence or absence of 10 μM KYT-1 or KYT-36 for 1 hour. After washing with PBS, *T. forsythia* was used for infection.

In experiments to examine the effect of gingipains on macrophages, THP-1 cells were preincubated with *P. gingivalis* VDS (equivalent to an MOI of 100) for 1 hour in the presence or absence of 10 μM KYT-1 or KYT-36. The cells were then infected for 1 hour with CFSE-labelled *T. forsythia* in the presence or absence of *P. gingivalis* VDS and gingipain inhibitors.

Infected cells were detached with trypsin/EDTA, and the fluorescence intensity of the cells was analyzed by flow cytometry (FACSCalibur). To measure the internalization of *T. forsythia*, the cells were analyzed after quenching

extracellular fluorescence from adherent bacteria with 400 μg/ml trypan blue.

13. Intracellular persistence

THP-1 cells were infected with CFSE-labelled *T. forsythia* in the presence or absence of unlabelled *P. gingivalis* at an MOI of 100 for 1 hour. The infected cells were washed twice with PBS and incubated in media containing 300 μg/ml gentamicin (Sigma) and 200 μg/ml metronidazole (Sigma) for 1 hour to kill extracellular bacteria. The cells were then washed twice with PBS and further incubated in culture media for 0, 6, 12, 24, or 48 hours. At each time point, the cells were detached with trypsin/EDTA, and the fluorescence intensity of the cells was analyzed via flow cytometry after quenching the extracellular fluorescence from adherent bacteria with 400 μg/ml trypan blue.

14. Confocal microscopy

THP-1 cells (2 x 10^5 cells/well on coverslips in 24-well plates) were differentiated and infected with CFSE-labelled *T. forsythia* in the presence or absence of unlabelled *P. gingivalis* at an MOI of 100 for 1 hour. The infected cells were washed twice with PBS and fixed with 4% paraformaldehyde. The cells were stained with rhodamine phalloidin (Sigma) to visualize actin cytoskeleton and with Hoechst 33342 (Molecular Probes) to counterstain nuclei. The cells were observed under a confocal laser scanning microscope (LSM 700, Carl Zeiss, Jena, Germany).

In experiments to examine the persistence of *T. forsythia* in the infected macrophages, the cells were washed twice with PBS after 1 hour of infection. The cells were incubated in media containing 300 μg/ml gentamicin and 200 μg/ml metronidazole for 1 hour to kill extracellular bacteria, followed by further

incubation in media for 24 or 48 h. The cells were then washed twice with PBS and examined by confocal microscopy after fixation and staining as described above.

To examine phagosomal maturation, THP-1 cells (2 x 10^5 cells/well on coverslips in 24-well plates) were differentiated and infected with CFSE-labelled *P. gingivalis* or CFSE-labelled *T. forsythia* in the presence or absence of unlabelled *P. gingivalis* at an MOI of 100 for 1 or 3 hours. The cells were then stained with 50 nM LysoTracker Red (Molecular Probes) for 30 minutes. The cells were then washed twice with PBS and examined by confocal microscopy after fixation and staining as described above.

15. Coaggregation assay

The coaggregation activity between *T. forsythia* and *P. gingivalis* was quantitated as decrease in optical density at 660 nm (OD_{660}) as previously described [67] with slight modifications. Bacteria were harvested at 10,000 x g for 3 minutes at 4°C and washed with coaggregation buffer (0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.15 M NaCl, 3.1 mM NaN_3, and 1 mM Tris, pH 8.0). After the bacteria were resuspended in coaggregation buffer at 5 x 10^9 bacteria/ml, equal volumes (500 μl) of each bacterial suspension were mixed, and the initial OD_{660} of the mixtures was measured. To allow coaggregation of the two bacterial species, the mixtures were then left to stand for 90 minutes at room temperature, and the OD_{660} was measured. The precipitation of bacterial aggregates to the bottom of the tubes resulted in decreases in the optical density. The percent coaggregation was calculated as [(initial OD_{660} – OD_{660} at 90 minutes) / initial OD_{660} x 100]. To examine the effects of particular amino acids on coaggregation, L-arginine (Sigma), L-lysine (Sigma), histidine (Difco, Detroit, MI, USA), or glycine (Duchefa,

Haarlem, The Netherlands) was added to the bacterial mixtures at the indicated concentration.

16. RNA-based viability assay of intracellular *T. forsythia*

Viability of intracellular *T. forsythia* was assessed by analyzing *T. forsythia* 16S rRNA within infected cells as previously described [139]. THP-1 cells were infected with *T. forsythia* in the presence or absence of *P. gingivalis* at an MOI of 100 for 1 hour. The infected cells were washed twice with PBS and incubated in media containing 300 μg/ml gentamicin and 200 μg/ml metronidazole for 1 hour to kill extracellular bacteria. The cells were then washed twice with PBS and further incubated in culture media for 0, 24, or 48 hours. At each time point, total RNA from THP-1 cells was isolated with an easy-BLUE^{TM} total extraction kit, and cDNA was synthesized using an M-MLV Reverse Transcriptase kit and random primer (Promega). For real-time RT-PCR of *T. forsythia* 16S rRNA and human GAPDH, an equal amount of cDNA was mixed with primer pairs (50 nM each) and 10 μl of 2X Power SYBR® Green Master mix (Applied Biosystems) in a 20 μl reaction volume. Following initial denaturation at 95°C for 10 minutes, cDNA was amplified for 40 cycles of denaturation (95°C, 15 sec) and annealing/extension (57°C, 60 sec) in an ABI PRISM 7500 Fast Real-Time PCR System. Melting curve analysis of the PCR products was performed to ensure specificity of PCR amplification. The sequences of the forward and reverse primers (Cosmo GENETECH) used for real-time PCR of *T. forsythia* 16S rRNA were 5'-ATTGAAATGTAGACGACGGAGAGT-3' and 5'-TTACCTGTTAGCAACTG ACAGTCA-3', respectively. For real-time RT-PCR of human GAPDH, the primer pairs described earlier were used. The relative amount of live bacteria in host cells

was calculated by normalizing the amount of *T. forsythia* 16S rRNA against GAPDH mRNA in host cells by $2^{-\Delta\Delta CT}$ method.

17. Statistical analysis

A standard two-tailed *t* test was used for statistical analysis, and values of $P < 0.05$ were considered significant.

III. Results

1. Role of gingipains in caspase-1 activation

1.1 Low IL-1β and LDH levels without active caspase-1 in the culture supernatants at low MOIs are abrogated by high MOIs of *P. gingivalis* infection

Active caspase-1 (a tetramer of p20 and p10) is secreted into the extracellular space upon caspase-1 activation. Thus, the presence of the p20 or p10 subunits of active caspase-1 in culture supernatant is considered a sign of caspase-1 activation. When THP-1 cells were infected with live *P. gingivalis* ATCC 33277 at MOIs of 10, 50, 100, or 500, the subunit of active caspase-1(p20) was not detected in the culture supernatant of the *P. gingivalis*-infected cells (Fig. 3A), although mature IL-1β was slightly increased at low MOIs (Fig. 3A and B). In addition, high MOIs of *P. gingivalis* abolished IL-1β secretion in the culture supernatant although *P. gingivalis* induced the expression of pro-IL-1β mRNA in an MOI-dependent manner (Fig. 3B). As a positive control, it was shown that active caspase-1 (p20) and mature IL-1β were detected after infection with live *F. nucleatum*, a periodontopathogen known to activate the NLRP3 and AIM2 inflammasome [122]. The level of LDH in the culture supernatants, a commonly used marker of cell death, showed a similar pattern to that of mature IL-1β (Fig. 4A). However, when pore formation, another sign of pyroptosis, was assessed using a PI exclusion assay, it was found that *P. gingivalis* apparently induced the uptake of the membrane-impermeant PI in the infected cells in an MOI-dependent manner (Fig. 4B).

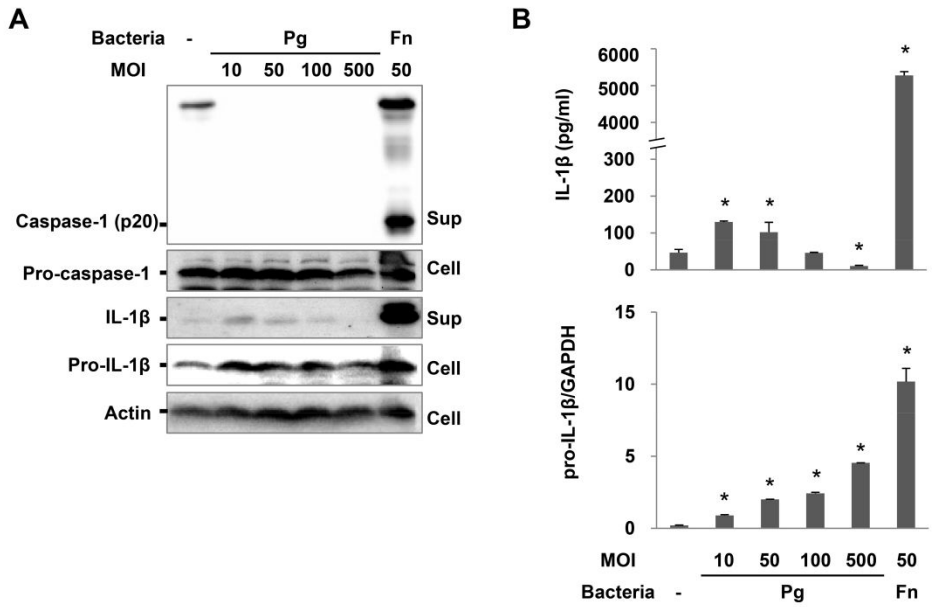


Figure 3. *P. gingivalis* induces minimal levels of IL-1β without active caspase-1 in culture supernatant. THP-1 cells were infected with live *P. gingivalis* at the indicated MOI for 6 hours. (A) Immunoblot analysis was performed to detect caspase-1 and IL-1β in the culture supernatants (sup) and pro-caspase-1, pro-IL-1β, and β-actin in the cell lysates (cell). (B) The concentration of IL-1β in the culture supernatants was determined by ELISA, and mRNA expression of pro-IL-1β was analyzed using real-time RT-PCR and was normalized to that of GAPDH. The experiments were performed three times, and the representative data are shown as the mean ± standard deviation. Pg, *P. gingivalis*; Fn, *F. nucleatum*. * $P < 0.05$ compared to the control.

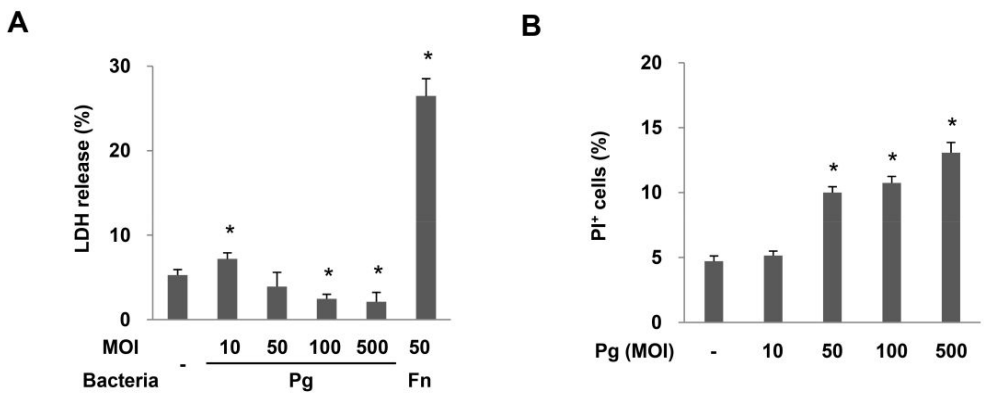


Figure 4. *P. gingivalis* induces pore formation with minimal levels of LDH in culture supernatant. THP-1 cells were infected with live *P. gingivalis* at the indicated MOI for 6 hours. (A) The levels of the cytoplasmic enzyme LDH in the culture supernatants were evaluated using the LDH cytotoxicity assay kit. (B) Propidium iodide (PI) uptake of the infected cells was analyzed using flow cytometry. The experiments were performed three times, and the representative data are shown as the mean ± standard deviation. Pg, *P. gingivalis*; Fn, *F. nucleatum*. * $P < 0.05$ compared to the control.

P. gingivalis–infected THP-1 cells were pulsed with ATP because it has been reported that *P. gingivalis* induces IL-1β secretion with the addition of exogenous ATP [122,140]. ATP is released from intracellular storage following cell distress, damage, or death, resulting in an elevation of extracellular ATP concentrations. The activation of the P_2X_7 receptor by high extracellular ATP concentrations leads to the activation of NLRP3 inflammasome and caspase-1 [141]. However, a pulse with ATP after *P. gingivalis* infection did not lead to the detection of active caspase-1 (p20) in the culture supernatants, although it slightly increased the IL-1β levels (Fig. 5A). In addition, *P. gingivalis* significantly induced ATP release from the infected cells in an MOI-dependent manner (Fig. 5B). The opposite regulation of pro-IL-1β mRNA expression and IL-1β secretion, the contrasting results for the PI uptake and LDH release, and the abrogation of caspase-1 detection induced by ATP all strongly imply that *P. gingivalis* proteases degrade IL-1β, LDH, and possibly caspase-1.

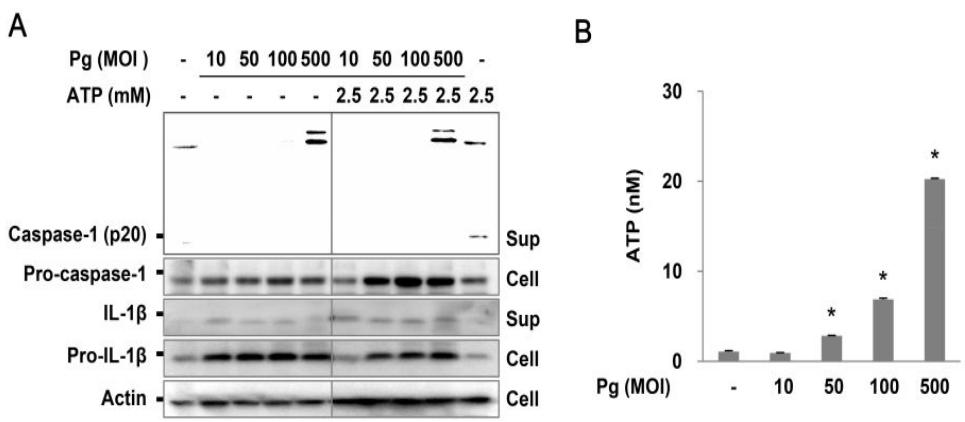


Figure 5. The absence of active caspase-1 is not due to lack of ATP release. (A) THP-1 cells were infected with live *P. gingivalis* at the indicated MOI for 6 hours and pulsed with 2.5 mM ATP for 30 minutes. The levels of caspase-1 and IL-1β in the culture supernatants (sup) and pro-caspase-1, pro-IL-1β, and β-actin in the cell lysates (cell) were analyzed by immunoblot analysis. (B) After THP-1 cells were infected with live *P. gingivalis* for 2 hours, the extracellular ATP concentration was measured using an ATP bioluminescence assay kit. The experiments were performed twice, and the representative data are shown as the mean ± standard deviation. Pg, *P. gingivalis*. * $P < 0.05$ compared to the control.

1.2. Low levels of IL-1β result from the potent proteolytic activity of *P. gingivalis*

To determine whether *P. gingivalis* has the potential to activate caspase-1, heat-killed bacteria was used to inactivate its proteases. After stimulation with heat-killed bacteria, THP-1 cells released significant amounts of active caspase-1 (p20) and IL-1β (Fig. 6A and B) as well as LDH (Fig. 6C) in a dose-dependent manner. Pre-treatment of the cells with the caspase-1 inhibitors z-YVAD-FMK and Ac-YVAD-CHO completely blocked IL-1β secretion (Fig. 6D), indicating that the maturation of IL-1β induced by heat-killed *P. gingivalis* is caspase-1-dependent. These results suggest that some heat labile components of *P. gingivalis*, possibly proteases, are responsible for the reduced levels of caspase-1, IL-1β, and LDH in the culture supernatants.

To confirm that *P. gingivalis* degrades the signature proteins of caspase-1 activation in the culture supernatants, culture supernatants of THP-1 cells that had been stimulated with heat-killed *P. gingivalis* were used as substrates for the bacterial proteases. The active caspase-1 (p20) and the mature IL-1β (p17) disappeared completely within 1 and 6 hours, respectively, of incubation with 1 x 10^9 bacteria/ml *P. gingivalis* (Fig. 7A). A smaller fragment of IL-1β was detected as a result of partial proteolysis. Likewise, most LDH in the culture supernatants was lost within 1 hour of incubation with *P. gingivalis* (Fig. 7B). Leupeptin, a cysteine protease inhibitor, was used for limiting proteolysis by the proteases of *P. gingivalis*. Preincubation of *P. gingivalis* with leupeptin was insufficient to prevent the degradation of IL-1β for 6 hours (Fig. 7C). In addition, neither caspase-1 nor IL-1β was detected in the culture supernatants after infection of THP-1 cells with leupeptin-preincubated *P. gingivalis* (Fig. 7D), indicating that *P. gingivalis* restored

its proteolytic ability within a few hours.

When THP-1 cells were infected in the presence of leupeptin with live *P. gingivalis* that had been preincubated with leupeptin. *P. gingivalis* evidently resulted in caspase-1 activation and IL-1β secretion (Fig. 8A and B). The level of LDH released from the macrophages after infection with live *P. gingivalis* was also significantly increased in the presence of leupeptin (Fig. 8C). Because *P. gingivalis* strains have different proteolytic activity, other strains of *P. gingivalis*, FDC 381, ATCC 49417, and W50 were used to determine whether this phenomenon is a common characteristic of *P. gingivalis* (Fig. 9A). Active caspase-1 was not detected in the culture supernatants of cells infected with any *P. gingivalis* strains at an MOI of 100. Infection with *P. gingivalis* ATCC 49417 and W50, which are known to be more virulent and genetically distant from *P. gingivalis* ATCC 33277 [25], resulted in a considerable amount of IL-1β in the culture supernatants. However, infection with a higher MOI (500) of the two strains lowered or abrogated the IL-1β levels, while the presence of leupeptin during infection led to an increase in the caspase-1 and IL-1β levels (Fig. 9B). These results indicate that the proteolytic depletion of the proteins is a common virulence mechanism shared by *P. gingivalis* strains.

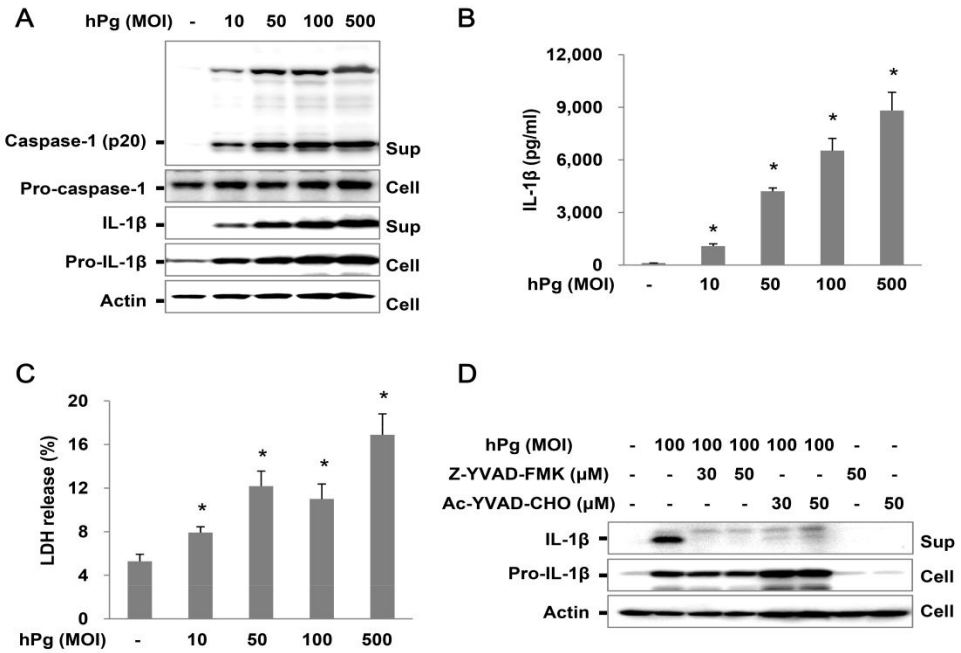


Figure 6. Heat-killed *P. gingivalis* induces caspase-1-dependent IL-1β secretion. **(**A-C) THP-1 cells were stimulated with heat-killed *P. gingivalis* at the indicated MOI for 6 hours. (A) The levels of caspase-1 and IL-1β in the culture supernatants (sup) and pro-caspase-1, pro-IL-1β, and β-actin in the cell lysates (cell) were analyzed by immunoblot analysis. (B) The concentration of IL-1β in the culture supernatants was determined by ELISA. (C) The levels of LDH in the culture supernatants were evaluated with the LDH cytotoxicity assay kit. (D) THP-1 cells were pre-treated with the caspase-1 inhibitor Z-YVAD-FMK or Ac-YVAD-CHO for 30 minutes before 6 hours of stimulation with heat-killed *P. gingivalis*. Immunoblot analysis was performed to detect IL-1β in the culture supernatants (sup) as well as pro-IL-1β and β-actin in the cell lysates (cell). The experiments were performed three times, and the representative data are shown as the mean ± standard deviation. * $P < 0.05$ compared to the control. hPg, heat-killed *P. gingivalis* ATCC 33277.

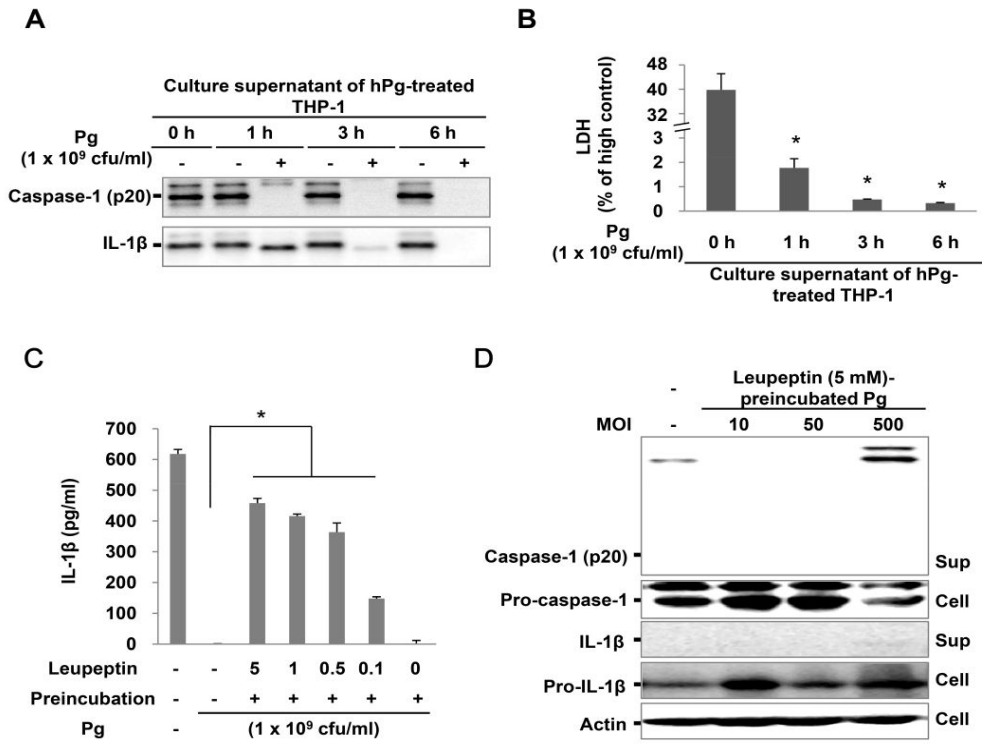


Figure 7. Live *P. gingivalis* proteolytically degrades caspase-1, IL-1β, and LDH. (A, B) Live *P. gingivalis* (1 x 10^9 cfu/ml equivalent to an MOI of 500) was incubated for the indicated time in the culture supernatants of THP-1 cells that had been stimulated with heat-killed *P. gingivalis*. The amounts of remaining active caspase-1 (p20) and IL-1β were determined by immunoblot analysis (A), and those of LDH were analyzed using an LDH cytotoxicity assay (B). * $P < 0.05$ compared to 0 h. (C) Live *P. gingivalis* was preincubated with 0.2 mM leupeptin, a cysteine protease inhibitor, for 1 hour and then incubated in the presence or absence of leupeptin (at the indicated concentration) for 6 hours with the culture supernatants of THP-1 cells that had been stimulated with heat-killed *P. gingivalis*. The amounts of remaining IL-1β in the culture supernatants were determined by ELISA. The experiments were performed twice, and the representative data are shown as the mean ± standard deviation. * $P < 0.05$ compared to *P. gingivalis* without

preincubation with leupeptin. (D) THP-1 cells were infected in the absence of leupeptin for 6 hours with *P. gingivalis* that had been preincubated with 5 mM leupeptin. Immunoblot analysis was performed to detect 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). Pg, *P. gingivalis*.

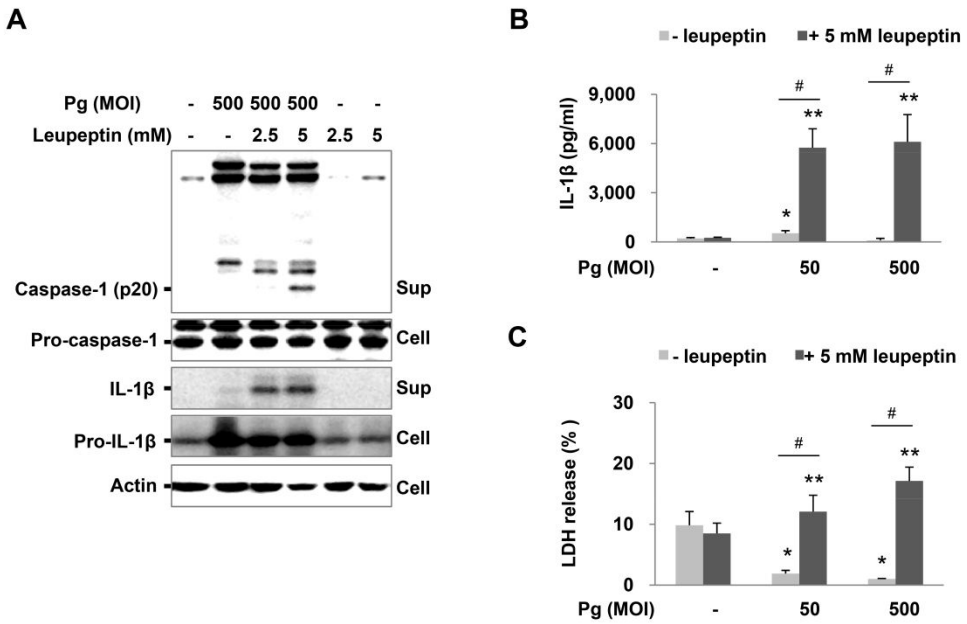


Figure 8. Live *P. gingivalis* induces secretion of caspase-1 and IL-1β as well as release of LDH in the presence of leupeptin. THP-1 cells were infected with *P. gingivalis* for 6 hours in the presence or absence of leupeptin. (A) Immunoblot analysis was performed to detect caspase-1 and IL-1β in the culture supernatants (sup) and pro-caspase-1, pro-IL-1β, and β-actin in the cell lysates (cell). (B) The concentration of IL-1β in the culture supernatants was determined by ELISA. (C) The levels of LDH in the culture supernatants were evaluated using the LDH cytotoxicity assay. The experiments were performed three times, and the representative data are shown as the mean ± standard deviation. * $P < 0.05$ compared to the control; ** $P < 0.05$ compared to the control containing 5 mM leupeptin; $^{\#}$ $P < 0.05$ compared between two groups. Pg, *P. gingivalis*.

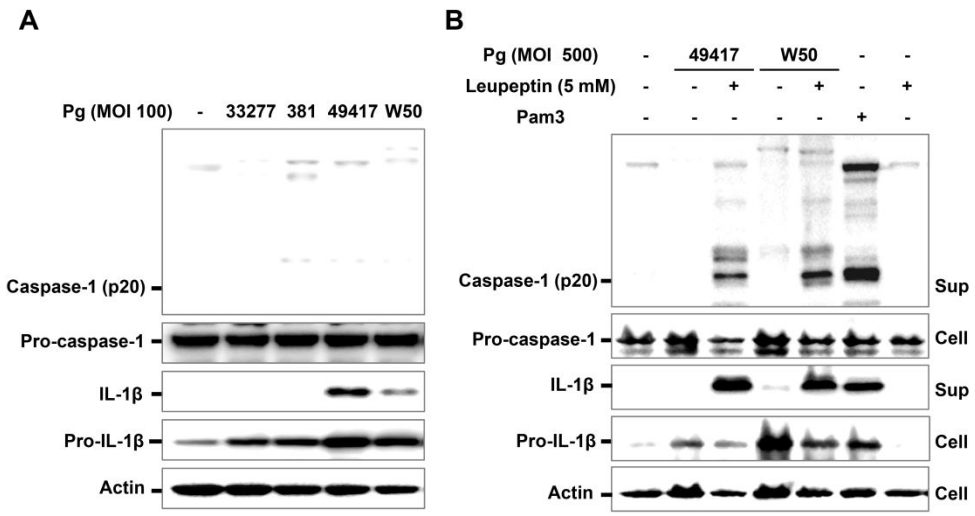


Figure 9. Proteolysis of secreted caspase-1 and IL-1β is a common feature of *P. gingivalis* infection. (A) THP-1 cells were infected with live *P. gingivalis* ATCC 33277, FDC 381, ATCC 49417, or W50 at an MOI of 100 for 6 hours. (B) THP-1 cells were infected with live *P. gingivalis* ATCC 49417 or W50 at an MOI of 500 in the presence or absence of leupeptin for 6 hours. The resulting levels of 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 analyzed by immunoblot analysis. Pg, *P. gingivalis*; Pam3, 0.5 μg/ml Pam3CSK4.

1.3. Rgps and Kgp of *P. gingivalis* wipe out caspase-1-dependent proteinaceous responses

Next, the roles of gingipains, the major cysteine proteases of *P. gingivalis*, in caspase-1 activation were investigated using gingipain mutant strains. Unlike wild-type *P. gingivalis* (ATCC 33277), infection with a *P. gingivalis* mutant strain lacking both Kgp and Rgps (KDP136) led to a substantial amount of active caspase-1 and IL-1β in the culture supernatants of both the THP-1 cells and the PBMC-derived macrophages (Fig. 10A and B). The levels of active caspase-1 and IL-1β induced by the mutant strain were comparable to those induced by heat-killed wild-type *P. gingivalis*. The mutant strains that lack either Kgp (KDP129) or Rgps (KDP133) induced lower IL-1β levels than KDP136, with either a low level or no detectable caspase-1 in the culture supernatants (Fig. 10A and B). Unexpectedly, KDP136 induced lower levels of LDH release from the infected cells than those induced by KDP129 and KDP133, while infection with wild-type *P. gingivalis* resulted in the lowest level of LDH in the culture supernatants (Fig. 10C). In the presence of leupeptin, which preserved IL-1β in the culture supernatants (Fig. 11A), the pre-treatment of the cells with the caspase-1 inhibitor Ac-YVAD-CHO significantly reduced the IL-1β secretion induced by *P. gingivalis*, regardless of the presence of a gingipain mutation (Fig. 11B) whereas secretion of IL-8, which is not dependent on caspase-1 activation, was not affected by the inhibitor (Fig. 11C). These results indicate that the maturation of IL-1β induced by live *P. gingivalis* is caspase-1-dependent.

To determine the role of each gingipain in the degradation of the signature proteins of caspase-1 activation, wild-type *P. gingivalis* and gingipain mutants were incubated in the culture supernatants of THP-1 cells that had been stimulated with

heat-killed *P. gingivalis*. The proteolytic depletion of IL-1β and LDH seems to be accelerated by the cooperative action of Rgps and Kgp, as their degradation by wild-type *P. gingivalis* was much faster than by KDP129 or KDP133 (Fig. 12A and B). An IL-1β fragment of a smaller size (approximately 15-16 kDa) was detected after incubation with wild-type or KDP129, indicating that Rgps result in the partial proteolysis of IL-1β. It was determined whether other proteins that are released from cells with caspase-1 activation and can augment inflammation were also degraded. HMGB1 is a DAMP that is released after caspase-1 activation and binds RAGE, TLR2, and TLR4 [142]. HMGB1 in the culture supernatants was also more rapidly decreased by the wild-type *P. gingivalis* than by the gingipain mutant strains (Fig. 12A), suggesting a combined action of Kgp and Rgps. Recent studies have reported that upon caspase-1 activation, inflammasome particles or ASC specks are released into the extracellular space and continue to process pro-caspase-1 and pro-IL-1β [143,144]. In addition, the released particles and specks amplify the inflammatory response by acting as particulate danger signals [143,144]. By degrading inflammasome components such as NLRP3, ASC, and pro-caspase-1 in the culture supernatants (Fig. 12A), *P. gingivalis* gingipains may stop the propagation of inflammation. Their degradation pattern was similar to HMGB1, while pro-IL-1β was degraded comparably by the gingipain-expressing wild-type, KDP129, and KDP133 (Fig. 12A).

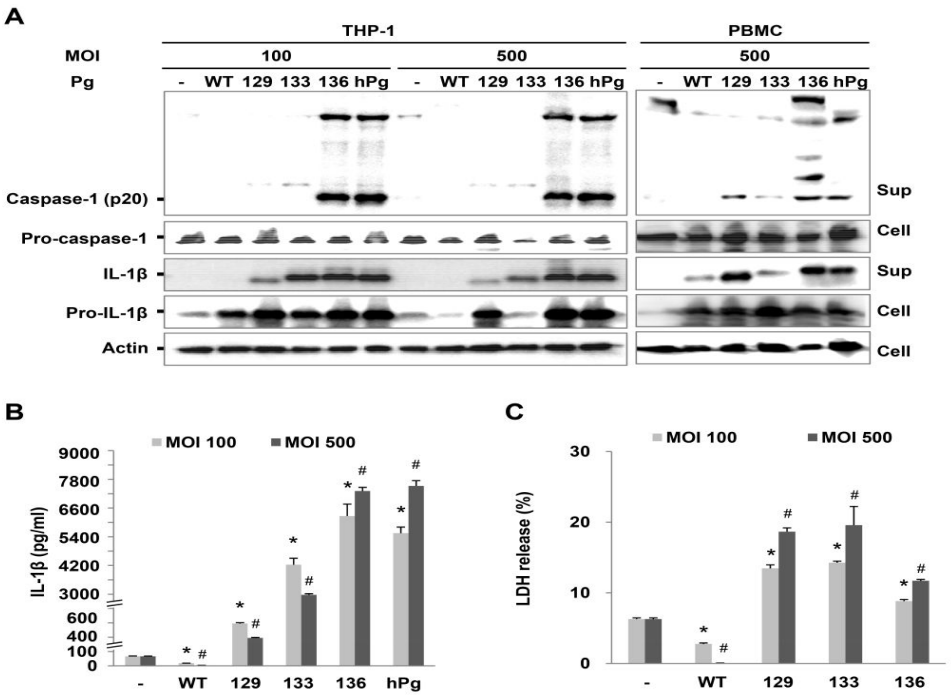


Figure 10. The secretion of caspase-1 and IL-1β is cooperatively diminished by Rgps and Kgp. (A) THP-1 cells or PBMC-derived macrophages were infected with wild-type *P. gingivalis* or gingipain mutant strains KDP129, 133, or 136 at an MOI of 100 or 500 for 6 hours. The levels of caspase-1 and IL-1β in the culture supernatants (sup) and pro-caspase-1, pro-IL-1β, and β-actin in the cell lysates (cell) were analyzed by immunoblot analysis. (B) The concentration of IL-1β in the culture supernatants of THP-1 cells was determined by ELISA. (C) The levels of LDH in the culture supernatants of the THP-1 cells were evaluated with the LDH cytotoxicity assay kit. The experiments were performed three times, and the representative data are shown as the mean ± standard deviation. * $P < 0.05$ compared to the control in the MOI of 100 set; $^{\#}$ $P < 0.05$ compared to the control in the MOI of 500 set. WT, *P. gingivalis* ATCC 33277; 129, KDP129 (*kgp*$^-$); 133, KDP133 (*rgpA*$^-$ *rgpB*$^-$); 136, KDP136 (*kgp*$^-$ *rgpA*$^-$ *rgpB*$^-$); hPg, heat-killed *P. gingivalis* ATCC 33277.

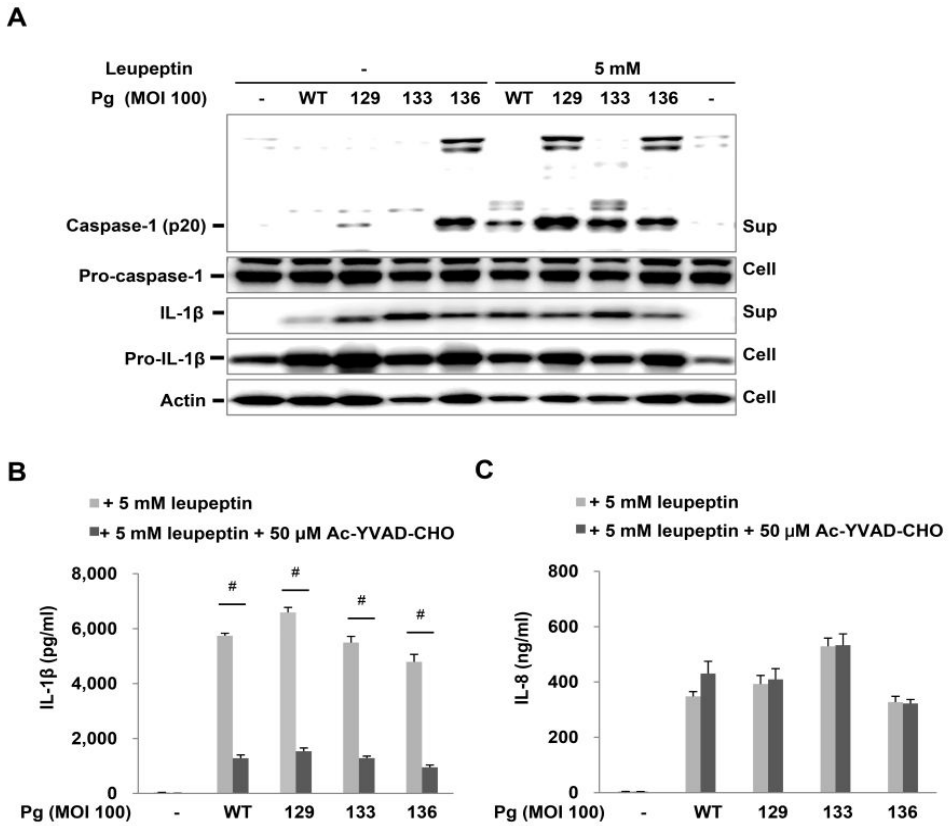


Figure 11. IL-1β secretion induced by live *P. gingivalis* is caspase-1-dependent. (A) THP-1 cells were infected with wild-type *P. gingivalis* or gingipain mutants for 6 hours in the absence or presence of leupeptin. The resulting levels of caspase-1 and IL-1β in the culture supernatants (sup) and pro-caspase-1, pro-IL-1β, and β-actin in the cell lysates (cell) were analyzed by immunoblot analysis. (B, C) THP-1 cells were pre-treated with 50 μM Ac-YVAD-CHO for 30 minute and then stimulated with wild-type *P. gingivalis* or gingipain mutant strains at an MOI of 100 in the presence of leupeptin for 6 hours. The concentration of IL-1β (B) and IL-8 (C) in the culture supernatants was assessed by ELISA. $^{\#}$ $P < 0.05$ compared between two groups. WT, *P. gingivalis* ATCC 33277; 129, KDP129 (*kgp*$^-$); 133, KDP133 (*rgpA*$^-$ *rgpB*$^-$); 136, KDP136 (*kgp*$^-$ *rgpA*$^-$ *rgpB*$^-$).

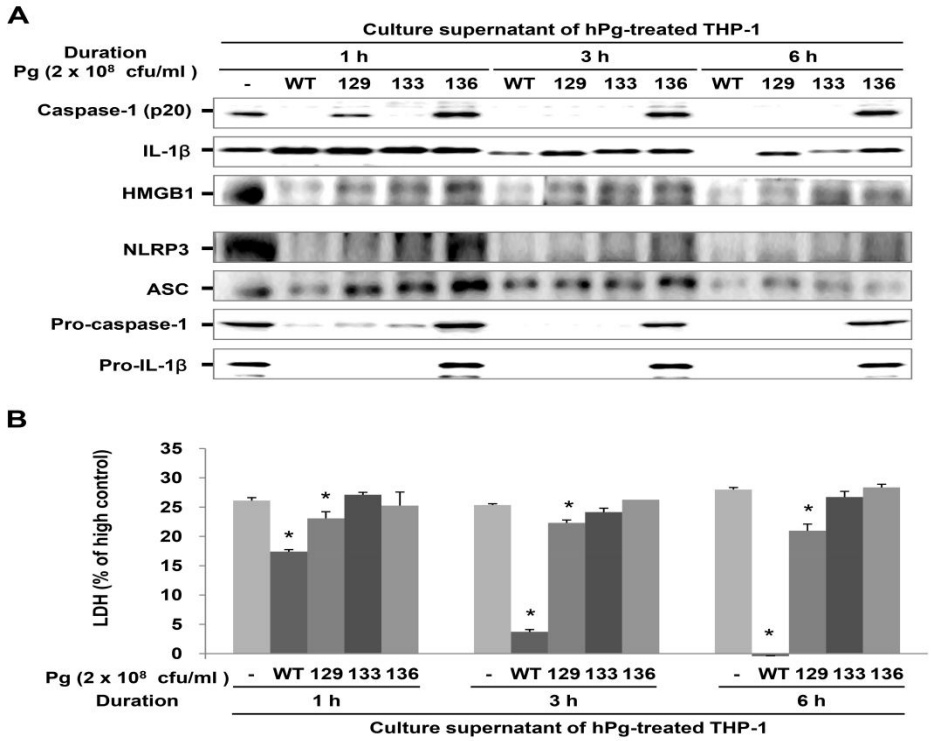


Figure 12. Rgps and Kgp cooperatively degrade the released proteins including inflammasome components. Wild-type *P. gingivalis* and gingipain mutants (2 x 10^8 cfu/ml equivalent to an MOI of 100) were incubated for the indicated time in the culture supernatants of THP-1 cells that had been stimulated with heat-killed *P. gingivalis*. (A) Immunoblot analysis was performed on the culture supernatants to determine the amounts of remaining caspase-1 (p20), IL-1β, and HMGB1 as well as inflammasome components including NLRP3, ASC, pro-caspase-1, and pro-IL-1β. (B) The loss of LDH in the culture supernatants was determined using an LDH cytotoxicity assay kit. * $P < 0.05$ compared to the group without Pg. Pg, *P. gingivalis*; WT, *P. gingivalis* ATCC 33277; 129, KDP129 (*kgp*$^-$); 133, KDP133 (*rgpA*$^-$ *rgpB*$^-$); 136, KDP136 (*kgp*$^-$ *rgpA*$^-$ *rgpB*$^-$); hPg, heat-killed *P. gingivalis*.

1.4. Gingipains differentially enhance caspase-1 activation by promoting ATP release

To investigate the role of gingipains in caspase-1 activation, an assessment that is not affected by the proteolytic depletion was used to determine caspase-1 activity in the infected macrophages. After infection with *P. gingivalis* at an MOI of 100 for 30 minutes or 2 hours, the active caspase-1 in the cells was stained with FAM-YVAD-FMK and analyzed by flow cytometry. Caspase-1 activity was higher after 30 minutes of *P. gingivalis* infection than after 2 hours of infection (Fig. 13A), which resulted from the rapid secretion of caspase-1 upon its activation. Both at 30 minutes and at 2 hours of infection, the gingipain-null mutant KDP136 induced the lowest level of intracellular caspase-1 activity, which did not result from more secretion of caspase-1 (Fig. 13B). *P. gingivalis* wild-type and KDP133 with Kgp induced similar levels of caspase-1 activation, while KDP129 with Rgps showed the highest caspase-1-activating potential (Fig. 13A). This result suggests that gingipains enhance the caspase-1-activating potential of *P. gingivalis*. In particular, the presence of Rgps seems to promote caspase-1 activation more effectively than the presence of both Rgps and Kgp.

To examine whether the gingipains affect ATP release from the infected THP-1 cells, the ATP concentration in the culture supernatants was measured. *P. gingivalis* wild-type, KDP129, and KDP133 induced more ATP release from the infected cells than that induced by the gingipain-null KDP136 (Fig. 14A). In addition, infection with KDP129 led to higher levels of ATP release than infection with the wild-type strain. Because of similar trends between caspase-1 activity and ATP release (Fig. 13A and 14A), it was examined whether the modulation of caspase-1 activity by gingipains is mediated by ATP release. Pre-treatment of THP-

1 cells with oxATP, a P_2X_7 receptor antagonist, before *P. gingivalis* infection significantly reduced the caspase-1 activity (Fig. 14B) and IL-1β secretion (Fig. 14C) in cells infected with wild-type *P. gingivalis*, KDP129, and KDP133. The decrease in active caspase-1 (p20) in the culture supernatants was not evident due to its low levels caused by the proteolytic depletion. Inhibition of caspase-1 activation and IL-1β secretion by oxATP in the KDP136-infected cells was also observed (Fig. 14C), although the decrease in caspase-1 activity caused by oxATP was minimal after 30 minutes of infection (Fig. 14B). This result might be attributed to low levels of ATP release and caspase-1 activation, and addition of exogenous ATP further increased activation of caspase-1 in the KDP136-infected cells (Fig. 15A and B). These results suggest that *P. gingivalis* gingipains affect caspase-1 activation by altering ATP release from the infected cells.

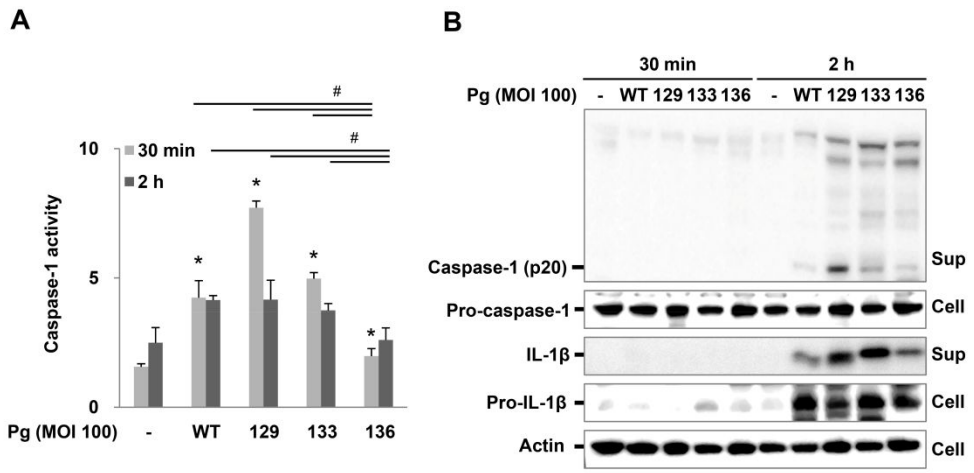


Figure 13. Gingipains differentially enhance caspase-1 activation in *P. gingivalis* infection. THP-1 cells were infected with wild-type *P. gingivalis* or gingipain mutants at an MOI of 100 for the indicated time. (A) The caspase-1 activity was quantified using a Caspase-1 assay kit and flow cytometry. (B) The levels of 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 analyzed by immunoblot analysis. The experiments were performed twice, and the representative data are shown as the mean ± standard deviation. * $P < 0.05$ compared to the control; # $P < 0.05$ compared between two groups. WT, *P. gingivalis* ATCC 33277; 129, KDP129 (*kgp*$^-$); 133, KDP133 (*rgpA*$^-$ *rgpB*$^-$); 136, KDP136 (*kgp*$^-$ *rgpA*$^-$ *rgpB*$^-$).

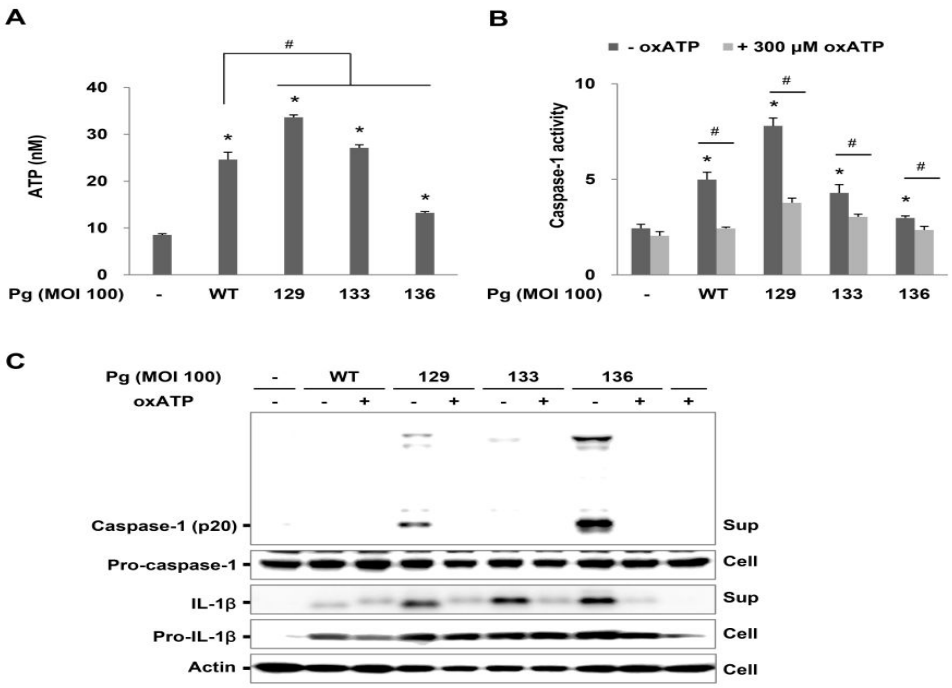


Figure 14. *P. gingivalis* gingipains modulate caspase-1 activation by affecting ATP release. (A) THP-1 cells were infected with wild-type *P. gingivalis* or gingipain mutants at an MOI of 100 for 50 minutes, and the extracellular ATP concentration was measured using an ATP bioluminescence assay kit. (B, C) THP-1 cells were preincubated with 300 μM oxATP, a P_2X_7 receptor antagonist, for 30 minutes before infection with *P. gingivalis*. (B) Caspase-1 activity was quantified after 30 minutes of infection using Caspase-1 assay kit and flow cytometry. (C) After 6 hours of infection, the levels of 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 analyzed by immunoblot analysis. The experiments were performed twice, and the representative data are shown as the mean ± standard deviation. * $P < 0.05$ compared to the control; # $P < 0.05$ compared between two groups. WT, *P. gingivalis* ATCC 33277; 129, KDP129 (*kgp*$^-$); 133, KDP133 (*rgpA*$^-$ *rgpB*$^-$); 136, KDP136 (*kgp*$^-$ *rgpA*$^-$ *rgpB*$^-$).

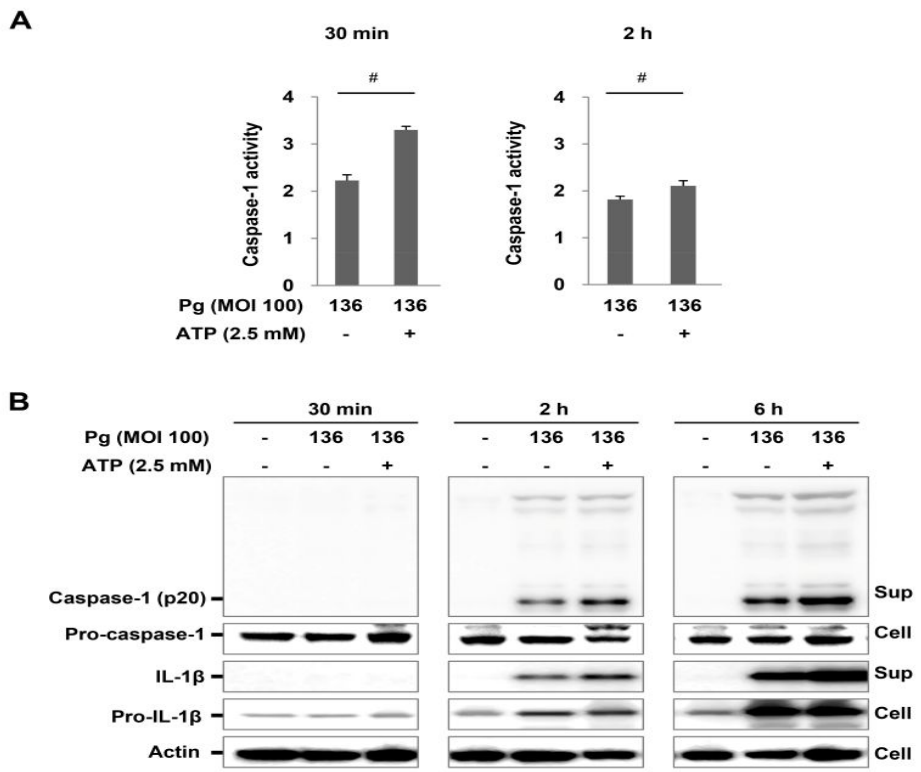


Figure 15. Exogenous ATP augments caspase-1 activation in KDP136-infected cells. THP-1 cells were infected with *P. gingivalis* KDP136 at an MOI of 100 for the indicated time and pulsed with 2.5 mM ATP for 15 minutes. (A) The caspase-1 activity was quantified using a Caspase-1 assay kit and flow cytometry. (B) The levels of 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 analyzed by immunoblot analysis. $^{\#}$ $P < 0.05$ compared between two groups. Pg, *P. gingivalis*; 136, KDP136 (*kgp*$^{-}$ *rgpA*$^{-}$ *rgpB*$^{-}$).

1.5. Gingipains indirectly facilitate caspase-1 activation in infected cells, possibly by processing the surface proteins of *P. gingivalis*

Because gingipains are important processing enzymes for multiple bacterial surface proteins such as major and minor fimbriae, hemoglobin receptors, and proteases including the gingipains themselves, the gingipain mutants have defects in these proteins [51,56]. Thus, the difference in caspase-1-activating potential among wild-type *P. gingivalis* and gingipain mutants may be attributable to differences in the processing and maturation of the surface proteins. A previous study demonstrated that soluble gingipains in membrane vesicle-depleted culture supernatants (VDS) can successfully generate an outer membrane protein in KDP136, which inherently lacks the mature form on the bacterial surface [58]. To examine how the processing of surface proteins by gingipains affects caspase-1 activation, the gingipain-null mutant KDP136 was grown in culture medium containing the VDS of KDP129, KDP133, or wild-type in the presence or absence of KYT-1 and KYT36 for Rgps and Kgp inhibition, respectively (Fig. 16A). KDP136 grown in VDS containing Rgps (of KDP129), Kgp (of KDP133), or both (of wild-type) induced more caspase-1 activation (Fig. 16B), IL-1β secretion (Fig. 16B and C), and LDH release (Fig. 16D) than that grown in VDS containing no gingipains (of KDP136). The observed enhancement was reversed by the presence of gingipain inhibitors. These results indicate that the protease activities of gingipains indirectly contribute to the caspase-1-activating potential of *P. gingivalis*, possibly by processing bacterial surface proteins, although changes in the surface morphology of the gingipain-null mutants were not examined.

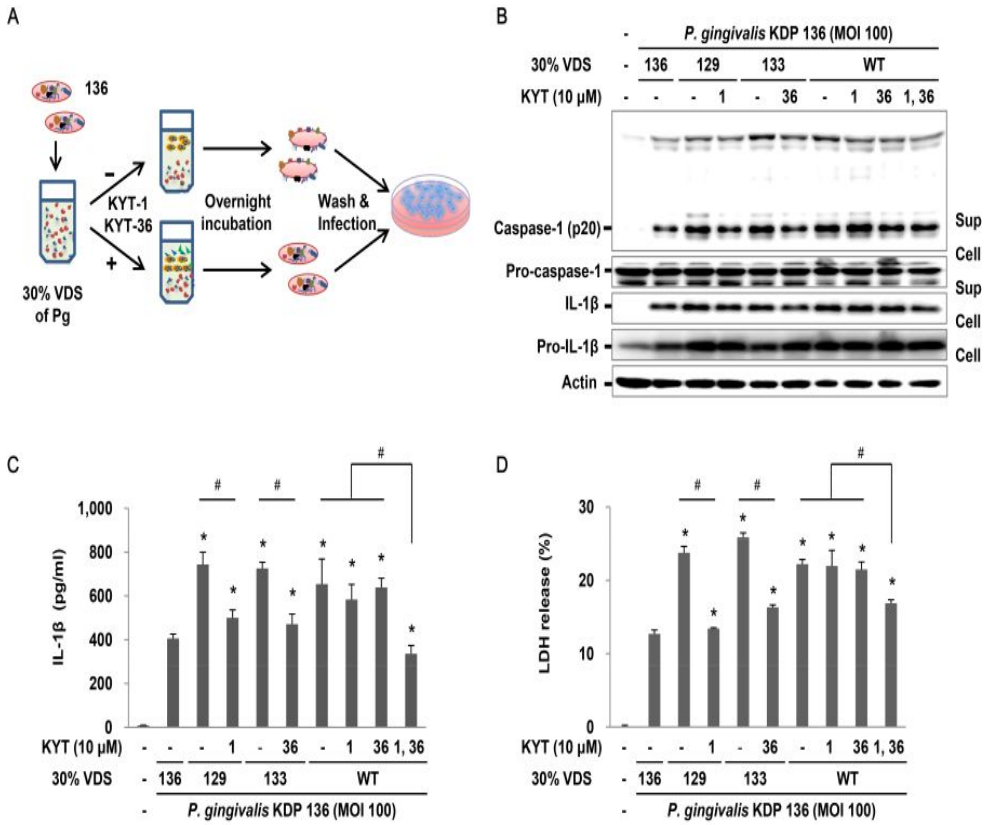


Figure 16. Treatment of gingipain-null mutant with exogenous gingipains enhances its ability to activate caspase-1. (A) The gingipain-null KDP136 was grown overnight in the bacterial culture media containing 30% membrane vesicle-depleted supernatants (VDS) of *P. gingivalis* gingipain mutants or wild-type with or without 10 μM KYT-1 (for Rgp inhibition), KYT-36 (for Kgp inhibition), or with both. THP-1 cells were then infected with the KDP136 at an MOI of 100 for 6 hours. (B) The levels of 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 analyzed by immunoblot analysis. (C) The concentration of IL-1β in the culture supernatants

was determined by ELISA. (D) The levels of LDH in the culture supernatants were measured using the LDH cytotoxicity assay. * $P < 0.05$ compared to the group of KDP136 preincubated with VDS of KDP136; $^{\#}$ $P < 0.05$ compared between two groups. WT, *P. gingivalis* ATCC 33277; 129, KDP129 (*kgp*$^{-}$); 133, KDP133 (*rgpA*$^{-}$ *rgpB*$^{-}$); 136, KDP136 (*kgp*$^{-}$ *rgpA*$^{-}$ *rgpB*$^{-}$).

1.6. Protease activity of gingipains impairs extracellular ATP release and caspase-1 activation

To examine the role of gingipain protease activity during the infection process in the modulation of ATP release and caspase-1 activation in the infected THP-1 cells, wild-type *P. gingivalis* was preincubated with KYT-36 for Kgp inhibition or KYT-1 for Rgp inhibition shortly before infection. The inhibition of Kgp significantly increased ATP release from *P. gingivalis*-infected THP-1 cells. Rgp-inhibited *P. gingivalis* also enhanced ATP release although the increase was less than that following Kgp inhibition (Fig. 17A). Unexpectedly, unlike the gingipain-null KDP136 (Fig. 13A), the inhibition of both Kgp and Rgps significantly increased ATP release (Fig. 17A). The difference might be attributable to the fact that although the protease activity of gingipains is absent or inhibited in both the gingipain-null mutant and the gingipains-inhibited wild-type *P. gingivalis*, the former does not have other features of gingipains such as adhesin domains and processing of bacterial proteins, whereas the latter has them. The inhibition of either or both gingipains led to enhanced caspase-1 activity in the infected THP-1 cells in a similar manner to the ATP release (Fig. 17B). Pre-treatment of THP-1 cells with oxATP before *P. gingivalis* infection significantly inhibited caspase-1 activity (Fig. 17B) and IL-1β secretion (Fig. 17C) that were enhanced by gingipain inhibition. These results indicate that the simultaneous proteolytic action of Kgp and Rgps during infection makes *P. gingivalis* induce less ATP release and caspase-1 activation. In addition, the high caspase-1-activating potential of the wild-type with both Kgp and Rgp inhibition compared to KDP136 supports the idea that some functions of Kgp and Rgps, other than direct protease activity during infection process, positively contribute to caspase-1 activation.

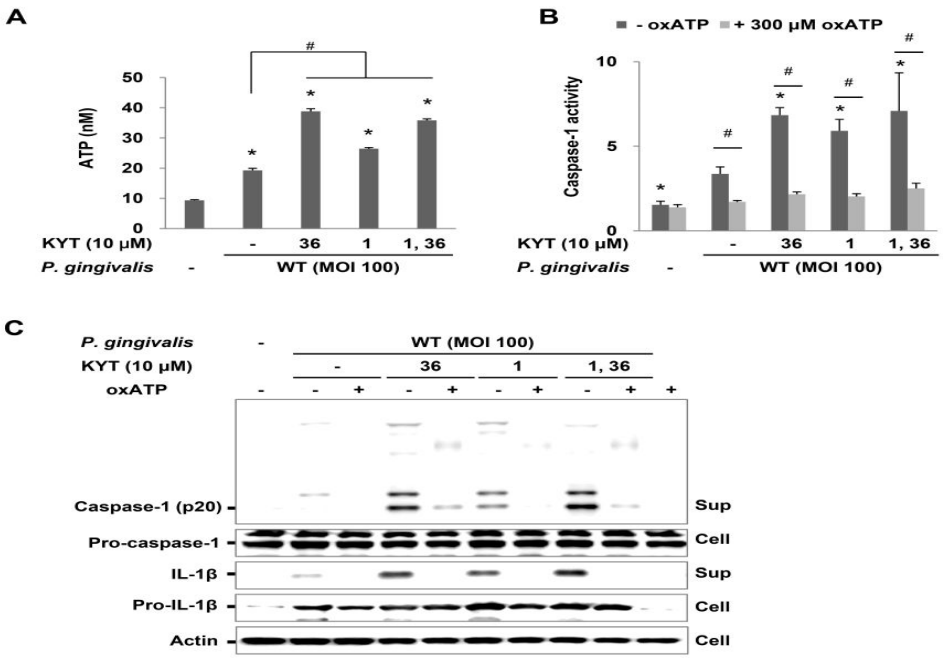


Figure 17. Inhibition of the protease activity of gingipains increases caspase-1 activation by augmenting ATP release. (A) Wild-type *P. gingivalis* was preincubated with 10 μM KYT-36 or KYT-1 for 1 hour to inhibit Kgp or Rgps, respectively. THP-1 cells were then infected with the wild-type *P. gingivalis* at an MOI of 100. The extracellular ATP concentration after 50 minutes of *P. gingivalis* infection was measured using an ATP bioluminescence assay kit. The experiments were performed twice, and the representative data are shown as the mean ± standard deviation. * $P < 0.05$ compared to the control; # $P < 0.05$ compared between two groups. (B, C) THP-1 cells were preincubated with 300 μM oxATP for 30 minutes before infection with *P. gingivalis* that had been preincubated with 10 μM KYT-36 or KYT-1 for 1 hour. (B) After 30 minutes of infection, caspase-1 activity was assessed using a Caspase-1 assay kit and flow cytometry. The experiments were performed twice, and the representative data are shown as the mean ± standard deviation. * $P < 0.05$ compared to WT; # $P < 0.05$ compared between two groups. (C) After 6 hours of infection, the levels of 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 analyzed by immunoblot analysis. WT, *P. gingivalis* ATCC 33277.

2. Role of gingipains in augmentation of *T. forsythia* phagocytosis

2.1. Phagocytosis of *T. forsythia* is facilitated by coinfection with *P. gingivalis*

To determine whether *P. gingivalis* coinfection influences phagocytosis of *T. forsythia*, THP-1 cells were infected with CFSE-labelled *T. forsythia* in the presence or absence of *P. gingivalis*. After 1 hour of infection at an MOI of 100, the percentage of macrophages that phagocytosed *T. forsythia* in the absence of *P. gingivalis* was minimal (ca. 4%). Coinfection with *P. gingivalis* even at an MOI of 10 doubled the percentage, and further increase in the MOIs of *P. gingivalis* significantly enhanced the phagocytosis of *T. forsythia* in an MOI-dependent manner (Fig. 18A and B). Mixed infection of *P. gingivalis* and *T. forsythia* at an MOI of 100 resulted in more phagocytosis of *T. forsythia* than did monoinfection with *T. forsythia* at an MOI of 500 (Fig. 18C).

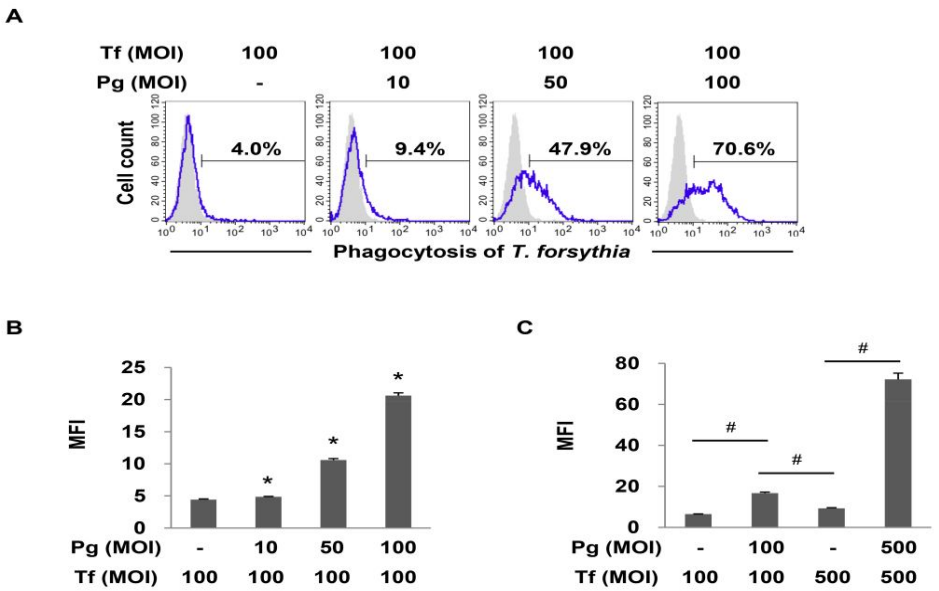


Figure 18. *P. gingivalis* promotes phagocytosis of *T. forsythia* in an MOI-dependent manner. THP-1 cells were infected with CFSE-labelled *T. forsythia* and unlabelled *P. gingivalis* at the indicated MOI for 1 hour. (A) Histograms of the infected macrophages (solid lines) are shown with the mean percentage of macrophages positive for phagocytosed *T. forsythia.* Uninfected cells are shown as the gray-filled histogram. (B, C) The mean fluorescence intensity (MFI) of the infected macrophages is shown as the mean ± standard deviation. The representative data of two independent experiments are shown. Tf, *T. forsythia*; Pg, *P. gingivalis*. * $P < 0.05$ compared to *T. forsythia* monoinfection; $^{\#}$ $P < 0.05$ compared between two groups.

2.2. *P. gingivalis* gingipains are essential for the enhancement of phagocytosis of *T. forsythia*

Gingipain mutant strains of *P. gingivalis* were used in the mixed infections to determine the role of gingipains in the enhancement of *T. forsythia* phagocytosis. The increase in the phagocytosis of *T. forsythia* was solely dependent on gingipains of *P. gingivalis*, because the increase was not observed when THP-1 cells were infected with *T. forsythia* in the presence of the gingipain-null mutant (KDP136) (Fig. 19A and B). Kgp- or Rgp-deficient mutants (KDP129 or KDP133, respectively) significantly facilitated the phagocytosis of *T. forsythia*, and the increase induced by the two mutant strains was similar. However, the increase in the proportion of *T. forsythia*-phagocytosing cells and the mean fluorescence intensity were about two-thirds and one-third of those induced by wild-type *P. gingivalis*, respectively (Fig. 19A and B). It was determined whether phagocytosis of *T. forsythia* is also enhanced by *P. gingivalis* in PBMC-derived macrophages. *T. forsythia* was more efficiently phagocytosed by PBMC-derived macrophages than by THP-1 cells, and *P. gingivalis* coinfection further increased *T. forsythia* phagocytosis in a gingipain-dependent manner (Fig. 19C and D). Confocal microscopy confirmed that CFSE-labelled *T. forsythia* was more abundantly internalized by THP-1 cells when the cells were coinfected with wild-type *P. gingivalis* than with KDP129 or KDP133, and the gingipain-null mutant KDP136 had no effect on the uptake of *T. forsythia* by macrophages (Fig. 19E). These results indicate that gingipains of *P. gingivalis* are major factors contributing to the augmentation of *T. forsythia* phagocytosis in mixed infections, and that both Kgp and Rgps are required to maximally boost phagocytosis.

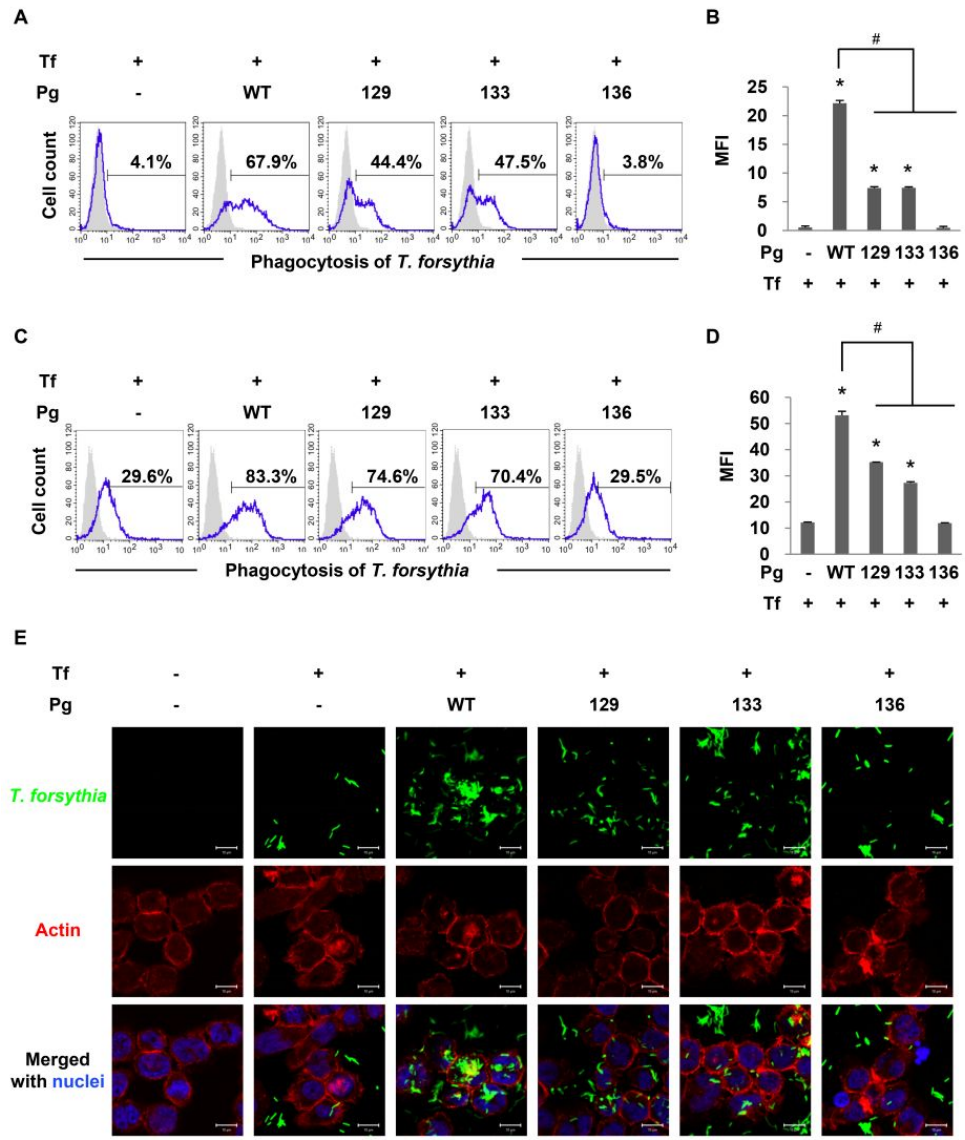


Figure 19. Gingipain mutations in *P. gingivalis* attenuate the enhancement of phagocytosis of *T. forsythia*. THP-1 cells (A, B, and E) and PBMC-derived macrophages (C, D) were infected with CFSE-labelled *T. forsythia* and unlabelled *P. gingivalis* at an MOI of 100 for 1 hour. (A, C) Histograms of the infected macrophages (solid lines) are shown with the mean percentage of macrophages positive for phagocytosed *T. forsythia*. The uninfected cells are shown as the gray-filled histogram. (B, D) The mean fluorescence intensity (MFI) of the infected

macrophages is shown as the mean ± standard deviation. The representative data of three independent experiments are shown. (E) The macrophages were fixed and examined under a confocal laser scanning microscope after staining F-actin (red) and nuclei (blue). CFSE-labelled *T. forsythia* is displayed in green. Tf, *T. forsythia*; Pg, *P. gingivalis*; WT, *P. gingivalis* ATCC 33277; 129, KDP129 (*kgp*$^-$); 133, KDP133 (*rgpA*$^-$ *rgpB*$^-$); 136, KDP136 (*kgp*$^-$ *rgpA*$^-$ *rgpB*$^-$). * $P < 0.05$ compared to *T. forsythia* monoinfection; $^{\#}$ $P < 0.05$ compared to coinfection with wild-type *P. gingivalis*.

2.3. *T. forsythia* augments phagocytosis of *P. gingivalis*

To examine whether *T. forsythia* coinfection influences phagocytosis of *P. gingivalis*, THP-1 cells were infected with CFSE-labelled *P. gingivalis* in the presence or absence of *T. forsythia*. Although *P. gingivalis* was phagocytosed well in monoinfection, *T. forsythia* coinfection further enhanced its phagocytosis in an MOI-dependent manner (Fig. 20A and B). When phagocytosis of gingipain mutants was assessed, the enhancement by *T. forsythia* was observed only in phagocytosis of wild-type *P. gingivalis* and KDP129, but not KDP133 nor KDP136 (Fig. 20C and D), indicating that the enhancement of *P. gingivalis* phagocytosis by *T. forsythia* is dependent on Rgps. It was also found that in contrast to the most substantial enhancement of *T. forsythia* phagocytosis by wild-type *P. gingivalis* (Fig. 19), wild-type *P. gingivalis* was less phagocytosed than KDP129 or KDP133 in monoinfection (Fig. 20C and D).

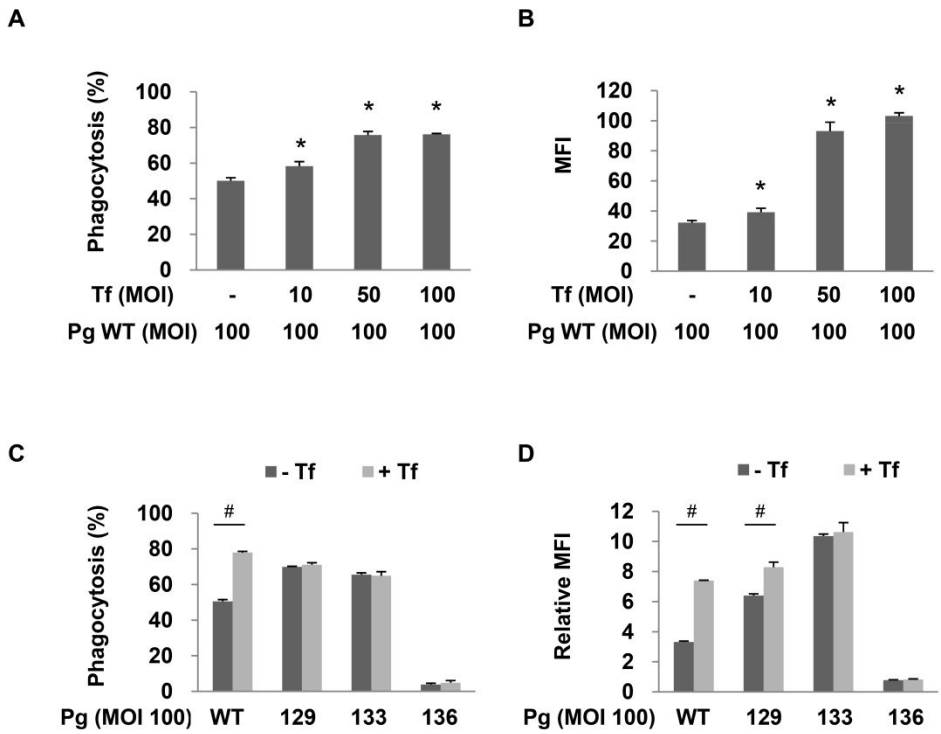


Figure 20. Phagocytosis of *P. gingivalis* is facilitated by *T. forsythia*. (A, B) THP-1 cells were infected with unlabelled *T. forsythia* and CFSE-labelled wild-type *P. gingivalis* at the indicated MOI for 1 hour. (C, D) THP-1 cells were infected with unlabelled *T. forsythia* and CFSE-labelled wild-type *P. gingivalis* or its gingipain mutants at an MOI of 100 for 1 hour. The percentage of macrophages positive for phagocytosed *P. gingivalis* (A, C) and the mean fluorescence intensity (MFI) of the infected macrophages (B, D) are shown as the mean ± standard deviation. The relative MFI is calculated as (MFI of the infected macrophages) / (the MFI of each *P. gingivalis* strain). Tf, *T. forsythia*; Pg, *P. gingivalis*; WT, *P. gingivalis* ATCC 33277; 129, KDP129 (*kgp*$^-$); 133, KDP133 (*rgpA*$^-$ *rgpB*$^-$); 136, KDP136 (*kgp*$^-$ *rgpA*$^-$ *rgpB*$^-$). * $P < 0.05$ compared to *P. gingivalis* monoinfection; $^{\#}$ $P < 0.05$ compared between two groups.

2.4. Coaggregation of *T. forsythia* with *P. gingivalis* that expresses gingipains contributes to the augmentation of phagocytosis of *T. forsythia*

To determine whether direct interaction of *T. forsythia* with *P. gingivalis* is required for the enhancement of *T. forsythia* phagocytosis, coaggregation between the two bacterial species and the effect of its inhibition on phagocytosis of *T. forsythia* were examined. *T. forsythia* coaggregated well (63% reduction of initial OD) with wild-type *P. gingivalis* (Fig. 21A), and the coaggregation was markedly inhibited by two basic amino acids, arginine and lysine (Fig. 21A). They also significantly alleviated the enhancement of *T. forsythia* phagocytosis by wild-type *P. gingivalis* in a dose-dependent manner (Fig. 21B). Histidine, the other basic amino acid, also moderately reduced the coaggregation and phagocytosis of *T. forsythia* in a concentration-dependent manner, although the inhibitory effect was weaker than those of arginine and lysine (Fig. 21A and B). In contrast, glycine, a neutral amino acid, had a minimal suppressive effect on phagocytosis of *T. forsythia*, although its inhibitory effect on coaggregation was comparable to that of histidine (Fig.21A and B). However, it is not clear whether the inhibitory effect of Arg, Lys, and His depends on their positive charges or whether Arg, Lys, and His residues in the outer membrane proteins are involved in the protein-protein interaction for the coaggregation.

Next, the coaggregating ability of gingipain mutants of *P. gingivalis* with *T. forsythia* was assessed. Coaggregation of *P. gingivalis* with *T. forsythia* was entirely dependent on gingipains because *P. gingivalis* KDP136 did not coaggregate with *T. forsythia* (Fig. 22A). The inhibitory effect of basic amino acids on coaggregation and on phagocytosis of *T. forsythia* in mixed infection with *P. gingivalis* KDP136 was negligible and similar to that observed in monoinfection

with *T. forsythia* (Fig. 22A and B). While *P. gingivalis* KDP129 or KDP133 coaggregated well with *T. forsythia*, the coaggregation was inhibited considerably by arginine and lysine, and weakly by histidine, but not by glycine (Fig. 22A). Likewise, phagocytosis of *T. forsythia* in mixed infection with either of the two mutants was suppressed extensively by arginine and lysine, and mildly by histidine, but not by glycine (Fig. 22B). These results indicate that the augmentation of *T. forsythia* phagocytosis in mixed infection with *P. gingivalis* requires direct interaction between the two species of bacteria, and that gingipains are key molecules required for the interspecies interaction.

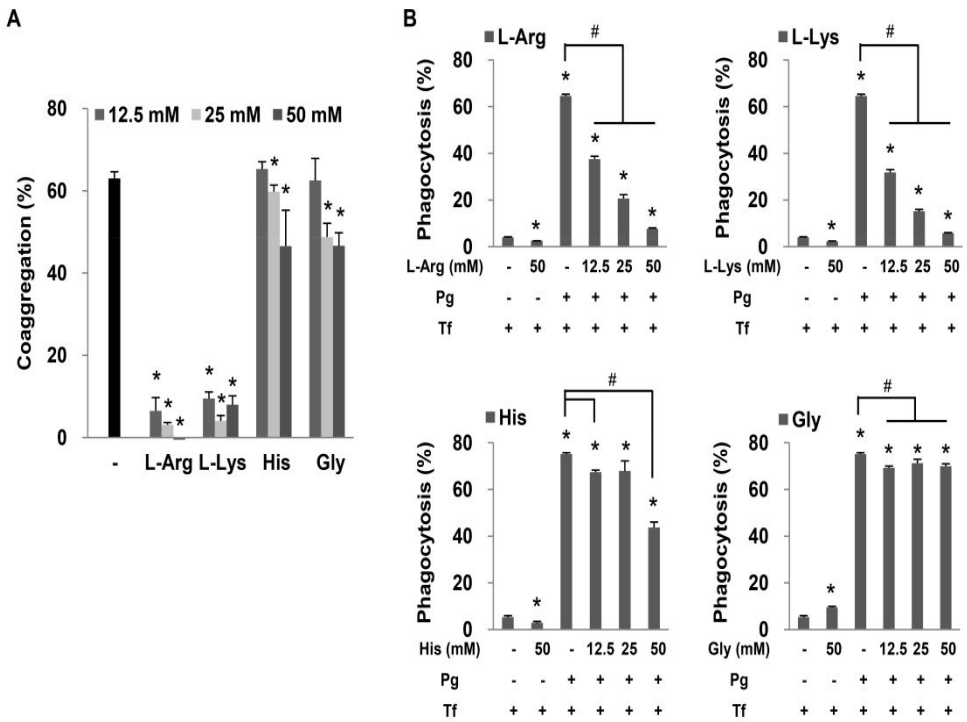


Figure 21. Inhibition of coaggregation reverses the boosting of *T. forsythia* phagocytosis by *P. gingivalis*. (A) Coaggregation activity between *T. forsythia* and wild-type *P. gingivalis* in the presence or absence of the indicated concentration of L-Arg, L-Lys, His, or Gly was measured as decrease (%) in OD_{660} after 90 min. * $P < 0.05$ compared to coaggregation in the absence of the amino acids. (B) Phagocytosis of CFSE-labelled *T. forsythia* by THP-1 cells was analyzed after 1 hour of infection at an MOI of 100 in the presence or absence of wild-type *P. gingivalis* and the indicated concentration of the amino acids. The percentage of macrophages positive for phagocytosed *T. forsythia* is shown as the mean ± standard deviation. The representative data of two independent experiments are shown. Tf, *T. forsythia* ; Pg, *P. gingivalis*. * $P < 0.05$ compared to *T. forsythia* monoinfection; $^{\#}$ $P < 0.05$ compared to coinfection with wild-type *P. gingivalis* in the absence of amino acids.

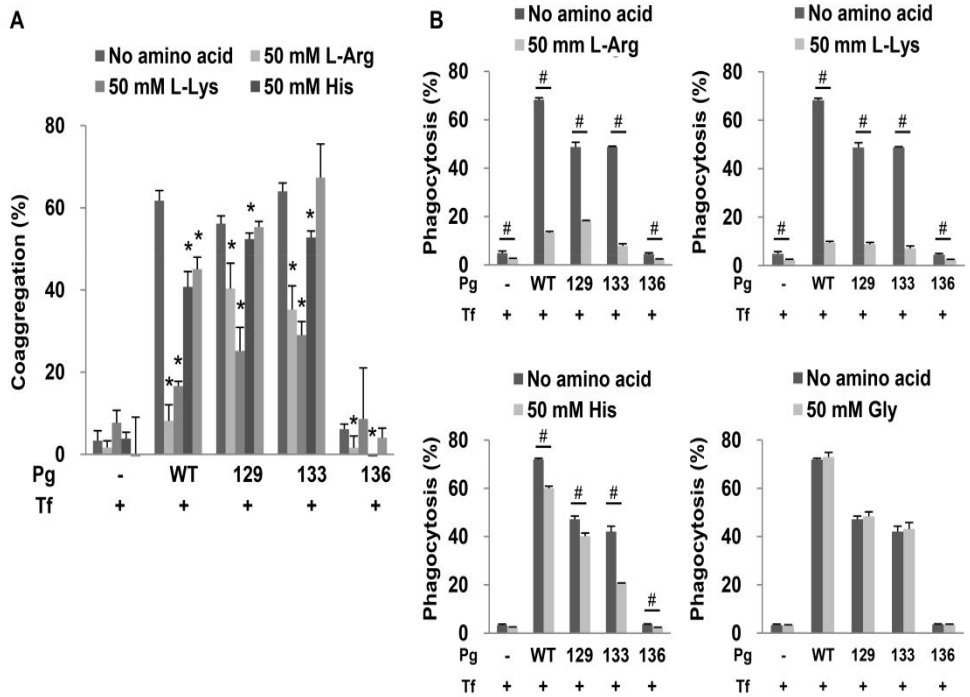


Figure 22. Gingipain-dependent coaggregation mediates the augmentation of phagocytosis of *T. forsythia*. (A) Coaggregation activity between *T. forsythia* and wild-type *P. gingivalis* or its gingipain mutants in the presence or absence of 50 mM L-Arg, L-Lys, His, or Gly was quantitated as decrease (%) in OD_{660} after 90 min. * $P < 0.05$ compared to coaggregation in the absence of the amino acids. (B) Phagocytosis of CFSE-labelled *T. forsythia* by THP-1 cells was analyzed after 1 hour of infection at an MOI of 100 in the presence or absence of *P. gingivalis* and 50 mM of the indicated amino acids. The percentage of macrophages positive for phagocytosed *T. forsythia* is shown as the mean ± standard deviation. The representative data of two independent experiments are shown. Tf, *T. forsythia*; Pg, *P. gingivalis*; WT, *P. gingivalis* ATCC 33277; 129, KDP129 (*kgp*$^-$); 133, KDP133 (*rgpA*$^-$ *rgpB*$^-$); 136, KDP136 (*kgp*$^-$ *rgpA*$^-$ *rgpB*$^-$). $^{\#}$ $P < 0.05$ compared between the two groups.

2.5. Protease activities of *P. gingivalis* gingipains are partially responsible for the facilitation of phagocytosis of *T. forsythia*

To investigate the role of gingipain protease activities in the enhancement of phagocytosis of *T. forsythia*, wild-type *P. gingivalis* was preincubated with KYT-36 or KYT-1 to inhibit Kgp or Rgps, respectively, shortly before infection. The inhibition of Kgp or Rgps of wild-type *P. gingivalis* significantly attenuated the enhancement of *T. forsythia* phagocytosis, with more reduction observed in the Kgp-inhibited *P. gingivalis* (Fig. 23A and B). The inhibition of both Kgp and Rgps further reduced the increment of *T. forsythia* phagocytosis. This result indicates that the protease activities of both Kgp and Rgps are required to maximally boost phagocytosis of *T. forsythia* in mixed infection with *P. gingivalis*, although other functions of gingipains may also be involved. Next, it was determined whether the protease activities of gingipains contribute to the enhanced phagocytosis via coaggregation between the two species of bacteria. The inhibition of Kgp or Rgps of wild-type *P. gingivalis* significantly lessened the coaggregation (Fig. 23C) in a manner similar to the reduction in phagocytosis of *T. forsythia* (Fig. 23A and B), suggesting that the protease activities of gingipains may contribute to the facilitation of phagocytosis of *T. forsythia* partly in a coaggregation-dependent manner.

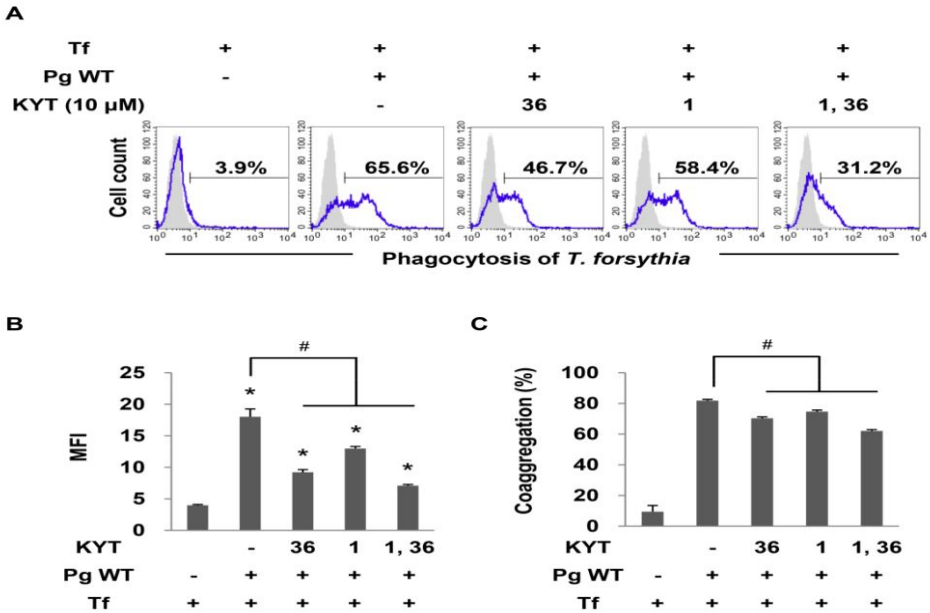


Figure 23. Protease activities of gingipains partially contribute to the enhancement of phagocytosis of *T. forsythia*. (A, B) THP-1 cells were infected at an MOI of 100 for 1 hour with CFSE-labelled *T. forsythia* and unlabelled wild-type *P. gingivalis* that had been preincubated with 10 µM KYT-36 (for Kgp inhibition) or KYT-1 (for Rgp inhibition) for 1 hour. Histograms with the mean percentage of macrophages positive for phagocytosed *T. forsythia* (A) and the mean fluorescence intensity (MFI) of the infected macrophages (B) are shown. * $P < 0.05$ compared to *T. forsythia* monoinfection; # $P < 0.05$ compared to coinfection with wild-type *P. gingivalis* without preincubation with KYT-1 or KYT-36. (C) Coaggregation activity between *T. forsythia* and wild-type *P. gingivalis* that had been preincubated with KYT-36 or KYT-1 was measured as decrease (%) in OD_{660} after 90 min. The representative data of two independent experiments are shown as the mean ± standard deviation. # $P < 0.05$ compared to coaggregation of *T. forsythia* and *P. gingivalis* without preincubation with KYT-1 or KYT-36. Tf, *T. forsythia*; Pg WT, *P. gingivalis* ATCC 33277.

2.6. Direct effect of soluble gingipains on *T. forsythia* is minimal

To investigate whether protease activities have a direct effect on *T. forsythia*, VDS of *P. gingivalis* was used. Preincubation of *T. forsythia* with VDS of *P. gingivalis* expressing gingipains led to a slight increase in *T. forsythia* phagocytosis, and this increase was reversed by the gingipain-specific inhibitor KYT-36 (Fig. 24A and B). In contrast, infection of macrophages with *T. forsythia* in the presence or absence of *P. gingivalis* VDS after preincubation of the cells with *P. gingivalis* VDS resulted in a minimal decrease in *T. forsythia* phagocytosis, which was not reversed by gingipain inhibitors (Fig. 24C and D). These results indicate that changes in macrophages induced by *P. gingivalis* VDS, possibly by some factors other than gingipains, do not contribute to the enhancement of *T. forsythia* phagocytosis. Taken together, these results suggest that gingipains might expose *T. forsythia* epitopes although the effect is minimal and that other mechanisms of protease activities of gingipains may be involved in the substantial increase of *T. forsythia* phagocytosis.

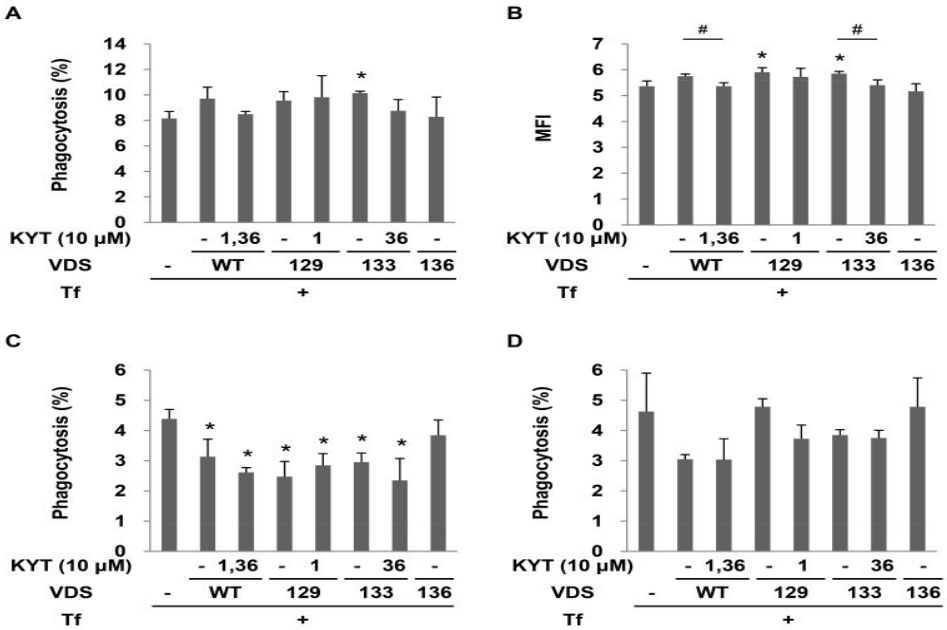


Figure 24. Preincubation of *T. forsythia* with culture supernatants containing gingipains minimally enhances phagocytosis of *T. forsythia*. (A, B) CFSE-labelled *T. forsythia* was preincubated with fresh culture media containing 30% VDS of *P. gingivalis* in the presence or absence of KYT-1 or KYT-36. THP-1 cells were then infected with the *T. forsythia* for 1 hour. (C, D) THP-1 cells were preincubated for 1 hour with VDS of *P. gingivalis* (equivalent to an MOI of 100) in the presence or absence of KYT-1 or KYT-36. The cells were then infected for 1 hour with CFSE-labelled *T. forsythia* in the presence (C) or absence (D) of *P. gingivalis* VDS. The percentage of macrophages positive for phagocytosed *T. forsythia* and the mean fluorescence intensity (MFI) of the infected macrophages are shown as the mean ± standard deviation. * $P < 0.05$ compared to *T. forsythia* without preincubation with *P. gingivalis* VDS; # $P < 0.05$ compared between the two groups. Tf, *T. forsythia*; Pg, *P. gingivalis*; WT, *P. gingivalis* ATCC 33277; 129, KDP129 (*kgp*$^-$); 133, KDP133 (*rgpA*$^-$ *rgpB*$^-$); 136, KDP136 (*kgp*$^-$ *rgpA*$^-$ *rgpB*$^-$).

2.7. More *T. forsythia* remain viable in the infected cells in *P. gingivalis* coinfection

To examine the effect of *P. gingivalis* on the persistence of phagocytosed *T. forsythia*, the presence of CFSE-labelled *T. forsythia* remaining in infected macrophages up to 48 hours post-infection was measured using flow cytometry and confocal microscopy. In monoinfection with *T. forsythia* or coinfection with *P. gingivalis* KDP136, the intracellular load of *T. forsythia* remained minimal from 0 to 48 hours post-infection. After infection with *T. forsythia* in the presence of *P. gingivalis* expressing gingipains, which resulted in more phagocytosis of *T. forsythia*, the percentage of macrophages with intracellular *T. forsythia* and the mean fluorescence intensity gradually declined over time (Fig. 25A), indicating clearance of the phagocytosed *T. forsythia*. However, more *T. forsythia* seemed to remain in the infected cells, even after 48 hours, if the cells were coinfected with gingipain-expressing *P. gingivalis* than if they were infected with *T. forsythia* alone or coinfected with the gingipain-null mutant, KDP136 (Fig. 25A and B).

To determine whether *P. gingivalis* infection modulates phagosomal maturation after *T. forsythia* phagocytosis, colocalization of CFSE-labelled bacteria with lysosomes was examined using LysoTracker Red. In *P. gingivalis* monoinfection, KDP129 and KDP133 induced more acidified phagolysosomes than the wild-type (Fig. 26). This is consistent with the phagocytosis results of *P. gingivalis* in monoinfection (Fig. 20C and D), indicating that phagocytosis of *P. gingivalis* induces phagosomal maturation. Most intracellular *P. gingivalis* colocalized with lysosomes. On the contrary, *T. forsythia* infection alone did not induce acidification of phagosomes (Fig. 27). In mixed infection with *T. forsythia* and *P. gingivalis*, acidification of phagosomes was more intense after coinfection

with *P. gingivalis* KDP129 and KDP133 than coinfection with the wild-type (Fig. 27), which is similar to those after *P. gingivalis* monoinfection (Fig. 26). While some intracellular *T. forsythia* colocalized with mature phagosomes after coinfection with *P. gingivalis*, the other intracellular *T. forsythia* did not. These results imply that *T. forsythia* alone does not induce phagosomal maturation and that phagosomes containing *T. forsythia* can undergo maturation when they also contain *P. gingivalis*.

RNA-based viability assay using *T. forsythia*-specific 16S rRNA revealed that the number of viable *T. forsythia* within the infected cells decreases over time, with 1-2% of initial bacterial load remaining viable after 48 hours (Fig. 28A, on a logarithmic scale; 28B, on a linear scale). This result indicates that clearance of *T. forsythia* can be accelerated by *P. gingivalis* coinfection. Nevertheless, the number of viable *T. forsythia* within macrophages was higher after coinfection with *P. gingivalis* expressing gingipains than after monoinfection with *T. forsythia* or coinfection with the gingipain-null mutant KDP136 (Fig. 28A and B).

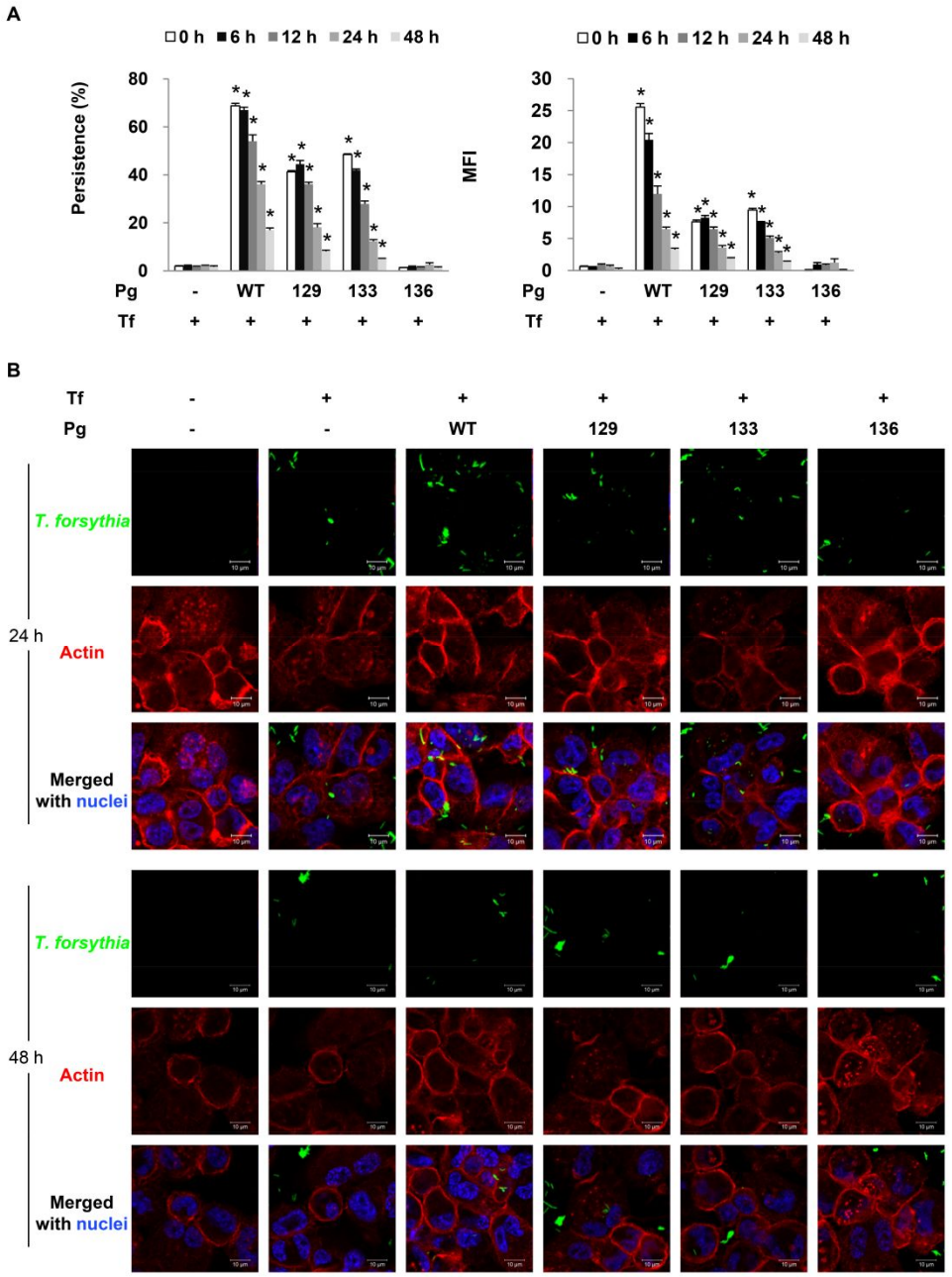


Figure 25. Most *T. forsythia* is cleared in macrophages, but with more *T. forsythia* remaining after coinfection with *P. gingivalis* than after monoinfection. After THP-1 cells were infected with CFSE-labelled *T. forsythia* and unlabelled *P. gingivalis* at an MOI of 100 for 1 hour, extracellular bacteria

were killed by incubation with 300 μg/ml gentamicin and 200 μg/ml metronidazole for 1 hour. The cells were then further incubated for the indicated time. (A) The percentage of macrophages positive for remaining *T. forsythia* and the mean fluorescence intensity (MFI) of the infected macrophages are shown as the mean ± standard deviation. The representative data of two independent experiments are shown. * $P < 0.05$ compared to *T. forsythia* monoinfection. (B) After 24 and 48 hours of infection, the cells were fixed and examined under a confocal laser scanning microscope after staining F-actin (red) and nuclei (blue). CFSE-labelled *T. forsythia* is displayed in green. The representative data of two independent experiments are shown. Tf, *T. forsythia*; Pg, *P. gingivalis*; WT, *P. gingivalis* ATCC 33277; 129, KDP129 (*kgp*$^-$); 133, KDP133 (*rgpA*$^-$ *rgpB*$^-$); 136, KDP136 (*kgp*$^-$ *rgpA*$^-$ *rgpB*$^-$).

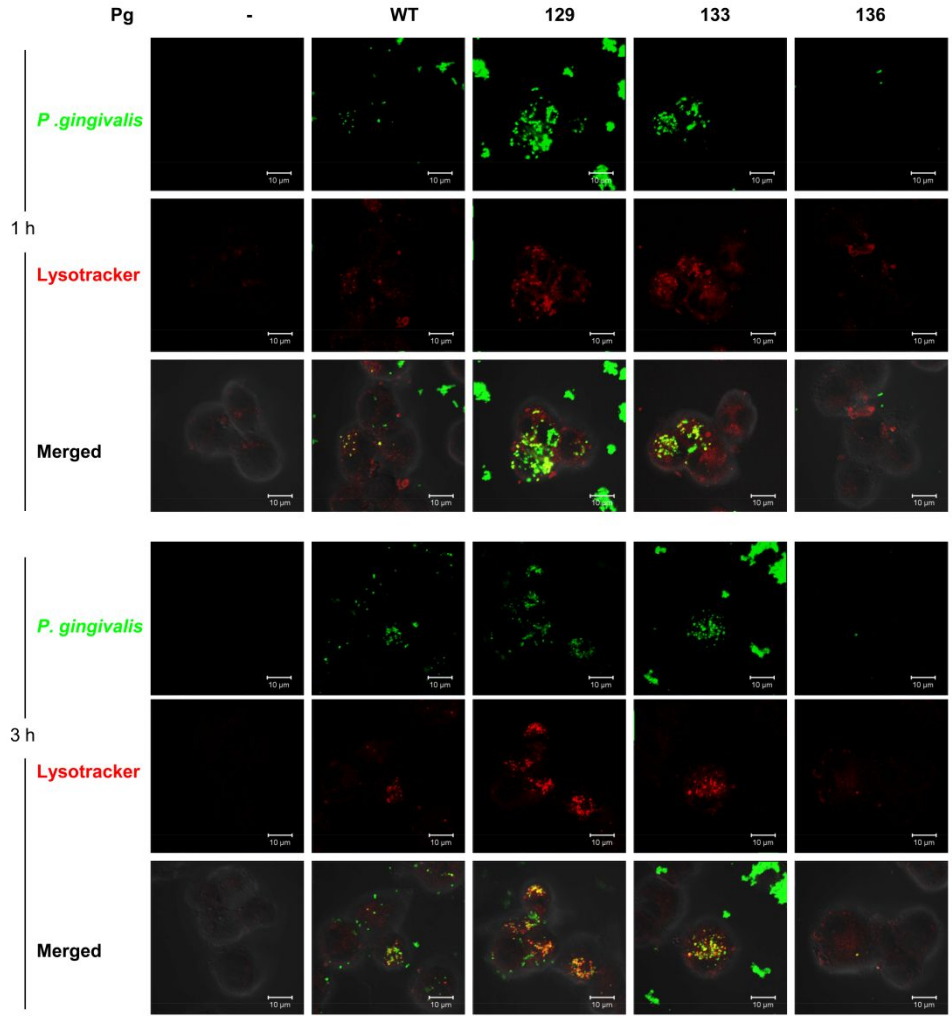


Figure 26. Phagocytosis of *P. gingivalis* induces phagosomal maturation. After THP-1 cells were infected with CFSE-labelled *P. gingivalis* at an MOI of 100 for 1 or 3 hours, the cells were stained with LysoTracker Red (red) for 30 minutes. The cells were fixed and examined under a confocal laser scanning microscope. CFSE-labelled *P. gingivalis* is displayed in green. Pg, *P. gingivalis*; WT, *P. gingivalis* ATCC 33277; 129, KDP129 (*kgp*$^-$); 133, KDP133 (*rgpA*$^-$ *rgpB*$^-$); 136, KDP136 (*kgp*$^-$ *rgpA*$^-$ *rgpB*$^-$).

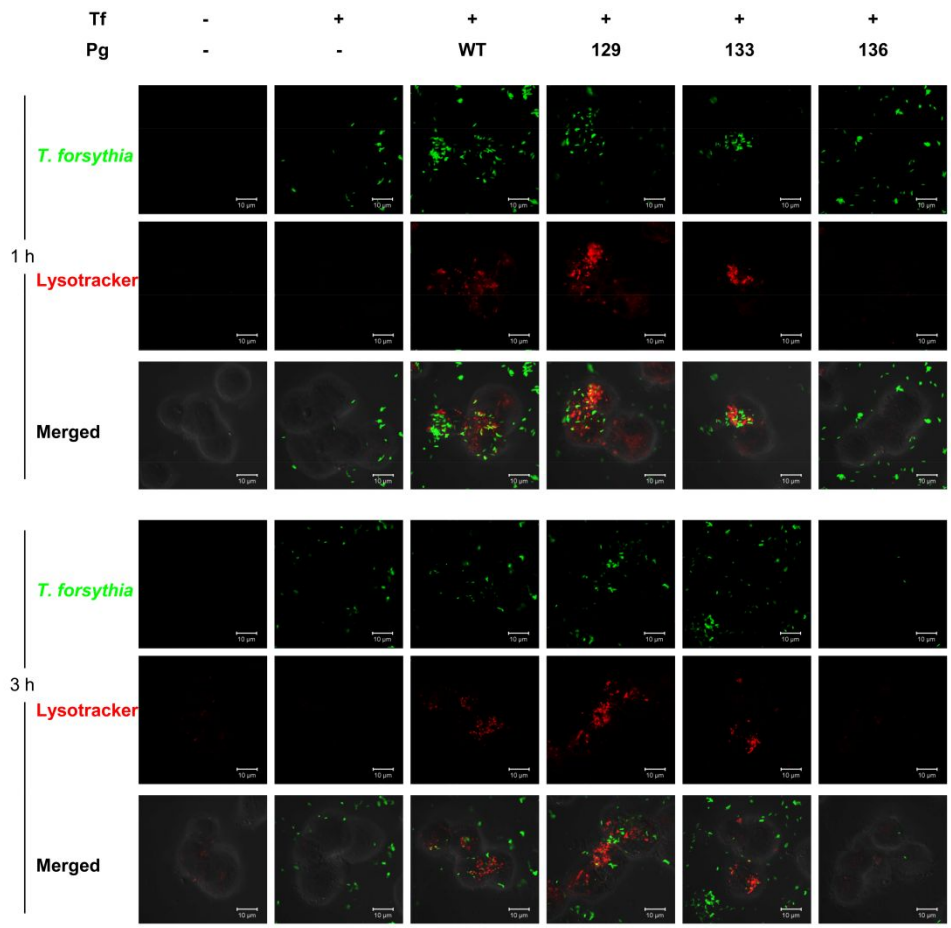


Figure 27. Some *T. forsythia* were found in mature phagosomes after coinfection with *P. gingivalis*. After THP-1 cells were infected with CFSE-labelled *T. forsythia* and unlabelled *P. gingivalis* at an MOI of 100 for 1 or 3 hours, the cells were stained with LysoTracker Red (red) for 30 minutes. The cells were fixed and examined under a confocal laser scanning microscope. CFSE-labelled *T. forsythia* is displayed in green. Tf, *T. forsythia*; Pg, *P. gingivalis*; WT, *P. gingivalis* ATCC 33277; 129, KDP129 (*kgp*$^-$); 133, KDP133 (*rgpA*$^-$ *rgpB*$^-$); 136, KDP136 (*kgp*$^-$ *rgpA*$^-$ *rgpB*$^-$).

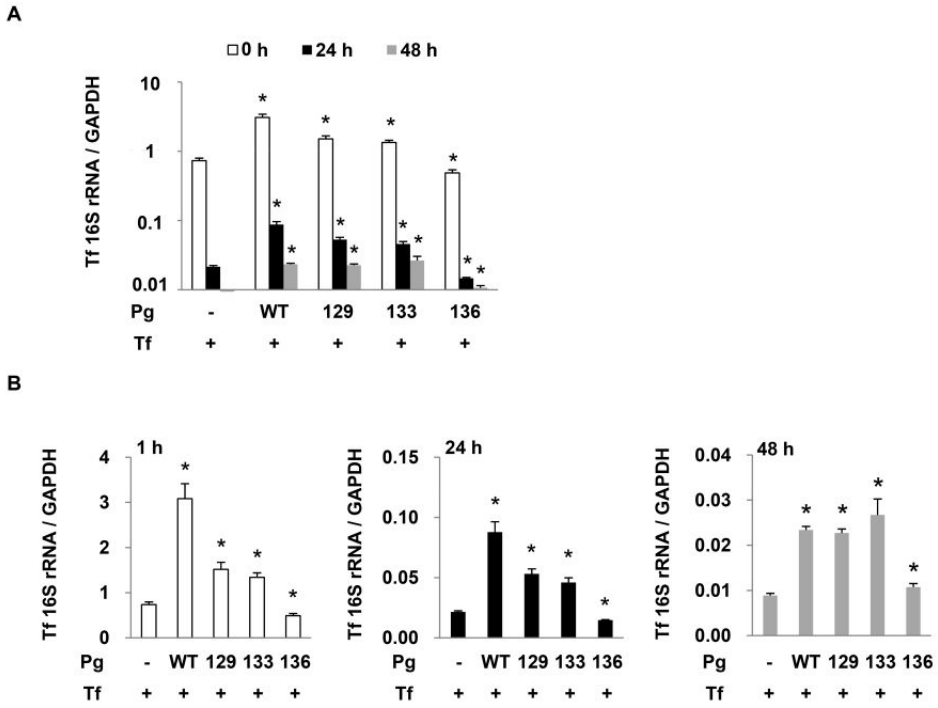


Figure 28. More *T. forsythia* remains viable within infected macrophages after coinfection with *P. gingivalis* expressing gingipains. After 1 hour of infection with *T. forsythia* in the presence or absence of *P. gingivalis* at an MOI of 100, extracellular bacteria were killed by incubation with 300 μg/ml gentamicin and 200 μg/ml metronidazole for 1 hour. The cells were then further incubated for the indicated time. Relative amount of *T. forsythia*-specific 16S rRNA in macrophages is shown as the mean ± standard deviation on a logarithmic scale (A) and on a linear scale (B). The representative data of two independent experiments are shown. * $P < 0.05$ compared to *T. forsythia* monoinfection. Tf, *T. forsythia*; Pg, *P. gingivalis*; WT, *P. gingivalis* ATCC 33277; 129, KDP129 (*kgp*$^-$); 133, KDP133 (*rgpA*$^-$ *rgpB*$^-$); 136, KDP136 (*kgp*$^-$ *rgpA*$^-$ *rgpB*$^-$).

2.8. More *T. forsythia*-infected cells remain viable in the presence of *P. gingivalis*

Next, the effect of *P. gingivalis* coinfection on viability of macrophages was examined. Interestingly, the cell viability was increased by coinfection with wild-type *P. gingivalis* while it was decreased by *T. forsythia* monoinfection (Fig. 29). Coinfection with KDP129 or KDP133 also slightly increased the cell viability while KDP136 did not. Together, these results indicate that *P. gingivalis* coinfection leads to more viable *T. forsythia* within more macrophages even after 48 hours post-infection.

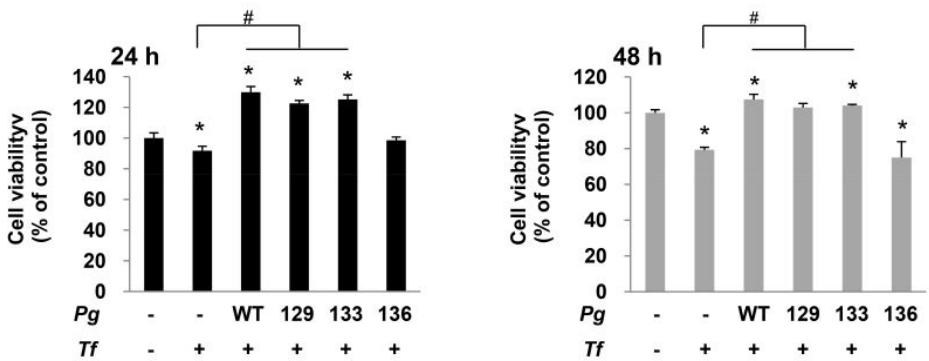


Figure 28. Coinfection with *P. gingivalis* improves the viability of macrophages infected with *T. forsythia*. The cell viability was assessed using the Cell Counting Kit-8 (CCK-8) after THP-1 cells were infected with *T. forsythia* in the presence or absence of *P. gingivalis* at an MOI of 100 for 24 or 48 hours. The representative data of two independent experiments are shown as the mean ± standard deviation. Tf, *T. forsythia*; Pg, *P. gingivalis*; WT, *P. gingivalis* ATCC 33277; 129, KDP129 (*kgp*$^-$); 133, KDP133 (*rgpA*$^-$ *rgpB*$^-$); 136, KDP136 (*kgp*$^-$ *rgpA*$^-$ *rgpB*$^-$). * $P < 0.05$ compared to the uninfected control; $^{\#}$ $P < 0.05$ compared to *T. forsythia* monoinfection.

IV. Discussion

1. Role of gingipains in caspase-1 activation

This study demonstrated that gingipains play complicated roles in *P. gingivalis* infection, resulting in contradictory effects on caspase-1 activation and its consequences. On one hand, gingipains positively contribute to the induction of caspase-1 activation in infected cells. *P. gingivalis* with gingipains induced caspase-1 activation more efficiently than the gingipain-null mutant, and the enhancement of the caspase-1-activating potential of *P. gingivalis* by gingipains was associated with increases in ATP release from the infected macrophages. Furthermore, the incubation of the gingipain-null mutant in the gingipain-containing culture supernatants augmented caspase-1 activation in the mutant-infected cells, which could be reversed by gingipain inhibitors. On the other hand, the protease activity of gingipains had some apparently contradictory effects. The inhibition of the protease activity of gingipains during *P. gingivalis* infection potentiated caspase-1 activation, which was also associated with an increase in ATP release from the infected cells. In addition, gingipains proteolytically depleted proteins that can propagate inflammation after being released as a result of caspase-1 activation.

Some previous studies have reported that *P. gingivalis* activates NLRP3 and AIM2 inflammasomes [121,135,136], while other studies have shown that *P. gingivalis* fails to activate caspase-1 [22,122,137]. The results in this study suggest that the protease activities of *P. gingivalis* have a substantial influence on the result and its interpretation. It was found that low MOIs, but not high MOIs, of *P. gingivalis* could induce low levels of IL-1β without detectable active caspase-1 in

the culture supernatants of infected cells. This induction could be a result of alternative IL-1β processing by *P. gingivalis* proteases because some microbial proteases including *Candida albicans* and *Staphylococcus aureus* have been demonstrated to process pro-IL-1β to active IL-1β [145-147]. However, further experiments using leupeptin precluded the possibility of IL-1β processing by *P. gingivalis* proteases because the inhibition of the bacterial proteases enhanced, rather than decreased, IL-1β levels. Moreover, the active subunit (p20) of caspase-1 was detected by preventing its proteolytic degradation, revealing that *P. gingivalis* infection induces caspase-1 activation.

This study showed that caspase-1 and LDH in the culture supernatants, which are commonly used to determine caspase-1 activation, are susceptible to proteolysis by gingipains. This proteolysis may partly explain discrepancies among the studies reporting inconsistent results regarding caspase-1 activation by *P. gingivalis* infection [22,121,122,135-137] because the expression and activity of gingipains can be different depending on strains and experimental conditions used; changes in the growth phase, availability of hemin, and oxidative stress levels could feasibly alter the results by affecting gingipains activity [148,149]. These findings also indicate that using only protein-based methods (e.g., western blot, ELISA, and LDH cytotoxicity assay) can be misleading in the analysis of caspase-1 activation by pathogens with potent proteolytic activity, supporting the need for the development of new analytical tools that can cumulatively assess caspase-1 activation without being affected by proteolysis.

Several pathogens use strategies to manipulate inflammasome activation by producing inhibitors that block inflammasome activation or by regulating the production or recognition of bacterial ligands that trigger inflammasomes [150]. It

has been reported that *P. gingivalis* differentially regulates the gene expression of inflammasome components in different cell types. While the expression of NLRP3 and AIM2 is increased by *P. gingivalis* infection in macrophages [121], *P. gingivalis* in a multi-species subgingival biofilm down-regulates the expression of NLRP3 in gingival fibroblasts although the expression of caspase-1 is not directly affected by the presence of *P. gingivalis* [151,152]. The biofilm challenge results in differential effects on the expression of caspase-1, ASC, and AIM2 depending on the bacterial burden [151]: increase at low bacterial load and decrease at high load. In the present study, the protein level of IL-1β showed a similar trend although the gene expression of IL-1β increased with the increasing MOIs, which may facilitate the pathogen survival by disrupting the host immune responses. The gene expression of IL-1β is also differently regulated depending on the cell types in response to *P. gingivalis*. While decreasing in gingival fibroblasts, it increases in macrophages and monocytes [121,152,153]. Notably, in monocytes, the up-regulation of IL-1β gene expression is partially dependent on gingipains, whereas that of IL-18 is not affected by gingipains [153].

P. gingivalis utilizes gingipains to modulate caspase-1 activation and to interfere with host defense mechanisms. An interesting observation from this study is that gingipains act as modulating factors of ATP release from *P. gingivalis*-infected cells. A recent study reported that ATP release, along with lysosomal disruption and cytosolic DNA, mediates caspase-1 activation in *P. gingivalis*-infected macrophages [121], which is consistent with the result in this study. ATP release occurs through both a lytic and non-lytic pathway, and mechanical stimuli or agonists of some receptors are known to induce ATP release [154]. Although how ATP release is triggered by *P. gingivalis* infection remains unknown, the

results of this study indicate that gingipains affect the ability of *P. gingivalis* to activate ATP release from the infected cells.

Gingipains have contradictory effects on caspase-1 activation through multilayered mechanisms, including adhesins and protease activity. Overall, gingipains, in particular Rgps, facilitate caspase-1 activation of *P. gingivalis*-infected cells because the gingipain-null mutant induced a lower degree of caspase-1 activation than that induced by *P. gingivalis* with gingipains. This result is the consequence of the combined effects of multiple roles of gingipains. First, this result could be due to hemagglutinin/adhesin domains of Kgp and RgpA (Fig. 30A) because a previous study demonstrated that the adhesin subunits of gingipains induce IL-1β secretion from macrophages [155]. In addition, increases in the caspase-1-activating potential of the gingipain-null mutant after incubation with gingipain-containing supernatants suggests that bacterial proteins processed by gingipains contribute to the caspase-1 activation in *P. gingivalis*-infected cells (Fig. 30B and 30B'). It is needed to elucidate which proteins contribute to caspase-1 activation among the bacterial proteins that are processed by gingipains.

Notably, the protease action of gingipains during the infection process seems to have opposite effects. The inhibition of Kgp or Rgps augmented caspase-1 activation by wild-type *P. gingivalis*, but the inhibition of both Kgp and Rgp did not further enhance caspase-1 activation compared to the inhibition of Kgp or Rgps. This result indicates that the combined proteolytic activity of Kgp and Rgps has inhibitory effects on caspase-1 activation. However, it is not clear whether Kgp and Rgps cleave the same proteins of host cells. Further research using overexpression of a gingipain in the mutant strain that is deficient in the other gingipain might give an answer to the question. The inhibitory effect of gingipain activity may be

attributed to the proteolytic cleavage of host cell proteins (Fig. 30C). A number of studies have reported that *P. gingivalis* gingipains dysregulate the host immune response by proteolytically inactivating or activating host cell surface receptors and proteins such as CD14, IL-6R, and protease-activated receptors [156-161]. Because the inhibition of gingipain protease activity increased ATP release from the infected cells, some host proteins that were rescued from cleavage by Kgp and Rgps may be associated with ATP release. Another possibility is that the protease action of Kgp and Rgps may expose some cryptic inhibitory receptors and that the inhibition of gingipains may prevent the exposure of such inhibitory receptors. Further research is needed to determine which host proteins are affected by Kgp or Rgps.

The proteolysis of host-derived proteins by gingipains is an important mechanism used by *P. gingivalis* to neutralize host defense responses and to evade immune systems. The degradation of IL-1β observed in this study (Fig. 30D) is consistent with the results of previous studies demonstrating the disruption of cytokine networks by *P. gingivalis* [44,162]. The transient appearance of a smaller fragment of IL-1β in this study may result from the partial proteolysis by Rgps at Arg^{120} or Arg^{127}, which can be inferred from the size of the fragment. Whether this fragment retains biological activity remains to be determined. Furthermore, this study assessed proteolysis of other proteinaceous proinflammatory mediators. Recent studies have demonstrated that HMGB1, inflammasome particles, and ASC specks amplify inflammatory responses after being released into the extracellular milieu following inflammasome activation [142-144]. Notably, all of these proteins were cleared by *P. gingivalis* through proteolysis, mainly by gingipains (Fig. 7D). The rate of degradation and susceptibility to each gingipain may depend on the number of Arg or Lys residues and the adjacent amino acids [163].

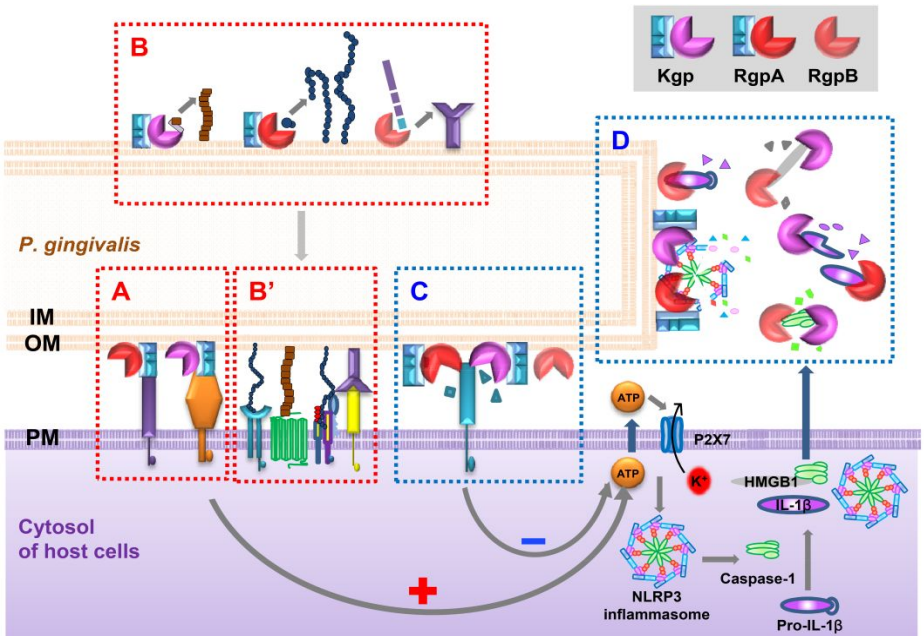


Figure 30. Plausible roles of gingipains in caspase-1 activation and its sequelae following *P. gingivalis* infection. The presence of gingipains in *P. gingivalis* promotes ATP release, thereby contributing to the activation of the NLRP3 inflammasome and caspase-1. Hemagglutinin/adhesin domains of Kgp and Rgps (A) and other bacterial surface proteins (B') processed by gingipains (B) would contribute to the enhancement of caspase-1 activation. However, the collaborative protease activity of Kgp and Rgps during the infection process, possibly through the cleavage of host proteins, decreases the level of ATP released from the infected cells, resulting in the attenuation of caspase-1 activation (C). Moreover, IL-1β, HMGB1, and inflammasome components, which are released from caspase-1-activated cells and amplify inflammation, are proteolytically depleted by gingipains (D). This depletion may prevent the recruitment of immune cells and the clearance of *P. gingivalis*, allowing for persistent infection and systemic dissemination. IM, inner membrane of *P. gingivalis*; OM, outer membrane of *P. gingivalis*; PM, plasma membrane of a host cell.

2. Role of gingipains in augmentation of *T. forsythia* phagocytosis

The present study demonstrated that coinfection with *P. gingivalis* boosted phagocytosis of *T. forsythia* by macrophages, and that both Rgps and Kgp of *P. gingivalis* were essential for the maximal boosting. The increase in phagocytosis of *T. forsythia* was dependent on the coaggregation between the two species of bacteria, which was also dependent on *P. gingivalis* gingipains. The contribution of gingipains seemed to involve both protease and non-protease activities. Coinfection with *P. gingivalis* did not prevent the clearance of the phagocytosed *T. forsythia*, but more *T. forsythia* remained in cells that were coinfected with gingipain-expressing *P. gingivalis* than in cells that were infected with *T. forsythia* alone.

Pathological tissue destruction in periodontal diseases results from host responses to polymicrobial biofilms accumulated in the subgingival area, and synergistic virulence of periodontal pathogens in animal models has been reported [79,164,165]. Therefore, it is important to understand the host responses not only to each bacterial species but also to mixed infections. The facilitated phagocytosis of *T. forsythia* and *P. gingivalis* by each other may contribute to their synergistic pathogenicity in vivo such as abscess formation and alveolar bone loss in murine models [79,80].

Gingipains are not only essential for the pathogenicity of *P. gingivalis* monoinfection but are also indispensable for the synergistic virulence of *P. gingivalis* and *T. forsythia*, including abscess formation [79], IL-6 production in macrophages [85], and stimulation of each other's growth [81,82], as well as inactivation of complement pathways [72]. In this study, the enhancement of *T. forsythia* phagocytosis by *P. gingivalis* was also completely dependent on the

presence of Kgp and Rpgs, because deficiency in either gingipain in *P. gingivalis* significantly decreased the enhancement, while the gingipain-null mutant did not have any effect on the phagocytosis. Protease actions of gingipains during the infection process were partly responsible for the ability of *P. gingivalis* to promote *T. forsythia* phagocytosis. However, direct effect of gingipain protease activities on *T. forsythia* seems miniscule, because preincubation of *T. forsythia* in the VDS of *P. gingivalis* only minimally increased phagocytosis of *T. forsythia*.

Gingipains seem to contribute to the enhancement of *T. forsythia* phagocytosis by mediating the interaction between the two species of bacteria, because inhibition of bacterial coaggregation significantly reduced the enhancement, and the lack of gingipains abrogated the coaggregation. The largest increase in *T. forsythia* phagocytosis by wild-type *P. gingivalis* despite less phagocytosis of itself than KDP129 or KDP133 suggests that the phagocytosis of *T. forsythia* was increased via complex interactions between the two bacterial species and macrophages. Phagocytosis of bacteria by macrophages involves multiple receptors and signaling pathways [166,167], and the bacterial coaggregate may harbor more ligands that can lead to receptor clustering and activation of the signaling pathways than does either individual bacterial species, possibly resulting in facilitation of phagocytosis. However, the exact mechanisms by which gingipains mediate the coaggregation of *P. gingivalis* and *T. forsythia* and by which the coaggregation promotes phagocytosis of *T. forsythia* remain to be elucidated.

Inhibition of coaggregation by amino acids, in particular arginine and lysine, suggests that protein-protein interactions may mediate the coaggregation. Previous studies have demonstrated the role of the adhesin domains of gingipains and the contribution of gingipains via processing of other adhesins to coaggregation of *P.*

gingivalis with other bacterial species, such as *T. denticola*, *Prevotella intermedia*, and *Actinomyces viscosus* [168-170]. These studies are consistent with the observations that gingipains are required for coaggregation and that inhibition of protease activities only partially reduces the coaggregation. Adhesins of *P. gingivalis* that are processed by gingipains and contribute to coaggregation with *T. forsythia* have to be elucidated.

It has been reported that the S-layer of *T. forsythia*, but not BspA, is involved in coaggregation with *P. gingivalis* [67,171]. In addition, *T. forsythia* S-layer delays immune responses in macrophages [69]. It also has been reported that glycosylation of *T. forsythia* S-layer protects the bacteria from binding to DCs and regulates cytokine expression in DCs and macrophages [70]. Therefore, *T. forsythia* S-layer or its glycosylation might contribute to the low phagocytosis rate of *T. forsythia* by hindering the bacteria from binding to macrophages, and protein expression or glycosylation of *T. forsythia* S-layer might be affected by *P. gingivalis*. However, further research to explore this possibility is needed.

Porphyromonas gingivalis infection impairs epithelial integrity and facilitates the invasion of oral bacteria into gingival tissues [172], and recruits macrophages to gingival tissues [92]. Therefore, the chance for *T. forsythia* to be phagocytosed by macrophages will further increase. Enhanced phagocytosis of *T. forsythia* by macrophages in coinfection with *P. gingivalis* can contribute to efficient clearance of the invading bacteria. However, more *T. forsythia* remained viable in macrophages coinfected with *P. gingivalis* after 24 and 48 hours than in monoinfected cells although *P. gingivalis* did not prevent the clearance of *T. forsythia*. In addition, macrophages coinfected with *T. forsythia* and *P. gingivalis* remained more viable than the cells infected only with *T. forsythia*. Considering

that macrophages can migrate from inflamed tissues to lymph nodes [173,174], the detection of periodontal pathogens, including *P. gingivalis* and *T. forsythia*, in lymph nodes draining the oral cavity [175], and possibly in distant sites such as atherosclerotic lesions and placenta [176,177], may be attributable to the intracellular bacteria that remains in infected macrophages. Given that oral infection with *P. gingivalis* increases macrophage infiltration in draining lymph nodes [92], coinfection with *P. gingivalis* may facilitate systemic dissemination of *T. forsythia*. Further research is required to determine whether the enhanced phagocytosis of *T. forsythia* contributes to host defenses by efficient clearance, or contributes to systemic dissemination of the invading bacteria.

To summarize, gingipain-dependent enhancement by *P. gingivalis* of *T. forsythia* phagocytosis was observed in macrophages, and this enhancement was mediated by coaggregation of the two bacterial species. More *T. forsythia* remained viable in macrophages after mixed infection with gingipain-expressing *P. gingivalis* than after monoinfection with *T. forsythia*. These results imply that the presence of *P. gingivalis* and its gingipains influences the clearance of *T. forsythia* and may significantly alter host immune responses to *T. forsythia*.

3. Pathological and clinical implication

Because gingipains are essential to the pathogenicity of *P. gingivalis*, they represent promising therapeutic targets for the prevention and treatment of periodontitis and other *P. gingivalis*-associated diseases. The present study demonstrates that several functions of Rgps and Kgp can differentially and contradictorily contribute to the pathogenicity.

A previous study has shown the increased expression of caspase-1 and other inflammasome components (NLRP3 and AIM2) in the gingival tissue of periodontal patients [121]. Gingipain-dependent caspase-1 activation in *P. gingivalis*-infected macrophages observed in this study may contribute to the exacerbation of inflammation through release of proinflammatory mediators.

Interestingly, the protease activities of gingipains exhibit inhibitory effects on caspase-1 activation while they positively contribute to the enhancement of *T. forsythia* phagocytosis. Attenuation of inflammasome activation and deactivation of proteinaceous proinflammatory mediators by the protease activity of gingipains may help *P. gingivalis* and coinfecting *T. forsythia* avoid detection by the host defense system. This avoidance may offer a survival advantage to *P. gingivalis* and *T. forsythia*, and possibly other co-infecting microorganisms in polymicrobial infections. In addition, *P. gingivalis* may contribute to dissemination of coinfecting *T. forsythia* by increasing the number of uncleared bacteria in infected macrophages, and by promoting migration of the macrophages into systemic circulation.

Due to complicated functions of gingipains, the preventive/therapeutic outcomes of gingipain inhibitors may vary depending on the therapeutic approaches. For example, transient inhibition of protease activities of gingipains

may not only enhance caspase-1 activation and the release of IL-1β and DAMPs, but it may also preserve proinflammatory mediators in infection sites. In contrast, the prolonged use of gingipain inhibitors or inhibitors targeting the expression of gingipains may lead to a decrease in caspase-1 activation. In mixed infection, inhibition of gingipains will decrease phagocytosis of *T. forsythia* within gingival tissue. This may result in ineffective clearance of the bacteria although it might reduce the possibility of the bacterial dissemination.

For the clinical application of the findings in this study, the following questions need be answered: How do gingipains modulate ATP release from *P. gingivalis*-infected macrophages?; whether are caspase-1-dependent effector mechanisms protective or deleterious in *P. gingivalis*-induced chronic inflammation?; whether is the enhancement of *T. forsythia* phagocytosis by *P. gingivalis* detrimental due to eliciting excessive inflammatory response, or protective through eliminating the infecting bacteria?; Does *P. gingivalis* increase the possibility of distant dissemination of *T. forsythia*? The answers to the questions will provide insight into which functions of gingipains should be targeted for effective prevention and control of *P. gingivalis*-associated diseases.

V. Conclusion

Considering that gingipains are one of major promising therapeutic targets in preventive and therapeutic strategies, it is of great importance to fully characterize the roles of gingipains in the pathogenicity of *P. gingivalis*. The present study demonstrated that gingipains play contradictory roles in caspase-1 activation in *P. gingivalis* monoinfection, and that gingipains are essential in augmentation of *T. forsythia* phagocytosis in mixed infection. These results illuminate the complicated roles of gingipains in the manipulation of host defense systems. This study provides clues to advance our understanding of the role of gingipains in the mechanism by which *P. gingivalis* disrupts host immune responses and acts as a keystone pathogen.

VI. References

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List of publications

This thesis is based on the following original papers.

Jung YJ, Jun HK, Choi BK. Contradictory roles of *Porphyromonas gingivalis* gingipains in caspase-1 activation. *Cell Microbiol* 2015; 17: 1304-1319.

Jung YJ, Jun HK, Choi BK. Gingipain-dependent augmentation by *Porphyromonas gingivalis* of phagocytosis of *Tannerella forsythia*. *Mol Oral Microbiol* (In press, DOI: 10.1111/omi.12139)

국문초록

Caspase-1 활성화와 *Tannerella forsythia*의 포식작용에서 *Porphyromonas gingivalis* Gingipain의 역할

정 영 정
서울대학교 대학원
치의과학과 면역및분자미생물치의학 전공

목적

*Porphyromonas gingivalis*는 치주염 병인에서 구강내 세균총 불균형을 유도하고, 이에 대한 숙주 면역 반응을 교란시키는 주요 병원균으로 작용한다. Arg-gingipains (RgpA, RgpB)과 Lys-gingipain (Kgp)은 종종 치주질환 및 연관질환에 대한 예방/치료제 개발에 있어 표적이 되는 이 세균의 주요 독성 인자이다. 이 연구의 목적은 1) 대식세포에서 *P. gingivalis*에 의한 caspase-1 활성화에 있어 gingipain의 역할을 규명하고, 2) *Tannerella forsythia*와의 혼합 감염에서 *P. gingivalis*가 대식세포에 의한 *T. forsythia* 포식작용에 미치는 영향을 알아보고, 이에 있어 gingipain의 역할을 규명하는 것이다.

방법

P. gingivalis 감염에 의한 대식세포에서의 caspase-1 활성화에 있어 gingipain의 역할을 살펴보기 위해, phorbol-12-myristate-13-acetate

(PMA)로 분화시킨 THP-1 대식세포 또는 말초혈액 단핵세포에서 유래된 대식세포를 *P. gingivalis* 또는 이의 gingipain 결핍 돌연변이주로 6시간 동안 감염시켰다. 면역블롯팅과, ELISA, lactate dehydrogenase (LDH) 세포독성검사를 이용하여 세포 배양액 내의 caspase-1, interleukin (IL)-1β, LDH를 분석하였다. Gingipain의 단백질 분해 효소 활성의 역할을 살펴보기 위해, *P. gingivalis*를 leupeptin (시스테인 단백질 가수분해 효소 억제제), 또는 gingipain-특이 억제제 (KYT-1, KYT-36)로 처리한 후, 대식세포에 감염시켰다. Caspase-1 활성 후 분비/유리되는 단백질을 *P. gingivalis*가 분해하는지 살펴보기 위해, 열 사멸 *P. gingivalis*로 자극한 대식세포의 배양액에 *P. gingivalis*를 배양한 후 분석하였다. *P. gingivalis*, gingipain 결핍 돌연변이주, 또는 gingipain 억제제로 전처리한 *P. gingivalis*를 대식세포에 감염시킨 후 세포 내 caspase-1 활성과 유리되는 ATP를 분석하였다. Gingipain에 의한 세균 표면 단백질 성숙/가공의 역할을 살펴보기 위해, gingipain 결핍 돌연변이주를 gingipain이 포함된 세균 배양액에서 배양한 후 대식세포에 감염시켜 caspase-1 활성화를 분석하였다.

P. gingivalis 혼합 감염이 대식세포에 의한 *T. forsythia* 포식작용에 미치는 영향을 살펴보기 위해, PMA로 분화시킨 THP-1 대식세포 또는 말초혈액 단핵세포에서 유래된 대식세포에 carboxyfluorescein diacetate succinimidyl ester (CFSE)로 표지한 *T. forsythia*를 *P. gingivalis*, 또는 gingipain 결핍 돌연변이주와 함께 1시간 감염시킨 후 *T. forsythia* 포식 정도를 유세포 분석기와 공초점 현미경을 이용하여 확인하였다. 두 세균 종간의 응집을 분석하였고, 세균 응집을 억제하는 아미노산이 *T. forsythia* 포식작용에 미치는 영향을 살펴보았다. Gingipain의 단백질 분해 효소 활성의 역할을 살펴보기 위해 KYT-1, KYT-36로 전처리한 *P. gingivalis*를 이용하였다. 세균 감염 및 항생제 처리

후 세포 내 *T. forsythia*의 지속/생존을 유세포 분석기, 공초점 현미경, 또는 16S 리보좀 RNA-기반 생존 분석법을 이용하여 확인하였다.

결과

낮은 MOI의 *P. gingivalis* 감염 후 세포 배양액에 IL-1β와 LDH가 적은 양으로 존재하나, caspase-1은 검출되지 않았으며, 높은 MOI에서는 IL-1β와 LDH도 검출되지 않았다. Caspase-1이 활성화된 세포에서 유리되는 단백질들이 gingipain에 의해 분해되는 것을 확인하였다. 그러나, gingipain을 발현하는 *P. gingivalis* 세균주로 대식세포를 감염시켰을 때, gingipain이 없는 세균주에 비해 세포 내 caspase-1 활성이 높았으며, 이는 감염된 세포로부터 ATP가 유리되는 것과 관련이 있었다. Gingipain을 발현하지 않는 세균주를 gingipain이 포함된 배양액에 배양한 후 대식세포를 감염시켰을 때, caspase-1 활성화가 증가하였다. 반면, Kgp 또는 Rgp의 단백질 분해 효소 활성을 억제하였을 때, 세포 내 caspase-1 활성이 증가하여, Kgp와 Rgp 단백질 분해 활성이 복합적으로 작용하여 억제효과를 나타냄을 확인하였다.

대식세포에서 *P. gingivalis* 혼합 감염이 *T. forsythia* 포식작용을 유의하게 증가시켰으며, Kgp 또는 Rgp 돌연변이주의 경우, 그 증가 정도가 감소하였다. 두 세균 종간의 응집에 gingipain이 관여함을 확인하였으며, 응집을 억제하였을 때 *T. forsythia* 포식작용의 증가가 약화되었다. Gingipain의 단백질 분해 효소 활성을 억제하였을 때, *T. forsythia* 포식작용이 감소하였다. 감염 24, 48시간 후 세포 내에서 대부분의 *T. forsythia*가 제거되었으나, *T. forsythia* 단독 감염에 비해 gingipain을 발현하는 *P. gingivalis*와의 혼합 감염 후 세포 내에 살아있는 *T. forsythia*가 더 많이 남아 있음을 확인하였다.

결론

Gingipain이 주요한 치료제 개발의 표적임을 고려할 때, *P. gingivalis*의 병독력 기전에서 gingipain의 역할을 이해하는 것이 중요하다. 본 연구 결과, *P. gingivalis* 단독 감염에 의한 caspase-1 활성화에 있어 gingipain이 복잡하고 상반되는 역할을 하며, *T. forsythia*와의 혼합 감염에서는 *T. forsythia* 포식작용을 증가시키는 데 핵심적 역할을 한다.

주요어: *Porphyromonas gingivalis*, Gingipains, 단백질 분해 효소, Caspase-1, *Tannerella forsythia*, 포식작용

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