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# Periodontopathic Bacterium *Fusobacterium nucleatum* Affects Matrix Metalloproteinase-9 Expression in Human Alveolar Epithelial Cells and Mouse Lung

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**Abstract.** *Background/Aim: Despite evidence of an association between pulmonary diseases and periodontopathic bacteria, the molecular mechanisms remain unknown. Matrix metalloproteinase-9 (MMP9) plays important roles in pneumonia, chronic obstructive pulmonary disease, and asthma; therefore, we assessed the effects of Fusobacterium nucleatum on MMP9 expression in mouse lung and A549 human alveolar epithelial cells. Materials and Methods: Heat-killed F. nucleatum was administered to the trachea of mice or added to A549 cell cultures. MMP9 expression was determined using real-time PCR and western blotting. The involvement of mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) in MMP9 expression was examined. Results: F. nucleatum induced expression of MMP9 in mouse lung and bronchoalveolar lavage fluid. In A549 cells, F. nucleatum induced production of MMP9 protein and mRNA in a density-dependent manner; this was inhibited by inhibitors of extracellular-regulated kinase 1/2 and NF-κB, but not of p38 and Jun N-terminal protein kinase. Conclusion: F. nucleatum may contribute to the onset of pulmonary diseases via MMP9 expression through extracellular-regulated kinase 1/2 and NF-κB activation.*



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Pulmonary diseases, including pneumonia and chronic obstructive pulmonary disease (COPD), are among the most common diseases that lead to increased morbidity and mortality worldwide (1). Pneumonia, an inflammatory condition of the lung parenchyma, is usually initiated by the introduction of bacteria into the lower airway (2). The aspiration of oropharyngeal secretions colonized with pathogenic bacteria is thought to be a major cause of pneumonia in the elderly. COPD, the third-leading cause of death worldwide (3), is characterized by emphysema and airflow limitation, symptoms that likely result from chronic inflammation in the lung periphery. The effective prevention of pulmonary diseases has important implications for clinical management and public health.

Periodontal disease, which is highly prevalent worldwide, is an inflammatory reaction induced by bacteria, including *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. This disease leads to the destruction of the periodontium, including the periodontal bone and connective tissue attachment. Recently, numerous studies have found that periodontal disease is associated with pulmonary diseases, including pneumonia, COPD, and asthma (4-7). Previous studies demonstrated that periodontopathic bacteria induced the production of proinflammatory cytokines in respiratory organs and by cell lines from respiratory tissue (8-10). However, the details of the relationship between periodontal disease and pulmonary diseases are not fully understood.

*F. nucleatum*, a Gram-negative anaerobic bacterium, is one of the most abundant species in the oral cavity; moreover, it has been implicated in various periodontal diseases, including gingivitis and periodontitis (11). Recent studies have implicated *F. nucleatum* in several systemic diseases, including pulmonary diseases, colorectal cancer, and preterm birth (11). *F. nucleatum* is reportedly frequently detected in the lower airway tract of patients with pneumonia (12-14).

In addition, in patients with severe COPD, the genus *Fusobacterium* was found to be increased in bronchoalveolar lavage fluid (BALF) (15) and sputum (16). Our previous studies demonstrated that heat-killed *F. nucleatum* strongly induced inflammatory cytokines in mouse lung and in respiratory epithelial cells (8, 9). Although it is possible that the tracheal aspiration of *F. nucleatum* plays a part in the pathogenesis of pulmonary diseases, little is known about the molecular mechanisms through which *F. nucleatum* affects the respiratory tract.

Matrix metalloproteinases (MMPs) are involved in extracellular matrix turnover and tissue repair (17). The dysregulation of MMPs leads to pathological conditions through tissue degradation (18). In particular, the dysregulation of MMP9 plays an important role in pulmonary diseases such as pneumonia (17), COPD, and asthma (19). MMP9 expression is regulated by mitogen-activated protein kinases [MAPKs, including p38-MAPK, c-Jun *N*-terminal protein kinase (JNK), and extracellular signal-regulated kinase (ERK)], and nuclear factor-kappa B (NF-κB) in various cell types (20, 21). MMP9 is one of the key executors of inflammatory reactions, which are capable of causing local tissue damage (17), processing cytokines (18), and promoting the infiltration of inflammatory cells (22). Thus, it is important to understand what causes the dysregulation of MMP9 expression in the lung. It is not known whether periodontopathic bacteria can induce the expression of MMP9 in the respiratory tract.

In the present study, we examined the effect of *F. nucleatum* on the expression of MMP9 in the mouse lung and human respiratory epithelial cells in order to evaluate the possible molecular basis linking periodontopathic bacteria to pulmonary diseases. Therefore, in this study, we assessed the effects of heat-killed *F. nucleatum* on the expression of MMP9 in mouse lung and respiratory epithelial cells. As *F. nucleatum*, an anaerobic bacterium, is considered to be incapable of surviving in the respiratory tract, we used heat-killed *F. nucleatum*.

## Materials and Methods

*Reagents*. p38-MAPK inhibitor (SB239063; 10 μM), JNK inhibitor (SP600125; 10 μM), ERK1/2 inhibitor (U0126; 10 μM) (Merck, Darmstadt, Germany), and NF-κB inhibitor (BAY11-7082; 10 μM) (Wako, Osaka, Japan) were added 60 min prior to the addition of *F. nucleatum*. All inhibitor stocks were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM.

*Bacterial culture*. *Fusobacteria nucleatu*m ATCC 25586 (American Type Culture Collection, Manassas, VA, USA) was grown in brain-heart infusion broth (Becton Dickinson, Franklin Lakes, NJ, USA). Bacterial cell cultures were grown under anaerobic conditions (80% $N_2$, 10% $H_2$, and 10% $CO_2$) at 37˚C using an anaerobic chamber (ANX-3; Hirasawa, Tokyo, Japan) for 24 h. The bacterial cell density was adjusted to $1.0\times10^{10}$ colony-forming units (CFU)/ml and then heat-killed at 60˚C for 1 h.

*Mice and inoculation with F. nucleatum*. Male C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). All experimental procedures were approved by the Nihon University Animal Care and Use Committee (AP18DEN031). At 13 weeks of age, the mice were anesthetized with isoflurane and intratracheally inoculated with 50 μl with $1.0\times10^{8}$ CFU of *F. nucleatum* once per day for 7 days. At 1 day after the final inoculation of *F. nucleatum*, BALF was recovered, and lung tissues were collected.

*Cell culture*. Human alveolar epithelial (A549) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37˚C in an atmosphere of 5% $CO_2$. For experiments, confluent A549 cells were prepared by seeding 24-well plates ($2.5\times10^{5}$ cells/well) overnight. Before the addition of *F. nucleatum*, the cells were washed with serum-free DMEM, and then the medium was replaced with serum-free DMEM. Subsequently, A549 cells were treated with different numbers of *F. nucleatum* ($1.0\times10^{7}$, $5.0\times10^{7}$, $1.0\times10^{8}$, CFU/ml) for 1 or 24 h. Phosphate-buffered saline (mock) was used as control.

*Real-time quantitative polymerase chain reaction*. Total RNA from mouse lung tissues or cultured cells were isolated using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). The RNA samples were reverse-transcribed using PrimeScript RT Master Mix (Takara Bio, Shiga, Japan). The primer sequences used for the amplification of each gene were as follows: Mouse *Mmp9*, forward 5'-GCCCTGGAACTCACAC GACA-3' and reverse 5'-TTGGAAACTCACACGCCAGAAG-3'; mouse *Actb*, forward 5'-GGTCAGAAGGACTCCTATGTGG-3' and reverse 5'-TGTCGTCCCAGTTGGTAACA-3'; human *MMP9*, forward 5'-ACTTTGACAGCGACAAGAAGTG-3' and reverse 5'-GGCACTGAGGAATGATCTAAGC-3'; human glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), forward 5'-ACCAGCC CCAGCAAGAGCACAAG-3' and reverse 5'-TTCAAGGGGTC TACATGGCAACTG-3'. The amplification and detection of the cDNA were accomplished using a TP-800 Thermal Cycler Dice Real-Time System (Takara Bio) with TB Grenn Premix Ex Taq (Takara Bio). Relative quantification of gene expression was determined by using the ΔΔCt method.

*Western blotting analysis*. BALF or A549 cell-culture supernatants were prepared, resolved by 5-20% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After non-specific binding was blocked by incubation of the membrane in 2% bovine serum albumin, the membrane was incubated with appropriate primary and secondary antibodies, washed thoroughly, and examined using ECL Prime (Cytiva, Tokyo, Japan). The primary antibodies were anti-MMP9 (Proteintech, Rosemont, IL, USA), anti-phospho-p38-MAPK, anti-p38-MAPK, anti-phospho-JNK, anti-JNK, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-NF-κB p65, anti-NF-κB p65, anti-IκBα (Cell Signaling Technology, Danvers, MA, USA), and anti-b-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Horseradish peroxidase-linked anti-rabbit IgG and horseradish peroxidase-linked anti-mouse IgG (Thermo Fisher Scientific, Rockford, IL, USA) were used as the secondary antibodies. The

bands were visualized using a ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA).

*Gelatin zymography*. The experimental procedures for gelatin zymography were performed as described in a previous study (23). Briefly, BALF or the conditioned medium were mixed with non-reducing sample buffer and resolved by electrophoresis on a 7.5% sodium dodecyl sulfate polyacrylamide gel containing 0.4% gelatin as an MMP substrate. The gels were incubated with reaction buffer (50 mM Tris–HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 1% Triton X-100) at 37˚C for 48 h, gelatinolytic activities were visualized as clear bands against a blue background. Gelatinolytic activity was evaluated qualitatively.

*Statistical analysis*. All experiments were repeated at least three times. Controls used were either phosphate-buffered saline (mock)-treated samples or a combination of DMSO and mock-treated samples. Statistical analyses were performed using KaleidaGraph (Synergy Software, Reading, PA, USA). Student's *t*-test was used for comparing the means of two groups. For comparisons of more than two groups, one-way analysis of variance with Tukey's multiple comparison test was used. Differences were regarded as significant at $p<0.05$. The data are expressed as the mean±standard error of the mean.

## Results

*Effects of F. nucleatum on MMP9 expression and activity in mice*. We investigated whether the intratracheal administration of *F. nucleatum* affected *MMP9* mRNA expression in the mouse lung from one day after the final administration of *F. nucleatum*. As shown in Figure 1A, *F. nucleatum*-induced the expression of *Mmp9* mRNA (5.54 ± 1.15-fold increase). Next, MMP9 protein expression in BALF was confirmed by western blotting (Figure 1B). The MMP9 protein level was increased by the administration of *F. nucleatum*. In addition, using gelatin zymography, we observed that MMP9 activity in BALF was increased by the administration of *F. nucleatum* (Figure 1C).

*Effects of F. nucleatum on MMP9 expression and activity in A549 cells*. Pulmonary epithelial cells are a major source of MMP9 (24). To investigate whether *F. nucleatum* up-regulated *MMP9* mRNA in A549 cells, we quantified the expression of *MMP9* mRNA using real-time polymerase chain reaction. A dose-dependent increase in *MMP9* mRNA expression was observed, with the mRNA expression increased by 12.27±2.54-fold at $10\times10^7$ CFU/ml (Figure 2A). Western blotting analysis showed that the protein level of MMP9 in the conditioned medium from *F. nucleatum*-treated A549 cells was increased (Figure 2B). In addition, gelatin zymographic analysis revealed that *F. nucleatum*-induced MMP9 activity (Figure 2C).

*F. nucleatum induces activation of MAPKs and NF-κB*. Several studies have indicated that *F. nucleatum* induces the expression of many genes *via* the activation of MAPKs and NF-κB in gingival fibroblasts and macrophages (25, 26). However, nothing is known about whether *F. nucleatum* activates MAPKs and NF-κB in respiratory cells. We therefore examined whether *F. nucleatum* could activate MAPKs and NF-κB in A549 cells. As shown in Figure 3, *F. nucleatum* increased the phosphorylation of p38-MAPK, JNK, and ERK1/2. NF-κB is usually sequestered in the cytoplasm by inhibitor of κB α (IκBα). NF-κB activation requires the degradation of IκBα; this results in the transport of NF-κB p65/50 into the nucleus. In addition to nuclear translocation, the phosphorylation of NF-κB p65 is also essential for its maximal transcriptional activity (27). As shown in the lower panel of Figure 3, *F. nucleatum* caused the phosphorylation of p65 and the degradation of IκBα after 60 min of stimulation. Our data suggest that *F. nucleatum* activated MAPKs and NF-κB in A549 cells.

*Involvement of ERK and NF-κB pathways in F. nucleatum-induced MMP9 expression*. The involvement of the signaling pathways in *F. nucleatum*-induced MMP9 expression was explored by using MAPKs and NF-κB inhibitors, including SB239063 (p38-MAPK inhibitor), SP600125 (JNK inhibitor), U0126 (ERK1/2 inhibitor), and BAY11-7082 (NF-κB inhibitor). The *F. nucleatum*-induced phosphorylation of p38-MAPK, JNK, ERK, and NF-κB p65 was reduced by SB239063, SP600125, U0126, and BAY11-7082, respectively (Figure 4A). As shown in Figure 4B, U0126 and BAY11-7082 reduced *F. nucleatum*-induced *MMP9* mRNA expression. In addition, U0126 and BAY11-7082 reduced *F nucleatum*-induced MMP9 expression and activity, as determined by western blotting (Figure 4C) and gelatin zymographic analysis (Figure 4D). In comparison, SB239063 and SP600125 had a negligible effect on *F. nucleatum*-induced MMP9 expression.

## Discussion

Periodontal pathogens, including *F. nucleatum*, are probably associated with pulmonary diseases, such as pneumonia and COPD exacerbation (11, 28, 29). However, little is known about the mechanism through which periodontal bacteria are involved in the pathogenesis of pulmonary diseases. In this study, we attempted to elucidate the effect of *F. nucleatum* on MMP9 expression in mouse lung and A549 cells. The intratracheal administration of *F. nucleatum* to mice induced MMP9 expression. In A549 cells, *F. nucleatum* increased MMP9 expression and intracellular signaling *via* the activation of ERK and NF-κB were involved in *F. nucleatum*-induced MMP9 expression. These observations suggest that *F. nucleatum* may play a role in the development of pulmonary diseases through MMP9 expression.

MMP9 expression is up-regulated in patients with pneumonia (1, 30), COPD, and asthma (19). The augmentation of MMP9 expression leads to the degradation of components of the lung

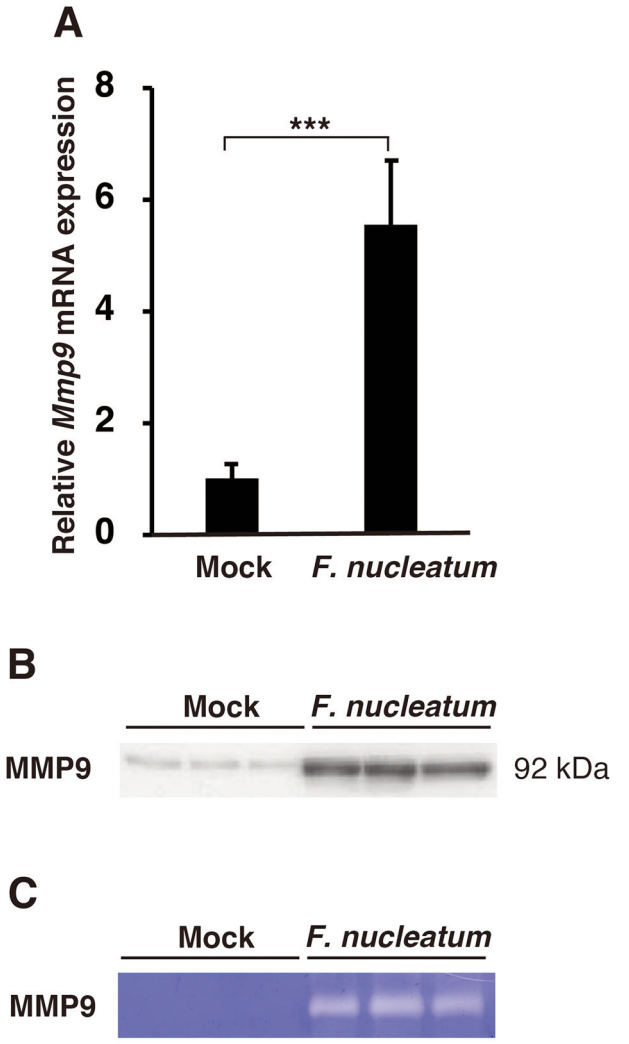


Figure 1. *Effects of Fusobacterium nucleatum on matrix metalloproteinase 9 (MMP9) expression in vivo. The mice were intratracheally administered either phosphate-buffered saline or F. nucleatum ($1.0\times10^8$ CFU/mouse) once per day for 7 days. A: The lungs were harvested at 1 day after the final administration of F. nucleatum. Mmp9 mRNA expression in the lung was determined by real-time polymerase chain reaction. The dats are presented as the mean±standard error of the mean; n=6. ***Significantly different at p<0.001. Bronchoalveolar lavage fluid (BALF) was collected at 1 day after phosphate-buffered saline or the final inoculation of F. nucleatum and analyzed by western blotting for MMP9 expression (B) and by gelatin zymography for MMP9 activity (C). Mock-treated samples were used as a control.*

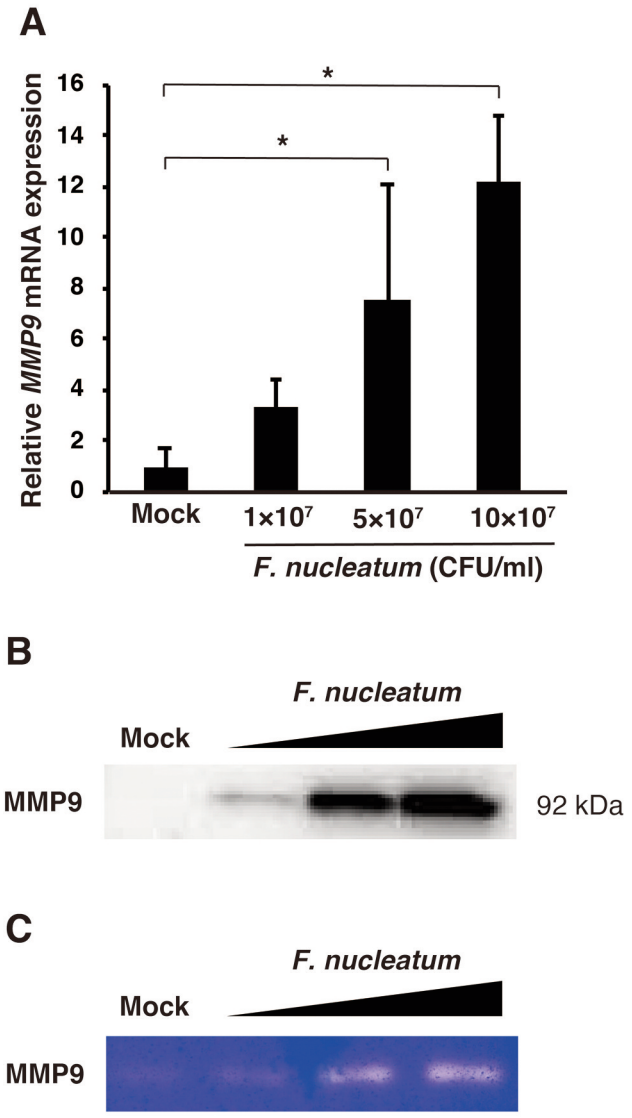


Figure 2. *Effect of Fusobacterium nucleatum on MMP9 expression in vitro. A549 cells were treated with $1.0\times10^7$, $5.0\times10^7$, or $1.0\times10^8$ CFU/ml F. nucleatum for 24 h. A: MMP9 mRNA expression was determined by real-time polymerase chain reaction. The data are presented as the mean±standard error of the mean; n=3. *Significantly different at p<0.05. The conditioned medium was analyzed by western blotting for MMP9 expression (B) and by gelatin zymography for MMP9 activity (C). Mock-treated samples were used as a control.*

extracellular matrix, such as elastin and collagen (1). In addition, MMP9 cleaves interleukin-8 (IL8) and IL1β into their active forms, affecting pathological processes (31). Major respiratory pathogens, including *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae*, have the capacity to induce MMP9 production (32, 33). These observations suggest that *F. nucleatum*-induced MMP9 expression is associated with the development of pulmonary diseases.

*F. nucleatum* has been shown to activate MAPKs (p38-MAPK, JNK, and ERK) and the NF-κB pathway in gingival fibroblasts and macrophages (25, 26); in this study,

the same findings were shown in lung epithelial cells. Although MMP9 expression appears to be regulated by all three of these MAPKs and NF-κB, nothing is known about the signaling pathways that mediate MMP9 expression induced by *F. nucleatum* in epithelial lung cells. In the current study, the specific inhibitors of ERK1/2 (U0126) and NF-κB (BAY 11-7082) effectively reduced *F. nucleatum*-induced MMP9 expression. In contrast, specific inhibitors of p38-MAPK (SB239063) and JNK (SP600125) did not alter *F. nucleatum*-induced MMP9 expression. These data suggest that ERK1/2 and NF-κB signaling are involved in *F. nucleatum*-induced MMP9 expression in A549 cells. IL1β-induced MMP9 expression was shown to be mediated through the activation of all three MAPKs and NF-κB in A549 cells (20). In contrast, although tumor necrosis factor-α can activate all these three MAPKs and NF-κB in A549 cells, it induced MMP9 expression *via* JNK, ERK1/2, and NF-κB signaling, but not *via* p38-MAPK signaling (34). Therefore, the signaling pathway inducing MMP9 expression may be different depending on the stimulus. Further studies are required to elucidate the signaling pathway for *F. nucleatum*-induced MMP9 expression in the lung *in vivo*.

The most recent evidence supports the idea that periodontal disease and poor oral hygiene are risk factors for the development of pneumonia and contribute to COPD exacerbation (2, 5). Periodontal treatment resulted in a significantly lower risk of pneumonia and frequency of COPD exacerbation (35, 36). Periodontopathic bacteria have been reported as etiological pathogens of pulmonary diseases. In particular, *F. nucleatum* was frequently detected in the lower airway tract from patients with pneumonia patients and in those with lung abscesses (13, 14, 37). In addition, the level of sputum antibodies against *F. nucleatum* were markedly elevated in patients with COPD exacerbation (29). Thus, *F. nucleatum* may be associated with the risk of pulmonary disease development. The salivary levels of *F. nucleatum* were increased in patients with periodontal diseases (38). Hence, it is quite possible that in patients with periodontal disease, aspiration of saliva, including *F. nucleatum*, may play an important role in the pathogenesis of pulmonary diseases. It appears that good oral hygiene and healthy periodontal conditions may prevent or reduce pulmonary diseases.

A major limitation of our study was that we used only heat-killed whole bacterial cells to investigate the effects of *F. nucleatum* on MMP9 expression. *F. nucleatum* expresses several virulence factors, including adhesins, lipopolysaccharide, and butyrate (39). In particular, fusobacterial adhesin FadA has been identified to bind host cells and to trigger the host immune responses (11, 39). Although we were able to verify the ability of *F. nucleatum* to induce MMP9 expression, further study is needed to identify, in detail, the mechanism of *F. nucleatum*-induced MMP9 expression that focuses on *F. nucleatum* virulence factors.

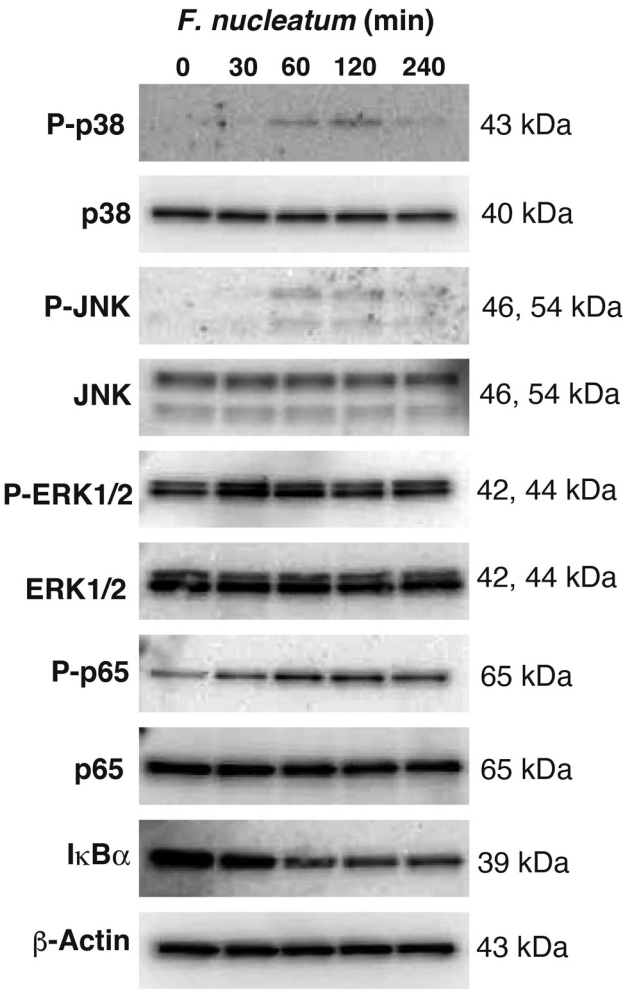


Figure 3. *Effects of Fusobacterium nucleatum on the activation of mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (NF-κB). A549 cells were treated with 1.0×10$^8$ CFU/ml F. nucleatum for the indicated time. Whole cell extracts were prepared and subjected to western blotting with p38-MAPK, phosphorylated p38-MAPK, c-Jun N-terminal protein kinase (JNK), phosphorylated JNK, extracellular signal-regulated kinase 1/2 (ERK1/2), phosphorylated ERK1/2, NF-κB p65, phosphorylated NF-κB p65, and NF-κB inhibitor IκBα.*

Although further studies are needed, this study has provided evidence suggesting that *F. nucleatum*, which can be aspirated into the lung, may cause the development and progression of pulmonary diseases through MMP9 expression. Pulmonary diseases can be prevented by periodontal treatment and professional oral care (10, 35, 36); therefore, it is considered that practicing good oral health to reduce the aspiration of oral bacteria into the lung can potentially prevent the development or progression of pulmonary diseases.

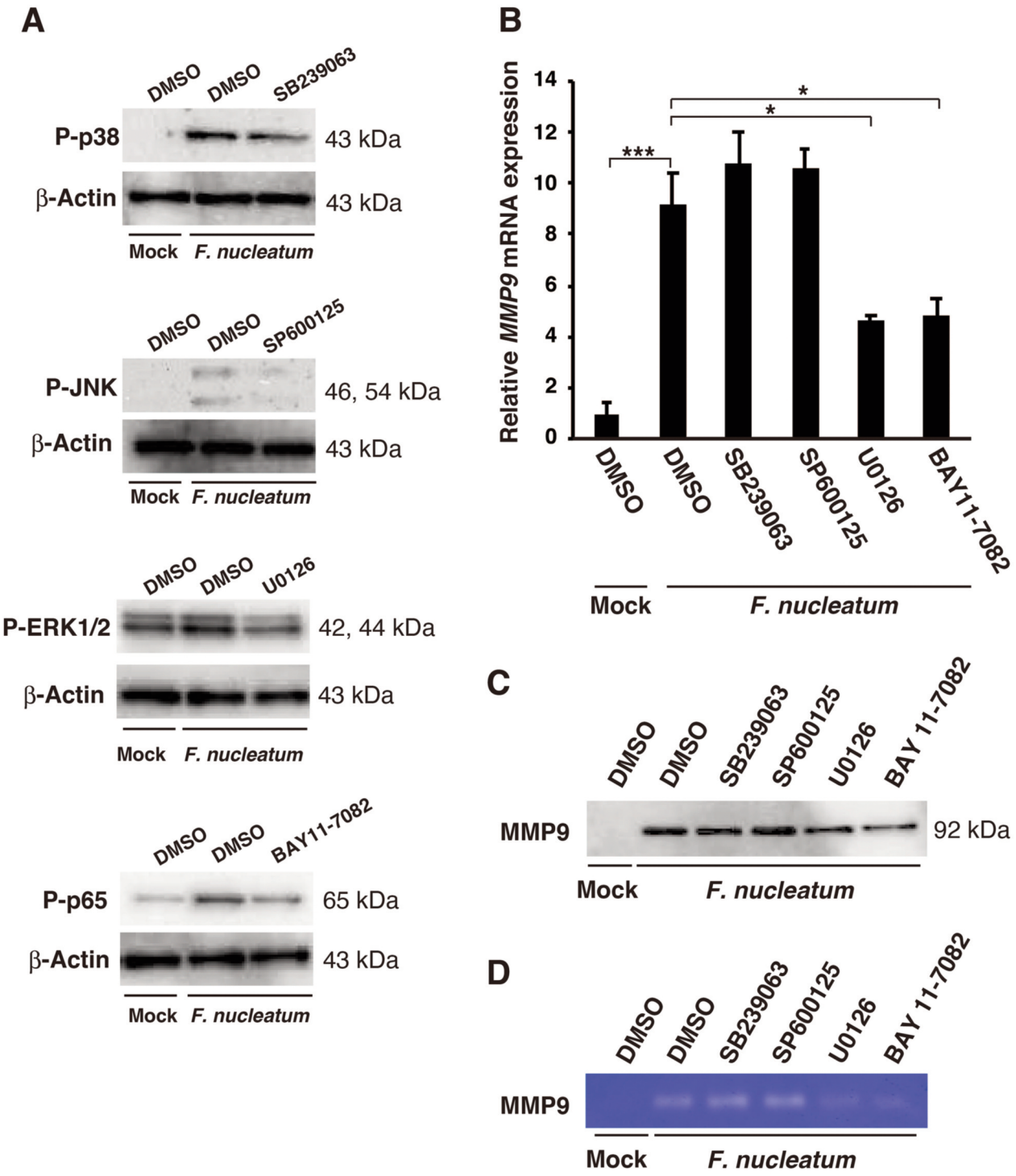


Figure 4. *Effects of various inhibitors on Fusobacterium nucleatum-induced matrix metalloproteinase 9 (MMP9) expression. A: A549 cells were pre-treated with 10 μM SB239063 (p38-MAPK inhibitor), 10 μM SP600125 (JNK inhibitor), 10 μM U0126 (ERK1/2), and 10 μM BAY11-7082 (NF-κB inhibitor) for 1 h, and then treated with $1.0\times10^8$ CFU/ml F. nucleatum for 1 h. p38-MAPK, c-Jun N-terminal protein kinase (JNK), extracellular signaling-regulated kinase 1/2 (ERK1/2), and NF-κB p65 phosphorylation was examined via western blotting. B: A549 cells were pre-treated with 10 μM SB239063, 10 μM SP600125, 10 μM U0126, and 10 μM BAY11-7082 for 1 h, and then treated with $1.0\times10^8$ CFU/ml F. nucleatum for 24 h. MMP9 mRNA expression was determined by real-time polymerase chain reaction. The data are presented as the mean±standard error of the mean; n=3. Significantly different at *p<0.05 and ***p<0.001. The conditioned medium was analyzed by western blotting for MMP9 expression (C) and by gelatin zymography for MMP9 activity (D). DMSO and mock-treated samples were used as controls.*

## Conflicts of Interest

The Authors declare no conflicts of interest.

## Authors' Contributions

RS, KS and NK designed and performed the experiments, analyzed the data, and wrote the article. SM, YG, TK, and YY contributed to the discussion, analyzed the data, and reviewed drafts of the article. KI was responsible for the study concept and article writing.

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