A Sialidase-Deficient *Porphyromonas gingivalis* Mutant Strain Induces Less Interleukin-1β and Tumor Necrosis Factor-α in Epi4 Cells Than W83 Strain Through Regulation of c-Jun N-Terminal Kinase Pathway

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Background: *Porphyromonas gingivalis* is one of the major periodontal pathogens. In a previous study, a mouse abscess model showed that sialidase deficiency of *P. gingivalis* weakened its virulence, but the mechanism behind this observation remains unknown.

Methods: A sialidase-deficient mutant strain (△PG0352) and a complemented strain (com△PG0352) were constructed. Epi4 cells were stimulated by wild-type strain *P. gingivalis* W83, △PG0352, or com△PG0352. Real-time polymerase chain reaction was carried out to detect expression of virulent genes in *P. gingivalis* and interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α in epi4 cells. Activities of sialidase, gingipains, and lipopolysaccharide (LPS) were compared among the different *P. gingivalis* strains. Levels of IL-1β and TNF-α in the epi4 cells supernatant were detected by enzyme-linked immunosorbent assay and levels of p38, extracellular signal-regulated kinase, c-Jun N-terminal kinase (JNK), and phospho-c-Jun were detected by western blotting.

Results: Compared with *P. gingivalis* W83 and com△PG0352, activities of Kgp and Rgp gingipains and amount of LPS decreased in △PG0352, whereas there were no differences in LPS activity among these three strains. Level of phospho-JNK was lower in epi4 cells stimulated by △PG0352. △pG0352 induced less IL-1β and TNF-α and more IL-8 in epi4 cells; differences in IL-1β and TNF-α could not be detected after JNK blocking.

Conclusion: A sialidase-deficient *P. gingivalis* mutant strain induces less IL-1β and TNF-α in epi4 cells than W83 strain through regulation of JNK pathway. *J Periodontol 2017;88: e129-e139.*

KEY WORDS

Epithelial cells; gingiva; JNK mitogen-activated protein kinases; neuraminidase; *Porphyromonas gingivalis*.

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Chronic periodontitis (CP) is one of the most frequently occurring infectious diseases in humans. It is characterized by constant interaction between bacteria in dental plaque and host defense mechanisms. Resorption of supporting alveolar bone and loss of tooth-surrounding soft tissue eventually lead to tooth exfoliation.[1] Multiple studies have shown that CP is a contributing risk factor for diseases, such as diabetes mellitus,[2] cardiovascular disease,[3] rheumatoid arthritis,[4] and low-weight premature birth.[5]

Development of dental plaque is the initial etiology of CP. There are hundreds of microbial species in a given dental plaque, including commensal and opportunistic oral species.[6] These opportunistic species are more important with regard to CP. Among them, *Porphyromonas gingivalis*, a Gram-negative bacteria, is considered a major etiologic agent involved in initiation and progression of CP.[7] *P. gingivalis* has a range of virulence factors, such as gingipain proteinases and lipopolysaccharide (LPS),[8] that enable it to adhere to and invade host tissues and to deregulate the immune system to promote survival

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inside the host.[9] LPS activates Toll-like receptors on the cell surface and induces cytokine and chemokine production in gingival epithelial cells (GECs), gingival fibroblasts, and macrophages.[10] Gingipains are a family of cysteine proteases that includes the arginine-specific proteinase Rgp and lysine-specific proteinase Kgp.[11] Gingipains not only degrade host tissue but also provide free amino acids (a source of carbon and nitrogen) for *P. gingivalis* growth and survival.[12] In addition, gingipains degrade antibacterial peptides, facilitating *P. gingivalis* evasion of host defenses.[13]

Sialidase is a type of enzyme that cleaves sialic acid from surfaces of eukaryotic cells and the surrounding environment. Sialidases are implicated in the pathogenicity of many bacteria, including *P. gingivalis*,[14,15] *Tannerella forsythia*,[16] *Treponema denticola*,[17] *Pseudomonas aeruginosa*,[18] *Streptococcus pneumoniae*,[19] and *Streptococcus oralis*.[20] Sialidases not only provide nutrients for bacterial growth and survival but also serve to modify macromolecules on surfaces of bacteria to interfere with host–bacterial interactions. *P. gingivalis* sialidase, encoded by the gene *PG0352*, has exo-α-neuraminidase activity. Inactivation of *PG0352* does not influence planktonic growth of *P. gingivalis*, but sialidase-deficient mutants of *P. gingivalis* fail to produce an intact capsule and show lower adherence to HeLa cells and lower virulence in a mouse abscess model;[14,15] the mechanism behind this phenotype remains unknown.

Bacteria that accumulate at the dentogingival junction initiate gingival inflammation that develops into periodontitis. During this developmental period, GECs maintain the physical barrier and release cytokines and chemokines to defend against periodontal pathogens, so GECs play an important role in homeostasis of periodontal tissue and are considered a key factor of innate immunity.[21] Either *P. gingivalis* or its virulence factors can influence GECs; they first activate pathogen recognition receptors and subsequently trigger downstream pathways, such as the mitogen-activated protein kinase (MAPK) pathway.[22] Mammalian MAPK pathways include mainly the extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 kinase pathways. Previous studies have shown that JNK and p38 are more responsive to inflammatory cytokine and chemokine stimulation.[23] Interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, and IL-8 are generally classified as proinflammatory cytokines and chemokines, which not only play important roles in inflammatory response but also regulate normal tissue homeostasis.[24]

In this study, sialidase gene (*PG0352*) was knocked out from *P. gingivalis* W83 (△PG0352), and a complemented strain (com△PG0352) was created to rescue loss of sialidase. Virulence factors of *P. gingivalis* were compared in these different *P. gingivalis* strains. Immortalized GEC line epi4 was stimulated with the different *P. gingivalis* strains, and effects of *P. gingivalis* sialidase on inflammatory response of GECs was determined.

MATERIALS AND METHODS

Experiments were done in the central laboratory, School of Stomatology, China Medical University (Shenyang, Liaoning, China), and approved by the institutional review board of China Medical University.

Bacterial Strains and Growth Conditions

P. gingivalis W83 was cultured anaerobically at 37°C in trypticase soy broth (TSB) supplemented with vitamin K (1 μg/mL) and hemin (5 μg/mL) or on TSB agar plates containing 5% defibrinated sheep blood. When necessary, clindamycin (5 μg/mL) and tetracycline (1 μg/mL) were added to the media. *Escherichia coli* DH5α strain was used for DNA cloning. *E. coli* was cultured in a broth‡ and on agar plates§ supplemented with appropriate antibiotics.

Construction of PG0352 *Mutant and Complemented Strains*

PG0352 mutant strain was prepared as previously described.[14] The 1-kb region upstream of *PG0352*, *ermF/AM* cassette from *pVA2198* plasmid, and the 1-kb region downstream from *PG0352* were inserted into a polymerase cloning system‖ to facilitate rearrangement. Primers used for *PG0352* mutant strain are listed in Table 1. For allelic-exchange mutagenesis, *PG0352-ermF/AM* plasmid was linearized with *Nde*I and electroporated into competent *P. gingivalis* W83 cells. Transformed cells were selected using blood TSB agar plates containing 5 μg/mL clindamycin.

For complementation of *PG0352* mutations, the *PG0352* open reading frame (ORF) and the *ragA* promoter were amplified from chromosomal DNA of *P. gingivalis* W83. Primers used for *PG0352* complemented strain are listed in Table 1. *Bam*HI restriction sites were designed at 5′-ends of both primers for *ragA*-F and *PG0352*-Com-R to facilitate subcloning of the polymerase chain reaction (PCR) fragment. *PG0352* ORF was fused to the *ragA* promoter by PCR using *ragA*-F and *PG0352*-Com-R primers. This DNA fragment was inserted into *Bam*HI-digested pT-COW and was used to transform *E. coli* DH5α cells. Purified, recombined plasmid was used to transform △PG0352 via electroporation. Transformants were selected using TSB agar plates with 5 μg/mL clindamycin and 1 μg/mL tetracycline.[25]

Epi4 Cell Culture

Epi4, an SV40 T-antigen-immortalized GEC line,[26] was maintained in medium¶ supplemented with

‡ Luria–Bertani broth, Gibco, Thermo Fisher Scientific, Waltham, MA.
§ Luria–Bertani agar plates, Gibco, Thermo Fisher Scientific.
‖ pGEM-T Easy vector, Promega, Madison, WI.
¶ HuMedia-KG2, Kurabo Industries, Osaka, Japan.

Table 1.

Primers Used in This Study

Description	Sequence (5′ to 3′)*
△PG0352 mutant strain	
PG0352 upstream region	F: GCTCTTTCAGCTTGGTATAGG
	R: AGATCTGACATAACGTCGAGTCTTCGC
ermF/AM cassette	F: AGATCTAGCTTCCGCTATTGCTTT
	R: AGATCTTTTATCTACATTCCCTTTAGT
PG0352 downstream region	F: AGATCTACGATCTCTTCGATGTCCGGC
	R: GACCTACCACGAATATCAACC
com△PG0352 complemented strain	
RagA-P	F:GGATCCTTGCAGAAATTTCTGCATTTGTGGT
	R:CGCCAAAAGAGTATTATTTGCCATAGACTTTTCTTTTGC GTTAAACTT
PG0352 com	F: ATGGCAAATAATACTCTTTTGGCGAAGA
	R: GGATCCTCATTGCCGGACATCGAAGAG
Real-time PCR	
kpg[28]	F: GCTTGATGCTCCGACTACTC
	R: GCACAGCAATCAACTTCCTAAC
rgpA[28]	F: CCGAGCACGAAAACCAA
	R: GGGGCATCGCTGACTG
rgpB[29]	F: TCGCTGATGAAACGAACTTGACGC
	R: TTCGAATACCATGCGGTTCTTAGC
16s rRNA[28]	F: AGGAACTCCGATTGCGAAGG
	R: TCGTTTACTGCGTGGACTACC
IL-1β[30]	F: ACGCTCCGGGACTCACAGCA
	R: TGAGGCCCAAGGCCACAGGT
IL-6[31]	F: AATCATCACTGGTCTTTTGGAG
	R: GCATTTGTGGTTGGGTCA
IL-8[31]	F: GACATACTCCAAACCTTTCCACC
	R: AACTTCTCCACAACCCTCTGC
TNF-α[32]	F: AAGCCTGTAGCCCATGTTGT
	R: CAGATAGATGGGCTCATACC
GAPDH[30]	F: GAAGGTGAAGGTCGGAGTC
	R: GAAGATGGTGATGGGATTTC

PCR = polymerase chain reaction; rRNA = ribosomal RNA.
* Engineered restriction enzyme sites are underlined.

antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin#) at 37°C with 5% CO_2.

Real-Time PCR

Gene expression levels of *kgp*, *rgpA*, and *rgpB* in *P. gingivalis* and IL-1β, IL-6, IL-8, and TNF-α in epi4 cells were detected using real-time PCR. Total RNA was isolated using a reagent,** and complementary DNA (cDNA) was synthesized as described previously.[27] Real-time PCR was performed in 20 μL reaction mixture containing 0.4 μL template cDNA, 10 μL of 2× premix reagent,†† 0.4 μL of 50× passive reference dye,‡‡ 0.8 μL of 10 μM primers, and 6.8 μL double-distilled H_2O. Primer sequences used for real-time PCR are listed in Table 1.[28-32] Amplification was performed in a fluorescence thermocycler§§ under the following conditions: initial denaturation at 94°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 5 seconds, with extension at 60°C for 34 seconds. The $2^{-\Delta\Delta Ct}$ method was used to evaluate variability of target genes. 16S rRNA for *P. gingivalis* or GAPDH for epi4 cells was used as internal control. Target gene expression in *P. gingivalis* W83 or in epi4 cells without stimulation by *P. gingivalis* was used as the calibrator.[33]

Sialidase Assay

Sialidase activity was detected using 4-methylumbelliferyl–D-*N*-acetylneuraminic acid (4-MUNANA)‖‖

Sigma-Aldrich, St. Louis, MO.
** TRIzol reagent, Life Technology, Thermo Fisher Scientific.
†† SYBR Premix Ex Taq, TaKaRa Bio, Mountain View, CA.
‡‡ ROX Reference Dye II, Applied Biosystems, Thermo Fisher Scientific.
§§ Applied Biosystems, Thermo Fisher Scientific.
‖‖ Sigma-Aldrich.

as a substrate in a filter paper spot test.[34,35] Whole-cell lysates of *P. gingivalis* were coincubated with 4-MUNANA, a fluorogenic sialidase substrate. Fluorescence was detected using a gel imaging system.[¶¶] Excitation and emission wavelengths were 302 and 548 nm, respectively.

Gingipain Activity Assay

Activities of arginine (Rgp) and lysine (Kgp) gingipains were determined using substrates N-α-benzoyl-DL-arginine p-nitroanilide hydrochloride (BAPNA) and N-(p-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt (ALNA),[##] respectively, according to previously described protocol.[36] Bacterial culture (2 mL) was harvested (optical density at 600 nm [OD_{600}] = 1.0) and centrifuged at 5,000 × g for 5 minutes at 4°C. The pellet was suspended in 2 mL reaction buffer. Prepared pellet suspension (100 μL) was added to an ice-cold 96-well microtiter plate. After 10 minutes of incubation at 37°C, 100 μL of 0.5 mM substrate solution was added, and OD_{405} values were measured at 0, 6, 30, 60, and 90 minutes at 37°C.

LPS Extraction and Activity Detection

Isolation and purification of *P. gingivalis* LPS was performed using a reagent*** protocol, as previously described.[37] Briefly, bacterial cultures were centrifuged at 6,500 rpm for 20 minutes, and the pellets were resuspended in a reagent.[†††] One-fifth volume of chloroform was added and mixed, the solution was centrifuged at 12,000 rpm for 10 minutes, and the top aqueous layer was retained as "crude LPS." Crude LPS was washed with 1 mL cold 0.35 M $MgCl_2$ in 95% ethanol and centrifuged at 5,000 rpm for 5 minutes at 4°C. The pellets were washed twice more with 1 mL cold 95% ethanol and once with 1 mL cold 100% ethanol and air-dried. To remove contaminating phospholipids, LPS was resuspended to 1% (weight/volume [w/v]) in 2:1 chloroform–methanol solution, centrifuged at 5,000 rpm for 5 minutes at 4°C, and air-dried. The final product was weighed and recorded as the amount of LPS.

Activities of the same amount of LPS from different *P. gingivalis* strains were measured using an endotoxin detection assay kit[‡‡‡] according to instructions of the manufacturer. Tachypleus amebocyte lysate solution, chromogenic substrate, and azo reagent were added to diluted standards and LPS solutions successively and incubated for the appropriate time at 37°C. The mixtures were transferred to a 96-well immunoassay plate. All standardized dilutions and samples were plated in triplicate. Finally, the plate was placed into a microplate reader, and the OD_{545} value was measured immediately. A standard curve was generated by plotting mean OD and activity of each standard dilution. Activity of each LPS solution was also calculated.

Enzyme-Linked Immunosorbent Assay (ELISA) for IL-1β, TNF-α, IL-6, and IL-8

Epi4 cells were seeded into six-well culture plates at 2×10^5 cells/well. The medium was changed (without antibiotics) after 48 hours, and epi4 cells were incubated for another 6 or 24 hours. A JNK inhibitor (10 μM)[§§§] was added for pretreatment (1 hour) in the JNK-inhibited groups. Epi4 cells were stimulated by *P. gingivalis* W83, ΔPG0352, or comΔPG0352 at a multiplicity of infection (MOI) of 100:1 for 6 and 24 hours.[38] The supernatant was collected, and levels of IL-1β, TNF-α, and IL-8 secreted by epi4 cells were determined using commercial ELISA kits[‖‖‖] according to instructions of the manufacturer. OD_{490} values of all samples were measured immediately. Concentration of each indicator was calculated according to each standard curve.

Western Blotting

Epi4 cells were infected with *P. gingivalis* W83, ΔPG0352, or comΔPG0352 (MOI = 100) for 24 hours. Cells (1×10^6) were harvested and rinsed with phosphate-buffered saline three times. Ice-cold 1% radioimmunoprecipitation assay lysis buffer was added, and, after 5 minutes on ice, lysates were centrifuged at 12,000 rpm for 10 minutes at 4°C. Protein concentration was determined using a protein assay kit.[¶¶¶] Proteins (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% (w/v) dried skimmed milk for 1 hour at room temperature and probed with antibodies[###] overnight at 4°C. p38 was detected using mouse monoclonal antibodies diluted to 1:1,000. ERK, JNK, phospho-p38 (p-p38), phospho-ERK (p-ERK), phospho-JNK (p-JNK), and phospho-c-Jun (p-c-Jun) were detected using rabbit polyclonal antibodies diluted to 1:500, 1:1,000, 1:500, 1:500, 1:1,000, and 1:1,000, respectively. Goat antimouse immunoglobulin (Ig)G and goat antirabbit IgG were used as secondary antibodies diluted to 1:2,000. Proteins were detected using enhanced chemiluminescence**** with a gel imaging system.[††††]

Statistical Analyses

All experiments were repeated at least three times. The Kolmogorov–Smirnov test was used to analyze normality of distribution for all variables. Data are

¶¶ ChemiDoc XRS System, Bio-Rad, Hercules, CA.
Sigma-Aldrich.
*** TRIzol reagent, Life Technology, Thermo Fisher Scientific.
††† TRIzol reagent, Life Technology, Thermo Fisher Scientific.
‡‡‡ Chromogenic Endpoint Tachypleus Amebocyte Lysate Kit, Xiamen Houshiji, Xiamen, China.
§§§ SP600125, Abcam, Cambridge, U.K.
‖‖‖ R&D Systems, Minneapolis, MN.
¶¶¶ Pierce Bicinchoninic Acid Protein Assay Kit, Thermo Fisher Scientific.
Abcam, Cambridge, U.K.
**** GE Healthcare, Uppsala, Sweden.
†††† Gel-Pro-Analyzer system, Media Cybernetics, Silver Spring, MD.

presented as mean ± SD. One-way analysis of variance (ANOVA) and a post hoc Student–Newman–Keuls test for multiple comparisons in ANOVA were used to compare differences in measurements between each group. Statistical analyses were performed using statistical software,[‡‡‡‡] and results were considered to be statistically significant at $P < 0.05$.

RESULTS

Deletion of PG0352 *Reduces Gingipain Activity and Amount of LPS but Does Not Affect* P. gingivalis *LPS Activity*

Sialidase activity of *P. gingivalis* W83, △PG0352, and com△PG0352 was examined. As shown in Figure 1A, *P. gingivalis* W83 and com△PG0352 were able to cleave the fluorogenic sialidase substrate 4-MUNANA, and there were no significant differences in fluorescence intensities between *P. gingivalis* W83 and com△PG0352; however, sialidase activity was not detected in △PG0352. Collectively, these results indicated that the *PG0352* gene was abrogated in △PG0352, and there was no obvious *PG0352* overexpression in com△PG0352.

Expression of *P. gingivalis* gingipains, including *kgp*, *rgpA*, and *rgpB*, was assayed. As shown in Figure 1B, expression levels of *rgpA* and *rgpB* decreased in △PG0352, but that of *kgp* did not change in △PG0352. *P. gingivalis* whole-cell gingipain activities were measured (Figs. 1C and 1D). Compared with *P. gingivalis* W83 and com△PG0352, activity levels of Kgp and Rgp significantly decreased in △PG0352. LPS was also compared among these strains; amount of LPS was significantly lower in △PG0352, but there were no significant differences in LPS activity among *P. gingivalis* W83, △PG0352, and com△PG0352 (Figs. 1E and 1F).

Sialidase Deficiency Moderates IL-1β, IL-8, and TNF-α Levels in Epi4 Cells Stimulated by P. gingivalis

To determine whether *PG0352* gene deletion moderates immuno-inflammatory responses of human GECs stimulated by *P. gingivalis*, epi4 cells were stimulated by *P. gingivalis* W83, △PG0352, or com△PG0352 at MOI 100:1 for 6 and 24 hours. Cytokine and chemokine levels secreted by epi4 cells, including IL-1β, IL-6, IL-8, and TNF-α, were evaluated by real-time PCR and ELISA. Compared with the unstimulated control group, gene expressions of IL-1β and TNF-α were higher when epi4 was stimulated by different strains in both 6 and 24 hours, whereas those of IL-8 were higher in 6 hours but lower in 24 hours. Compared with *P. gingivalis* W83 and com△PG0352 groups, IL-1β, IL-8, and TNF-α were lower in the △PG0352 group in both 6 and 24 hours (Figs. 2A and 2B). Compared with the unstimulated control group, epi4 cells stimulated by the different *P. gingivalis* strains elicited greater IL-1β and TNF-α production and suppressed IL-8 production in both 6 and 24 hours. Compared with the *P. gingivalis* W83 and com△PG0352 groups, epi4 cells stimulated by △PG0352 induced less IL-1β and TNF-α and more IL-8 in both 6 and 24 hours (Figs. 2C and 2D). Almost no IL-6 was detected in supernatants of epi4 cells in either the unstimulated control group or the bacterially stimulated cells (data not shown).

Sialidase-Deficient Mutant Strain Induces Less Phosphorylated JNK in Epi4 Cells Than P. gingivalis *W83*

Western blotting was used to detect levels of p38, ERK1/2, and JNK, along with the corresponding p-p38, p-ERK1/2, and p-JNK. As shown in Figure 3, compared with the control group, epi4 cells that were stimulated by the different *P. gingivalis* strains expressed increased levels of p-p38 and p-JNK. Notably, p-JNK level was lower in epi4 cells stimulated by the △PG0352 strain compared with the *P. gingivalis* W83 and com△PG0352 strains. There were no differences in levels of p-p38 and p-ERK1/2 among epi4 cells stimulated by the *P. gingivalis* W83, △PG0352, and com△PG0352 strains.

Alterations of IL-1β and TNF-α Secretion in Epi4 Cells Stimulated by P. gingivalis *Sialidase-Deficient Mutant Strain Are Mediated Through JNK Pathway*

Results indicated that compared with the *P. gingivalis* W83 strain, the △PG0352 strain induced lower amounts of p-JNK in epi4 cells. To determine whether this finding was mechanistically important to the △PG0352 strain's decreased capacity for cytokine production, a JNK inhibitor[§§§§] was used to block the JNK pathway before stimulation with *P. gingivalis*. As shown in Figures 4A and 4B, the same JNK inhibitor blocked expression of p-c-Jun, the downstream protein of JNK. Compared with the groups without pretreatment with the JNK inhibitor, IL-1β and TNF-α decreased in all stimulated cells with pretreatment with the JNK inhibitor, regardless of stimulating strain, and there were no differences among epi4 cells stimulated by different strains (Figs. 4C and 4D).

DISCUSSION

A previous study showed that sialidase deficiency reduces pathogenicity of *P. gingivalis*.[14] To clarify

‡‡‡‡ SPSS version 11.5, SPSS, Chicago, IL.
§§§§ SP600125, Abcam.

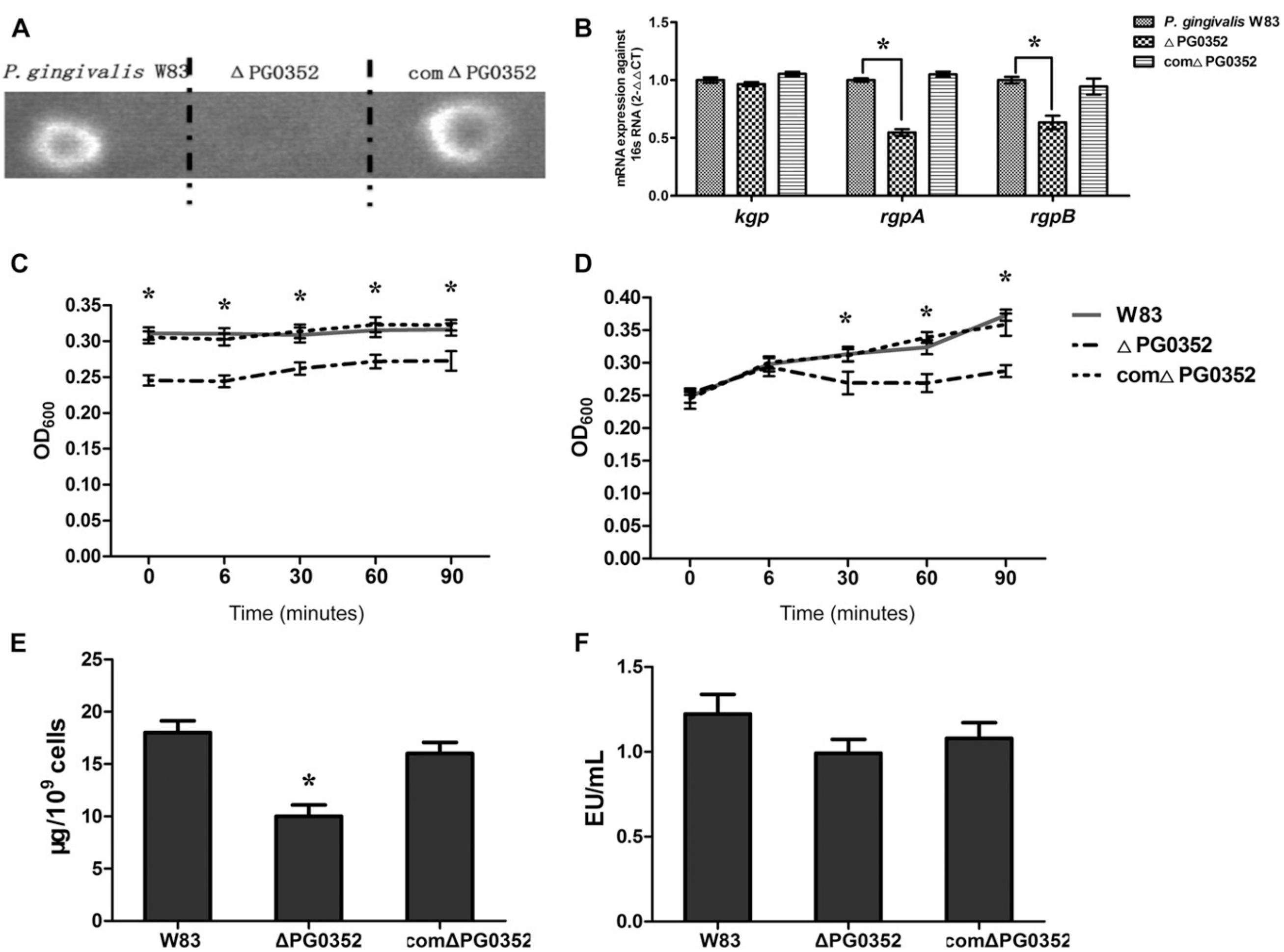

Figure 1.
Comparisons of virulence factors between P. gingivalis *W83, ΔPG0352, and comΔPG0352.* ***A)*** *Filter paper spot assay using whole-cell lysates of different* P. gingivalis *strains with 4-MUNANA substrate.* ***B)*** *Gene expression levels of* kgp, rgpA, *and* rgpB *in* P. gingivalis *as detected by real-time PCR.* ***C)*** *Lysine gingipain (Kgp) activity detected using BAPNA substrate.* ***D)*** *Arginine gingipain (Rgp) activity detected using ALNA substrate.* ***E)*** *Amounts of LPS from different* P. gingivalis *strains.* ***F)*** *Activities of the same amount of LPS from different* P. gingivalis *strains detected by limulus assay.* **P* <0.05. *mRNA = messenger RNA.*

the mechanism of this reduction, relationship between *P. gingivalis* sialidase and its virulence factors need to be defined. Major virulence factors of *P. gingivalis* include gingipains and LPS. *P. gingivalis* produces multiple proteases, the most noteworthy being a set of cysteine proteases referred to as gingipains. One type of gingipain cleaves at lysine residues (lysine gingipain: Kgp), whereas two other family members cleave proteins at arginine residues (arginine gingipains A and B: RgpA and RgpB).[39] Gingipains have diverse functions. They are indispensable for nutrient uptake[40] and are involved in interactions between *P. gingivalis* and host cells, including *P. gingivalis* adhesion, invasion, survival and host cell autophagy, along with microbial clearance and infection control.[36,41] In the present study, *rgp* gene expression and activities of Kgp and Rgp decreased in △PG0352, but there was no difference in *kgp* gene expression among the different *P. gingivalis* strains, suggesting that sialidase deficiency affected activities of Kgp and Rgp in different ways. It appears that *PG0352* is involved in posttranslational processing in Kgp but messenger RNA regulation of *rgp*; of course, a role for *PG0352* in posttranslational regulation of Rgp cannot be ruled out. Sialidase can assist in biosynthesis and sialylation of bacterial macromolecules; Curtis et al.[42] found that gingipains had a certain level of sialic acid, and Vanterpool et al.[43] found that sialylation is involved in gingipain maturation. Sialic acid is required for biosynthesis and sialylation of gingipains, but *P. gingivalis* cannot obtain sialic acid by *de novo* biosynthesis; the processes need sialidase to release sialic acid from a range of host sialoglycoconjugates, so activities of gingipain decrease in sialidase-deficient mutant strain.

LPS is a kind of endotoxin in *P. gingivalis*. It might activate receptors on the surface of GECs and is

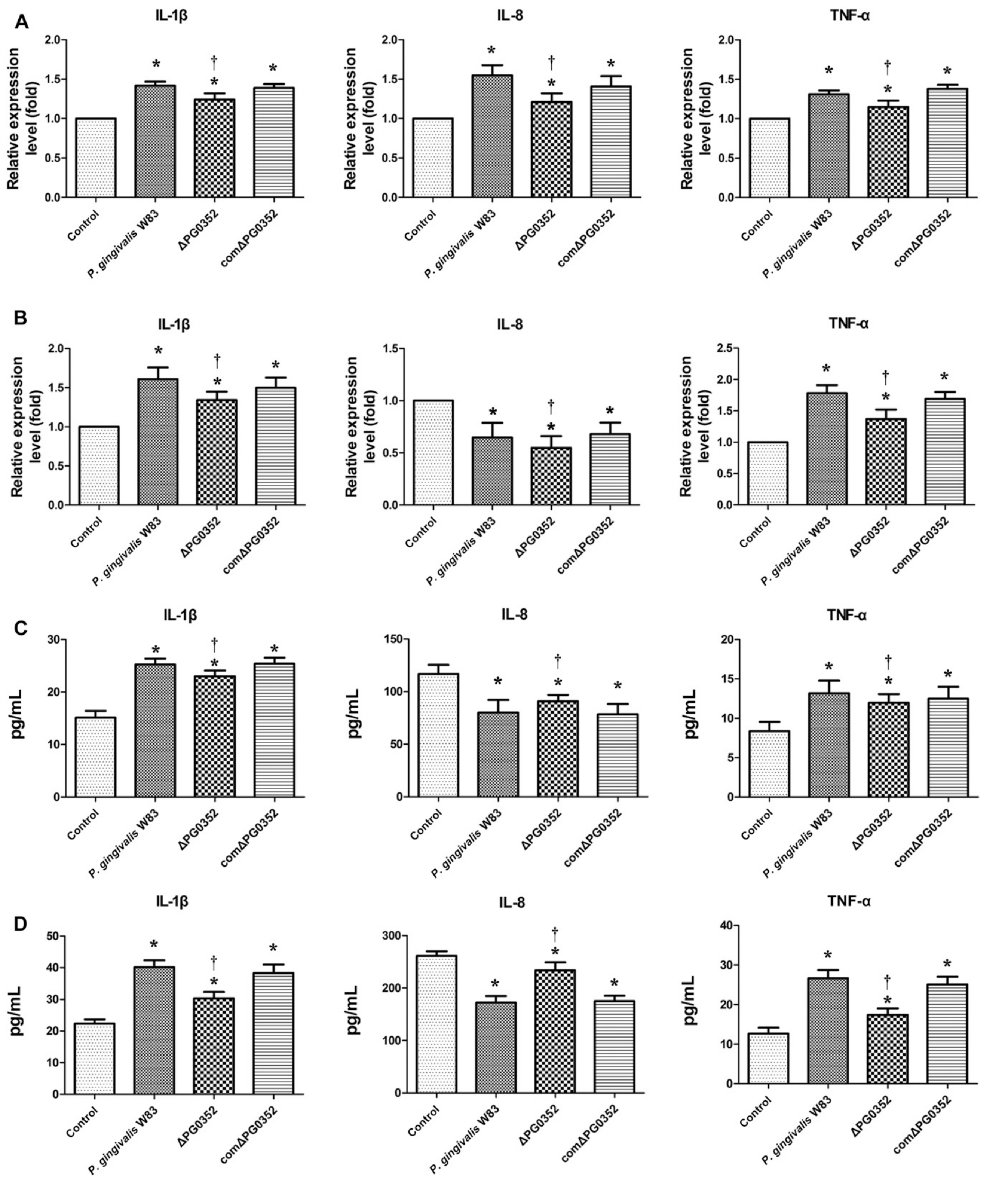

Figure 2.
Real-time PCR and ELISA for IL-1β, IL-8, and TNF-α in epi4 cells stimulated by different P. gingivalis *strains. Real-time PCR for 6* ***(A)*** *and 24* ***(B)*** *hours; gene expressions of IL-1β, IL-8, and TNF-α in stimulated groups reflect fold changes compared with control. ELISA for 6* ***(C)*** *and* ***(D)*** *24 hours.*
*****P *<0.05 versus control group;* †P *<0.05 versus* P. gingivalis *W83 group.*

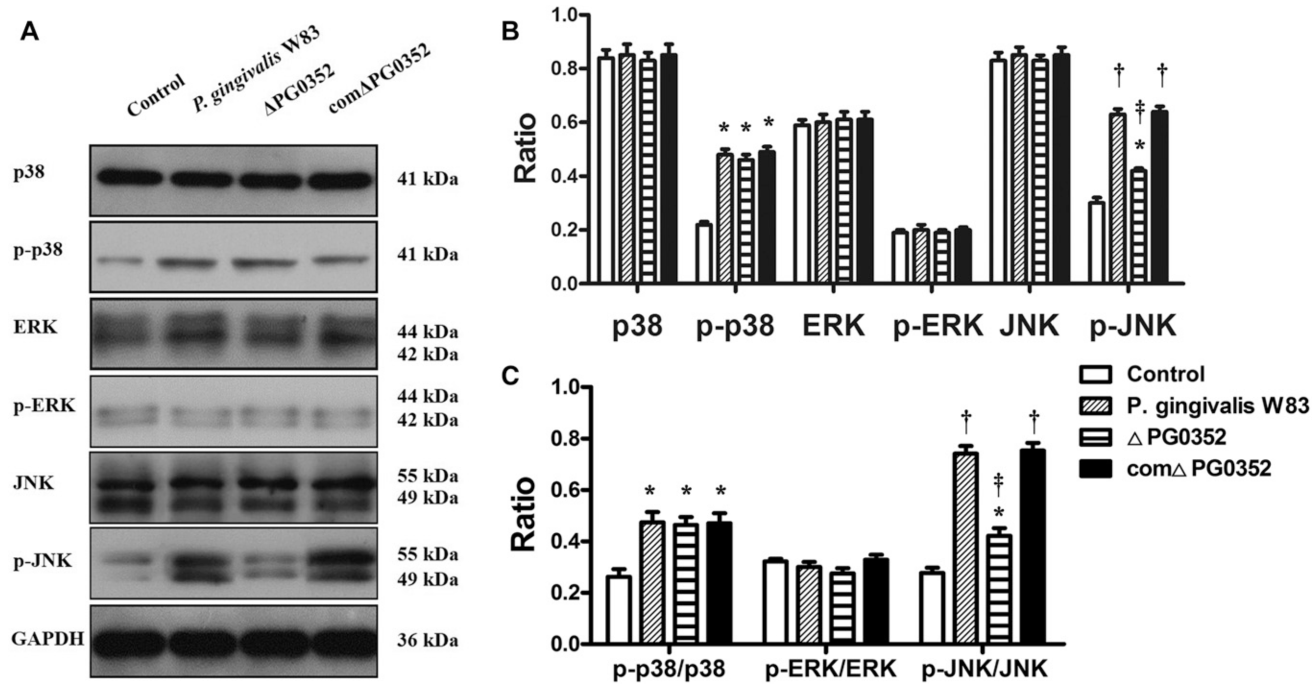

Figure 3.
Expression of MAPK pathways as detected by western blotting after epi4 cells were stimulated by P. gingivalis *W83, ΔPG0352, or comΔPG0352.* ***A)*** *Bands of western blotting.* ***B)*** *Ratio plot of each protein to GAPDH.* ***C)*** *Ratio plot of phosphoprotein to total protein.* *P <0.05 versus control group; †P <0.05 versus P. gingivalis W83 group; ‡P <0.01 versus control group.

involved in immuno-inflammatory responses, including eliciting proinflammatory cytokines and chemokines in host cells.[44] In the present study, sialidase deficiency reduced amount of LPS in *P. gingivalis* but did not affect LPS activity, suggesting that sialidase provided sialic acid for LPS biosynthesis or sialylation modification. However, the effect of sialic acid on LPS was not involved in lipid A activity, which determined LPS activity, so the results of limulus assay showed that amounts of LPS were reduced but LPS activities were unaffected in the sialidase-deficient mutant strain.

This study sought to clarify whether sialidase deficiency moderated immuno-inflammatory responses of human GECs stimulated by *P. gingivalis*. GECs, the superficial layer of the gingival epithelium, are the first cells that come into contact with microorganisms; therefore, GECs are considered to be the first line of defense against initiation and progression of CP. Epi4 is an immortalized GEC line that is obtained by transfecting human GECs with the SV40 T-antigen gene using calcium phosphate.[45] In the present study, epi4 cells were stimulated with different *P. gingivalis* strains, and the role of *P. gingivalis* sialidase in the organism's evasion from host immune responses of GECs was determined. Both IL-1β and TNF-α levels increased in epi4 cells stimulated by different strains of *P. gingivalis*, but increased expression levels in the △PG0352 group were lower than those in the *P. gingivalis* W83 and com△PG0352 groups, suggesting that compared with *P. gingivalis* W83 and com△PG0352, △PG0352 induced less of an inflammatory response in epi4 cells.

It is noteworthy that IL-8 expression decreased in epi4 cells when stimulated by the different *P. gingivalis* strains and that this decrease was less profound in the △PG0352 group compared with the *P. gingivalis* W83 and com△PG0352 groups. IL-8, considered to be an important secondary proinflammatory chemokine, can be induced by many types of cells in response to microbial infection. IL-8 can stimulate host immune responses by migrating and accumulating leukocytes at the site of infection. Many microorganisms can induce IL-8 in host tissues,[46,47] whereas *P. gingivalis* inhibits IL-8 secretion in oral epithelial cells.[48] There are two reasons: one is that gene expression of IL-8 increases at the early phase (6 hours) and is paralyzed at the late phase (24 hours); the other is that *P. gingivalis* proteases, such as Kgp and Rgp, can degrade IL-8 protein. Compared with *P. gingivalis* W83 and com△PG0352, Kgp and Rgp activity levels decreased in △PG0352, indicating that *P. gingivalis*-mediated IL-8 inhibition was lower in the △PG0352 group and that IL-8 level in the △PG0352 group was indeed higher than in the

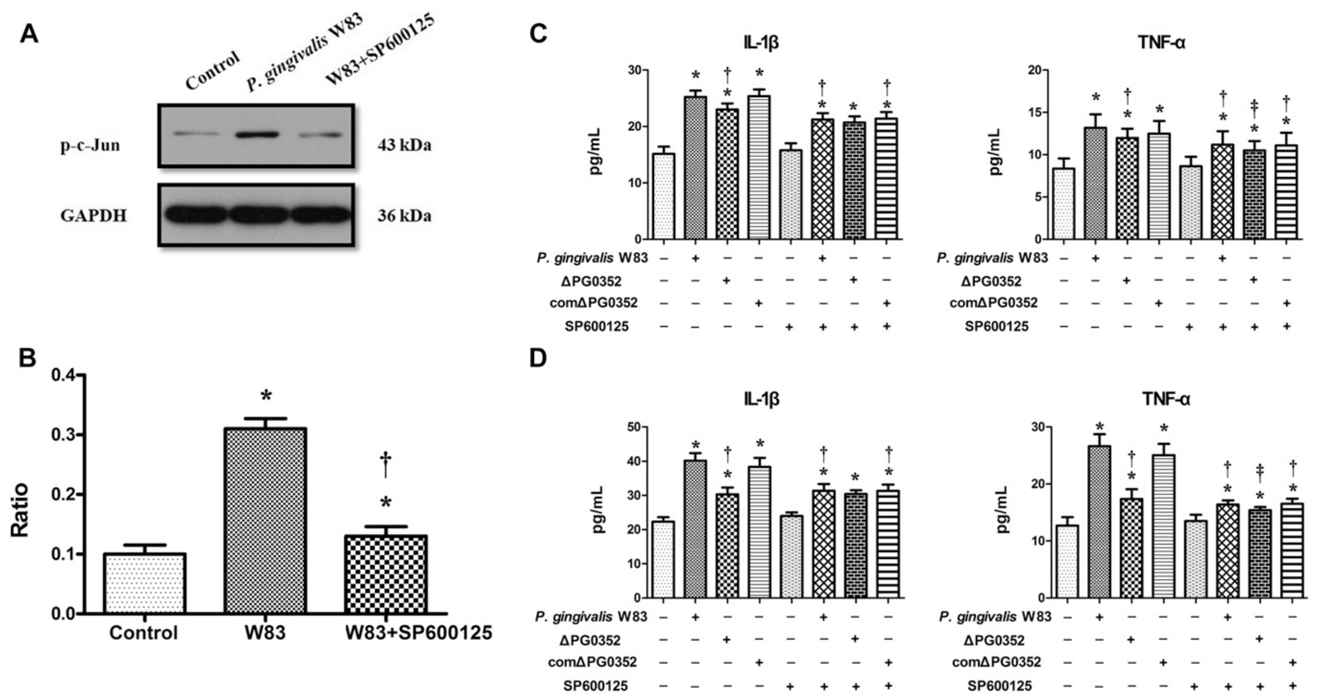

Figure 4.
Effects of JNK inhibitor on phospho-c-Jun, IL-1β, and TNF-α levels in epi4 cells stimulated by P. gingivalis *W83, ΔPG0352, or comΔPG0352.* ***A)*** *Western blotting for phospho-c-Jun.* ***B)*** *Ratio plot of phospho-c-Jun to GAPDH.* ***C)*** *ELISA for 6 hours.* ***D)*** *ELISA for 24 hours.* *****P *<0.01 versus unstimulated cells;* †P *<0.05 versus cells stimulated with* P. gingivalis *W83 without pretreatment with the JNK inhibitor;* ‡P *<0.05 versus cells stimulated with ΔPG0352 without pretreatment with the JNK inhibitor.*

P. gingivalis W83 and com△PG0352 groups. These results suggest that sialidase deficiency in *P. gingivalis* can result in accumulation of more leukocytes at the site of infection, and the infection is thus more easily cleared by immune response in the periodontitis process.

Finally, to determine why the different *P. gingivalis* strains induce diverse responses in host cells, inflammatory signaling pathways were measured in epi4 cells. There are three parallel MAPK signaling pathways, including the p38, ERK, and JNK signaling pathways.[49] Different microbial pathogens that infect distinct host cells may activate different signaling pathways. Results of the present study showed that *P. gingivalis* could not alter protein levels of p38, ERK, and JNK but increased p-p38 and p-JNK, whereas levels of p-ERK were weak regardless of *P. gingivalis* stimulation. In addition, it is noteworthy that, compared with *P. gingivalis* W83 and com△PG0352, epi4 cells stimulated by △PG0352 expressed lower p-JNK levels. Furthermore, there were no differences in IL-1β and TNF-α levels in epi4 cells stimulated by the different *P. gingivalis* strains when JNK signaling was blocked using the JNK inhibitor. These results suggest that epi4 cells stimulated by the different *P. gingivalis* strains induced differing amounts of IL-1β and TNF-α because of differences in p-JNK levels.

CONCLUSIONS

Evidence in this study clearly demonstrated that sialidase deficiency in *P. gingivalis* modulates immuno-inflammatory responses of human GECs caused by *P. gingivalis* exposure by abrogating the increased JNK signaling pathway. Sialidase is a transmembrane protein; it may be involved in the interaction between *P. gingivalis* and GECs directly. Although results of this study found that sialidase deficiency in *P. gingivalis* reduced its virulence by affecting gingipain activity and amount of LPS, these virulence factors also moderate immuno-inflammatory response in epithelial cells. This work helps to understand how *P. gingivalis* causes periodontitis and serves as groundwork for treatment of periodontitis.

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