Porphyromonas gingivalis Mfa1 fimbria induces both IL-6 and IL-8 production by human bronchial epithelial cells via Toll-like receptor 2

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This doctoral thesis was prepared using the original article "*Porphyromonas gingivalis* Mfa1 fimbria putatively binds to TLR2 and induces both IL-6 and IL-8 production in human bronchial epithelial cells" (Yuwa Takahashi, Marni E. Cueno, Noriaki Kamio, Toshimitsu Iinuma, Yoshiaki Hasegawa, and Kenichi Imai. Biochem Biophys Res Commun 589 (2022), 35-40.) with new unpublished data (Fig. 3, Fig. 4A and Fig. 4B).

Abstract

Porphyromonas gingivalis (Pg) is a major periodontal pathogen involved in periodontal disease development and progression. Moreover, Pg has two fimbriae surface proteins (FimA and Mfa1) that are genetically distinct and make-up the fimbrial shaft which in-turn form crucial attachment to oral bacteria and multiple host cells. However, unlike FimA, Mfa1 attachment to non-periodontal cells has not been fully elucidated. Considering Pg contributes to periodontal disease development while periodontal disease is proposed to influence pulmonary disease development it is postulated that Pg (possibly via Mfa1) can potentially adhere to the respiratory epithelial cell surface and, consequently, lead to an inflammatory response putatively related to pulmonary disease development. Unfortunately, this idea was never fully explored. Initially, molecular docking was simulated and performed both luciferase and neutralization assays to confirm Mfa1-related functional interaction. Subsequently, BEAS-2B cells were treated with purified Mfa1 and performed cytokine quantification through real time-PCR and ELISA to establish Mfa1-related functional response. Results showed that both Mfa1-TLR2 and Mfa1-TLR4 docking is possible, however, only Mfa1-TLR2 showed a functional interaction. Additionally, it was observed that both IL-8 and IL-6 gene

expression and protein levels were induced confirming Mfa1-related functional response. Taken together, this study proposes that BEAS-2B human bronchial epithelial cells are able to recognize Pg Mfa1 and induce both IL-8 and IL-6 inflammatory responses. Considering a Pg-related periodontal disease scenario, this study proposes the following sequence of events: (1) Pg may aspirate down the human airway which in-turn would expose the human bronchial epithelial cells to the bacteria; (2) Pg presence does not trigger an infection reaction along the human bronchial epithelial cells, thus, TLR4 was not exposed to the cell surface; (3) Pg (due to Mfa1) could putatively be recognized by TLR2 along the human bronchial epithelial cell surface, thereby, stimulating an inflammatory response via IL-8 and IL-6 induction; and (4) Prolonged Pg exposure and accumulation along the human bronchial epithelial cells would continuously induce an inflammatory response that may contribute to pulmonary disease development.

Introduction

Porphyromonas gingivalis (Pg) is a Gram-negative bacteria categorized as a key periodontal pathogen that has several virulence factors and among them are two fimbriae surface proteins, namely: FimA and Mfa1 [1]. Both fimbriae are genetically distinct and build-up the fimbrial shaft [2]. Moreover, these two fimbriae form crucial attachment with oral biofilm-associated bacterial cells and multiple types of host cells [1, 3-5]. However, unlike the extensively studied FimA fimbria [6, 7], Mfa1 attachment to non-periodontal cells has not been fully elucidated. Considering Pg contributes to periodontal disease development [8, 9] while periodontal disease is proposed to influence pulmonary disease development [10], it is postulated that Pg (possibly via Mfa1) can potentially adhere to the respiratory epithelial cell surface and, consequently, lead to an inflammatory response putatively related to pulmonary disease development. A better understanding of the putative Mfa1 attachment to human respiratory epithelial cells may shed light on how Pg-related periodontal disease (via Mfa1) can contribute to inducing an inflammatory response within respiratory cells and, subsequently, lead to novel therapeutic strategies in treating and/or preventing pulmonary disease development.

Materials and Methods

Molecular docking

Protein crystal structures of Pg Mfa1 (PDB ID: 5NF2) [6], human TLR2 (PDB ID: 2Z7X) [11], and human TLR4 (PDB ID: 2Z63) [12] were used for molecular docking simulation, whereas, ClusPro [13] was used to establish Mfa1-TLR2 and Mfa1-TLR4 docking. Additionally, to facilitate the correctness of the docking simulation among all the possible docking simulations, PrankWeb [14] was utilized to determine the probable protein-protein docking region along the Mfa1 protein surface.

In vitro cell culture and treatment

For the human cell cultures, BEAS-2B human bronchial epithelial cells were obtained from ATCC (Manassas, VA, USA). BEAS-2B cells were incubated *in vitro* in 37°C Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) and supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Rockford, IL, USA), penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}). To serve as a reference for TLR2 and TLR4 stimulation, HEK293 human embryonic kidney cells were used. HEK293 cells were stably transfected with either human TLR2 (293/TLR2;

InvivoGen, San Diego, CA, USA) or TLR4 (293/TLR4-MD2-CD14; InvivoGen) and incubated *in vitro* in 37°C in DMEM and supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific). Similarly, blasticidin antibiotic (10 μg mL^{-1}) was likewise added to maintain transfectant selection. Additionally, lipoteichoic acid (LTA) which was derived from *Staphylococcus aureus* (Sa) as a TLR2 ligand and lipopolysaccharide (LPS) which was derived from *Escherichia coli* (Ec) as a TLR4 ligand were purchased from Sigma and used as positive control. Fimbriae were purified from Pg mutant strains derived from ATCC33277 according to standard protocols [15-17]. Mfa1 and FimA fimbriae were purified from JI-1 and SMF1, in which *fimA* and *mfa1* were deleted, respectively [18, 19].

Cytokine quantification

IL-8 and IL-6 were selected for downstream analyses since both proinflammatory cytokines are known to be stimulated by pathogens along respiratory epithelial cells [20]. For cytokine gene expression analyses, real-time PCR was performed. Two treatment set-ups were performed for analyses: (1) Mfa1-treated human bronchial epithelial cells were incubated at a 24 h timeframe (1, 3, 6, 12, 24 h intervals); and (2) increasing Mfa1 (0.125, 0.25, 0.5, and 1 μg mL^{-1}), Pg FimA (0.25, 0.5, 1, 5, 10, 50 μg

mL^{-1}), and Pg LPS (0.5 and 1 μg mL^{-1}) were independently incubated in a fixed human bronchial epithelial cell culture concentration (4×10^5 CFU mL^{-1}), consequently, RNA was extracted from these cell lines using QIAshredder and RNeasy Mini Kit (QIAGEN, Alameda, CA, USA). Subsequently, cDNA was synthesized using an RNA PCR kit (PrimeScript; Takara Bio, Shiga, Japan) and the resulting cDNA mixture was utilized for real-time PCR analysis using SYBR Premix Ex Taq solution (Takara Bio). Primer sequences used are as follows: IL-8, forward (5′ -CTT GTC ATT GCC AGC TGT GT-3′) and reverse (5′ -TGA CTG TGG AGT TTT GGC TG-3′); IL-6, forward (5′ -TTC GGT CCA GTT GCC TTC TC -3′) and reverse (5′ -GAG GTG AGT GGC TGT CTG TG-3′); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward (5′ -ACC AGC CCC AGC AAG AGC ACA AG-3′) and reverse (5′ -TTC AAG GGG TCT ACA TGG CAA CTG-3′). PCR assays were performed using a TP-800 Thermal Cycler Dice Real-Time System (Takara Bio) following these thermal cycling conditions: 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 1 min. PCR results were verified via melting curve analyses and all PCR experiments were performed in triplicates. Similarly, calculated gene expression levels were normalized to GAPDH mRNA levels.

For cytokine protein level measurements, IL-8 and IL-6 concentrations in the Mfa1-treated human bronchial epithelial cell culture supernatants were measured using commercially available ELISA kits (R&D Systems; Minneapolis, MN, USA) according to the manufacturer's recommendations. Three treatment set-ups were performed for analyses: (1) comparison of Mfa1-, FimA-, and LPS-treated cell lines with all treatment concentration; (2) Mfa1-treated human bronchial epithelial cells were incubated at a 24 h timeframe (1, 3, 6, 12, 24 h intervals); and (3) increasing Mfa1 amounts (0.125, 0.25, 0.5, and 1 μg mL^{-1}) were incubated in a fixed human bronchial epithelial cell culture concentration (4×10^5 CFU mL^{-1}). All experiments were performed in triplicate, and data are presented as the mean ± SD.

Luciferase reporter assay

HEK293, 293-TLR2, and 293-TLR4 cells were incubated overnight in 12-well plates with 4×10^5 cells mL^{-1}growth and, subsequently, cells were transfected with reporter plasmids via the Lipofectamine 2000 transfection reagent (ThermoFisher Scientific) following manufacturer's instructions. Reporter plasmids used for transfection include: 200 ng of 5xκB-luc plasmid (luciferase gene expression is under NF-κB control); and 10 ng of an internal pRL-TK control plasmid (*Renilla reniformis* luciferase is under TK

promoter control). After transfection, Mfa1 (0.25 and 0.5 μg mL^{-1}), LTA (5 μg mL^{-1}), and Ec LPS (200 ng mL^{-1}) were incubated with the transfected cells for 24 h. Consequently, Passive Lysis Buffer (Promega, Madison, WI, USA) was used for cell harvesting and the extracts were assessed for luciferase activity using a Dual-Luciferase Assay System (Promega) with luciferase activity normalized using the internal control.

Cytokine neutralization assay

BEAS-2B cells were pre-incubated with neutralizing antibodies against human TLR2 and TLR4 (R&D Systems) prior to Pg stimulation. IgG antibody was used as control (Invivogen). Optimized anti-TLR2 and anti-TLR4 concentrations used were 0.05, 0.5 and 5 μg mL^{-1}, whereas, anti-IgG concentration used was 5μg mL^{-1}.

Statistical analyses

All data are expressed as mean ± SEM. Statistical analyses were performed by one-way ANOVA with Tukey's *post hoc* analysis using KaleidaGraph (Synergy Software, PA, USA). Difference were regarded as significant at $P < 0.05$.

Results

Mfa1 docks in human TLR2 and TLR4 via the predicted protein-protein interaction sites

Both TLR2 and TLR4 have been found to be significant host receptors of Pg [21]. To determine whether Mfa1 can bind to both human TLR2 and TLR4 receptors, the putative protein-protein interaction site within Mfa1 was established and, likewise, simulated molecular docking. Mfa1 (Fig. 1A), human TLR2 (Fig. 1B), and human TLR4 (Fig. 1C) protein crystal structures are shown prior to docking. Interestingly, the predicted Mfa1 protein-protein interaction site (Fig. 1A) is also where the proline-rich region is located and has been proposed to be involved in protein-protein interaction [22]. This would suggest that the putative protein-protein interaction site is potentially where the Mfa1 protein would dock to either TLR2 or TLR4. As seen in Mfa1-TLR2 (Fig. 1D) and Mfa1-TLR4 (Fig. 1E) docking, it was found that the predicted Mfa1docking orientation differs between TLR2 (outside the concave surface) and TLR4 (inside the concave surface). Moreover, we attributed the difference in Mfa1-TLR2 (Fig. 1F) and Mfa1-TLR4 (Fig. 1G) docking orientation to the putative protein-protein interaction site. Taken together, these results potentially established that: (1) Mfa1 can potentially dock to human TLR2 and TLR4 via

the predicted protein-protein interaction site; and (2) Mfa1 docking orientation differs between Mfa1-TLR2 and Mfa1-TLR4 docking.

Mfa1 functionally interacts with the TLR2 receptor

NF-κB is a transcription factor known for IL-8 and IL-6 induction [23]. To determine if Mfa1 functionally interacts with TLR2 and TLR4 leading to NF-κB activation, luciferase assay was performed. As seen in Figure 2A, NF-κB activation was not observed suggesting that Mfa1 was unable to bind to a receptor consistent with HEK293 cells not having TLR2 and TLR4 receptors [24]. In contrast, 293-TLR2 cells showed NF-κB activation with Mfa1 treatment following a density-dependent manner (Fig. 2B), whereas, 293-TLR4 cells exhibited no NF-κB activation with Mfa1 treatment (Fig. 2C). This putatively suggests that Mfa1 binds to TLR2 receptor but not to TLR4 receptor. It has already been established that TLR4 alone cannot confer LPS responsiveness, however, the TLR4-MD-2 complex allows TLR4 to functionally interact with LPS [25]. As seen in Figure 3, MD-2 complexes with TLR4 inside the concave surface. Interestingly, this is also where Mfa1 binds (Fig. 1E, 1G). In this regard, it is suspected that the inability of Mfa1 to bind to TLR4 is due to MD-2 occupying the same binding site [26]. Subsequently, to elucidate whether Mfa1 binding to BEAS-2B cells resulted to IL-8 and IL-6 induction, neutralization assay was performed. As

shown in Figure2D (IL-8) and 2E (IL-6), TLR4 neutralization had no effect in inhibiting IL-8 and IL-6 induction following Pg exposure, whereas, TLR2 neutralization inhibited both IL-8 and IL-6 induction following Pg exposure. These results further highlight that Mfa1-TLR2 binding occurred while no Mfa1-TLR4 binding occurred. Taken together, these results putatively elucidated the following: (1) Mfa1 functionally interacts with TLR2 and not TLR4 along the bronchial epithelial cell; and (2) Mfa1 binding to TLR4 is possibly affected by protein-protein complex formation inside the TLR4 concave surface.

Mfa1 potentially induces IL-8 and IL-6 proinflammatory cytokine response

Pg is known to cause dysbiosis of the periodontal flora which in-turn trigger proinflammatory cytokine responses [27]. To determine whether Mfa1 can induce proinflammatory cytokine production in bronchial epithelial cells, both IL-8 and IL-6 gene expression and protein levels were measured. Initially, comparison of both IL-8 and IL-6 protein levels was made among bronchial epithelial cells independently treated with Mfa1 and other known Pg virulence factors (FimA and LPS). It was found that only Mfa1 was able to stimulate both IL-8 (Fig. 4A, *left panel*) and IL-6 (Fig. 4B, *left panel*), whereas, both Pg LPS (Figs. 4A and 4B, *left panels*) and FimA (Figs. 4A and 4B, *right panels*) were unable to stimulate IL-8 and IL-6 protein levels. This would imply that Mfa1 can induce a

functional response from bronchial epithelial cells possibly ascribable to Mfa1-TLR2 binding (Fig. 1D). Moreover, it was likewise observed that the length of Mfa1 exposure to bronchial epithelial cells could affect IL-8 and IL-6 gene expression (Fig. 4C, 4D) and protein levels (Fig. 4E, 4F), respectively. Noticeably, there is a synthesis delay in both IL-8 and IL-6 mRNA and protein accumulation which is expected during state transition [28]. Nevertheless, it was observed that both IL-8 and IL-6 induction and, likewise, this establishes that the best quantitative correlation of protein levels is at 24 h post Mfa1 treatment to the mRNA levels at 3 h. Similarly, it was found that increasing Mfa1 amounts subsequently increases both IL-8 and IL-6 gene expression (Fig. 4G, 4H) and protein levels (Fig. 4I, 4J). This would insinuate that Mfa1 concentration directly influences both IL-8 and IL-6 induction. Taken together, these results postulate the following: (1) Mfa1 can stimulate both IL-8 and IL-6 from bronchial epithelial cells; and (2) Mfa1 functional response is both time- and dose-dependent.

Discussion

Periodontal diseases are hypothesized to contribute to pulmonary disease development attributable to oral bacteria (such as Pg) aspirating down the lower respiratory tract [10, 29]. Considering Pg plays a role in periodontal disease development [8, 9], it is believed that Pg presence along respiratory epithelial cells (such as bronchial epithelial cells) may trigger an inflammatory response which in-turn may contribute to pulmonary disease development. Throughout this study, the possible functional interaction and response of Pg to human bronchial epithelial cells was elucidated.

TLRs belong to a family of trans-membrane proteins generally found in eukaryotic cells and function in pathogen detection by recognizing pathogenic ligands with specific structural features which consequently activates downstream signaling pathways associated with immune responses [30]. However, TLRs are less widespread along epithelial cells since TLR proteins may not be detected regardless of TLR mRNA presence in order to avoid unnecessary immune response especially when dealing with commensal organisms [31]. In fact, airway epithelial cells particularly express TLR2 and TLR4 at low levels and would only increase during infection [32]. Additionally, both TLR2 and TLR4 responses along the airway epithelial cells are likewise influenced

by either the low or absent expression of MD2 and CD36 [33, 34] which may explain why bacterial LPS is unable to stimulate a functional response (particularly from TLR4) along the airway epithelial cells consistent with an earlier work [25]. Moreover, TLR4 along the airway epithelium is often intracellularly located [35] which would insinuate that TLR4 is not immediately available for binding until an infection occurs. In this regard, it is postulated that the putative Mfa1-TLR2 interaction lead to both IL-8 and IL-6 induction insinuating that Pg can putatively serve as an inflammatory stimulant along the human bronchial epithelial cells without necessarily causing an infection. Similarly, it is suspected that TLR4 was not significantly present along the human bronchial epithelial cells, thereby, no IL-8 and IL-6 induction were observed. Furthermore, considering TLR4 would only be available on the airway epithelial cell surface during an infection [35], it is hypothesized that Pg did not prompt an infection reaction and would mean that Pg presence alone may be enough to trigger an inflammatory response. Admittedly, additional work is needed to further prove this hypothesis.

In summary, the overall results putatively established that BEAS-2B human bronchial epithelial cells are able to recognize Pg Mfa1 and induce both IL-8 and IL-6 inflammatory responses. Considering a Pg-related periodontal disease scenario, this

study proposes the following sequence of events: (1) Pg may aspirate down the human airway which in-turn would expose the human bronchial epithelial cells to the bacteria; (2) Pg presence does not trigger an infection reaction along the human bronchial epithelial cells, thus, TLR4 was not exposed to the cell surface; (3) Pg (due to Mfa1) could putatively be recognized by TLR2 along the human bronchial epithelial cell surface, thereby, stimulating an inflammatory response via IL-8 and IL-6 induction; and (4) Prolonged Pg exposure and accumulation along the human bronchial epithelial cells would continuously induce an inflammatory response that may contribute to pulmonary disease development.

Conclusion

The purpose of this study was to elucidate the influence of Pg Maf1 fimbria on production of inflammatory cytokines in human bronchial epithelial cells. Thus, the following conclusions were drawn:

(1) Mfa1 can stimulate both IL-8 and IL-6 from bronchial epithelial cells.

(2) Mfa1 functionally interacts with TLR2 and not TLR4 along the bronchial epithelial cell.

(3) Prolonged Pg exposure and accumulation along the bronchial epithelial cells would continuously induce an inflammatory response that may contribute to pulmonary disease development.

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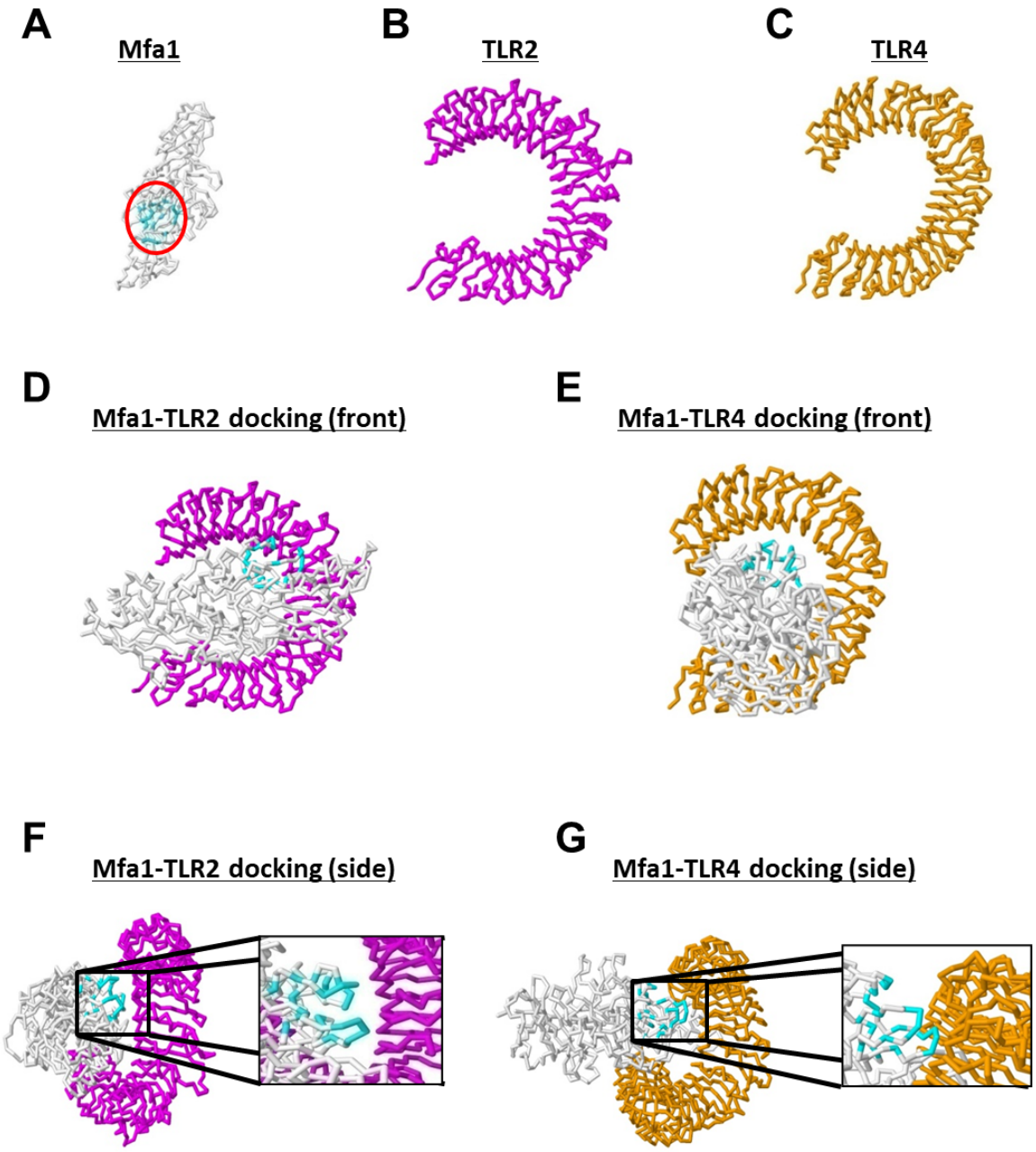


Figure 1. *Porphyromonas gingivalis* Mfa1 binds to human TLR2 and TLR4 via the predicted protein-protein interaction sites. Crystal structures of (A) *P. gingivalis* Mfa1, (B) human TLR2, and (C) human TLR4 are shown. Predicted Mfa1 protein-protein interaction sites are circled red and residues involved are colored cyan. (D) Mfa1-TLR2 and (E) Mfa1-TLR4 frontal view of docking are presented. (F) Mfa1-TLR2 and (G) Mfa1-TLR4 side view of docking are shown.

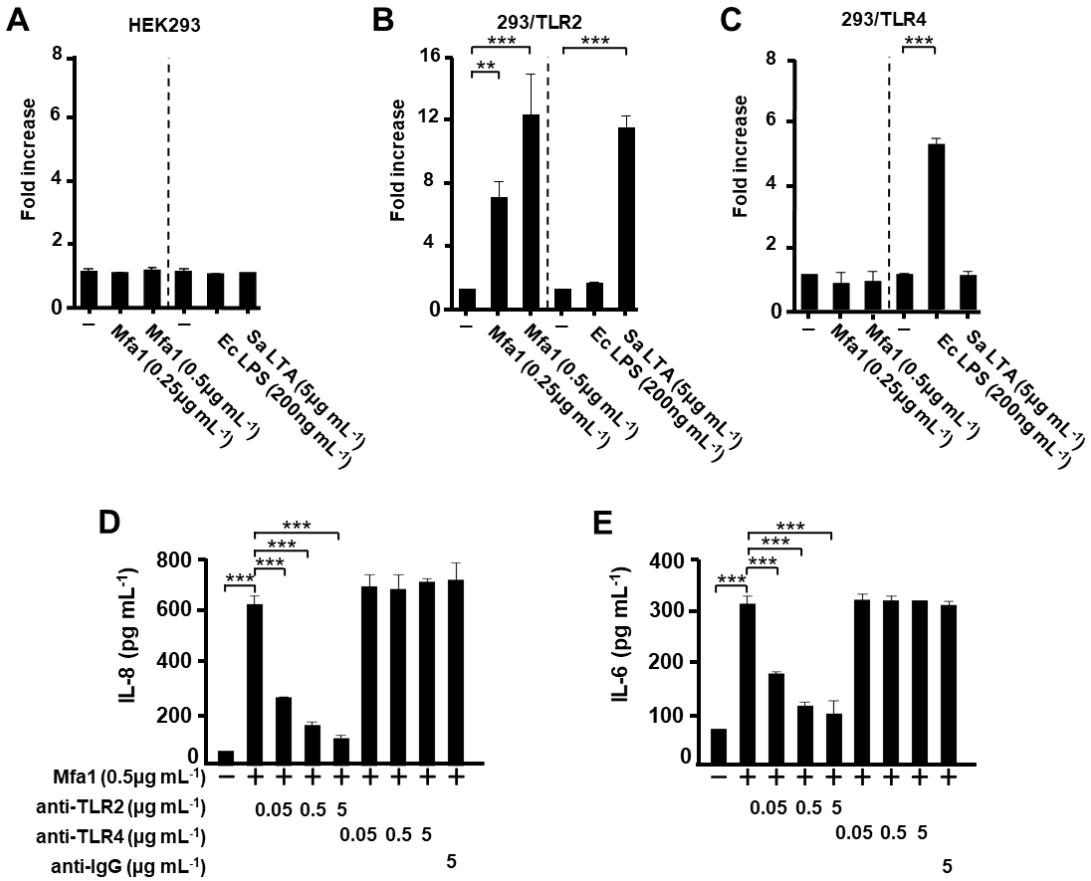


Figure 2. *Porphyromonas gingivalis* Mfa1 functionally interacts only with the human TLR2 receptor. Luciferase assay showing NF-κB activation of (A) HEK293 cells, (B) 293-TLR2, and (C) 293-TLR4 in the presence of *P. gingivalis* Mfa1 are presented. Treatments of increasing Mfa1 doses, *E. coli* (Ec) LPS, and *Staphylococcus aureus* (Sa) LTA are indicated. Neutralization assay showing secreted (D) IL-8 and (E) IL-6 levels from *P. gingivalis* Mfa1-treated BEAS-2B cells and antibodies used for control (anti-IgG) and treatment (anti-TLR2 and anti-TLR4) are indicated. These experiments were conducted in triplicate and data are presented as the mean ± SD; $n = 3$ (**, $p < 0.01$; ***, $p < 0.001$).

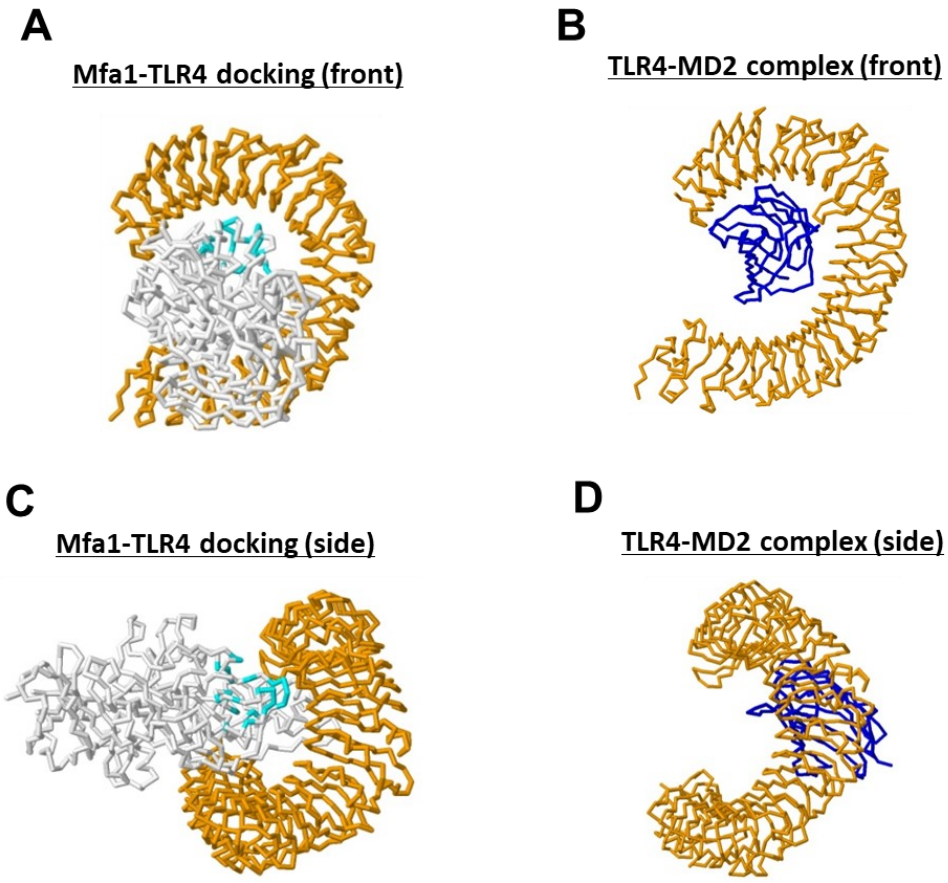
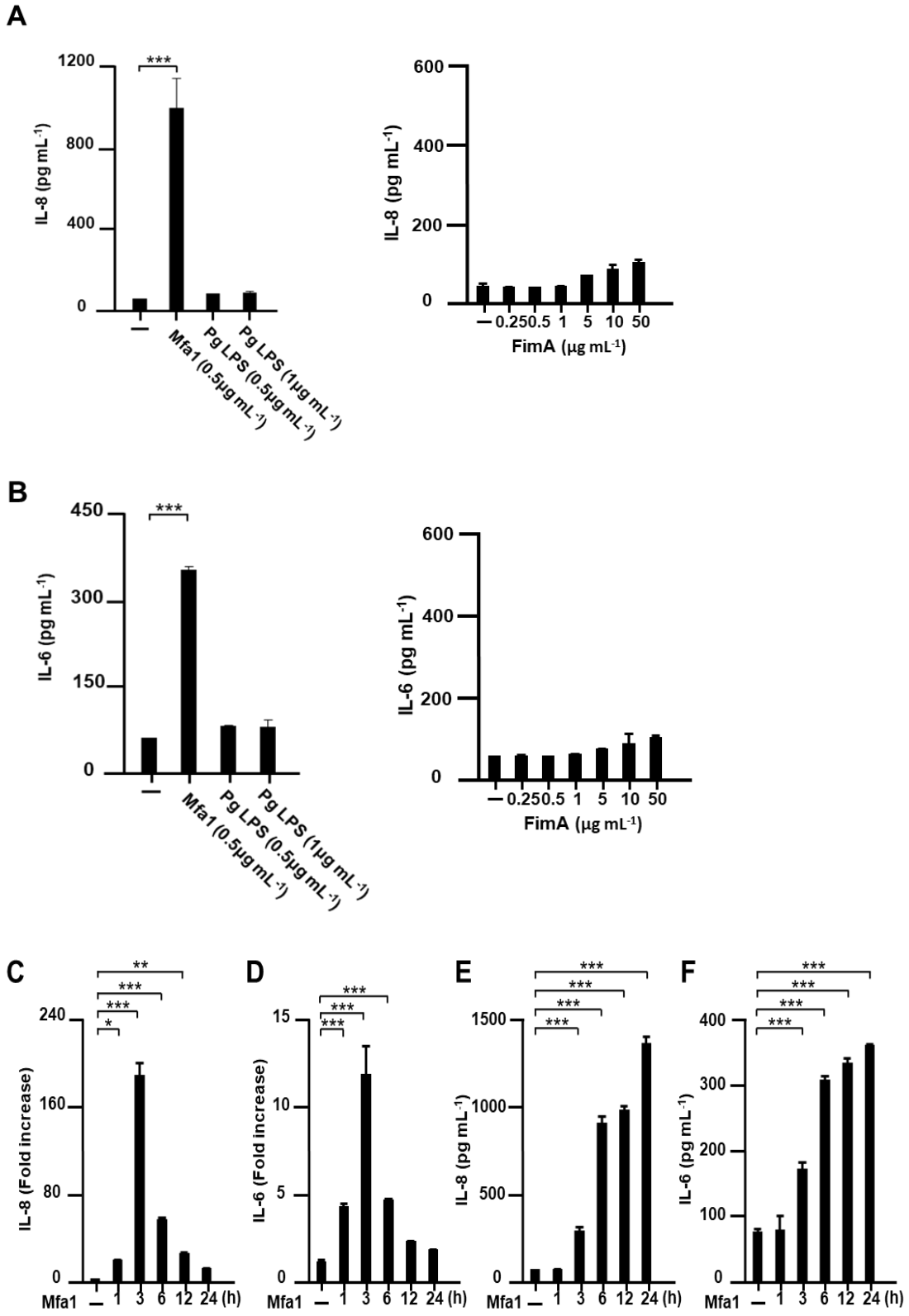


Figure 3. Docking comparison of Mfa1-TLR4 and TLR4-MD2. Frontal view of docking between (A) Mfa1-TLR4 and (B) TLR4-MD2 complex are shown. Side view of docking between (C) Mfa1-TLR4 and (D) TLR4-MD2 complex are presented.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IL-8 (pg mL-1)
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***
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Mfa1 (0.5μg mL-1)
Pg LPS (0.5μg mL-1)
Pg LPS (1μg mL-1)
600
400
200
0
—0.250.5 1 5 10 50
FimA (μg mL-1)
B
IL-6 (pg mL-1)
450
300
150
0
***
—
Mfa1 (0.5μg mL-1)
Pg LPS (0.5μg mL-1)
Pg LPS (1μg mL-1)
600
400
200
0
—0.250.5 1 5 10 50
FimA (μg mL-1)
C
IL-8 (Fold increase)
240
160
80
0
**
***
***
*
Mfa1 — 1 3 6 12 24 (h)
D
IL-6 (Fold increase)
15
10
5
0
***
***
***
Mfa1 — 1 3 6 12 24 (h)
E
IL-8 (pg mL-1)
1500
1000
500
0
***
***
***
***
Mfa1 — 1 3 6 12 24 (h)
F
IL-6 (pg mL-1)
400
300
200
100
0
***
***
***
***
Mfa1 — 1 3 6 12 24 (h)

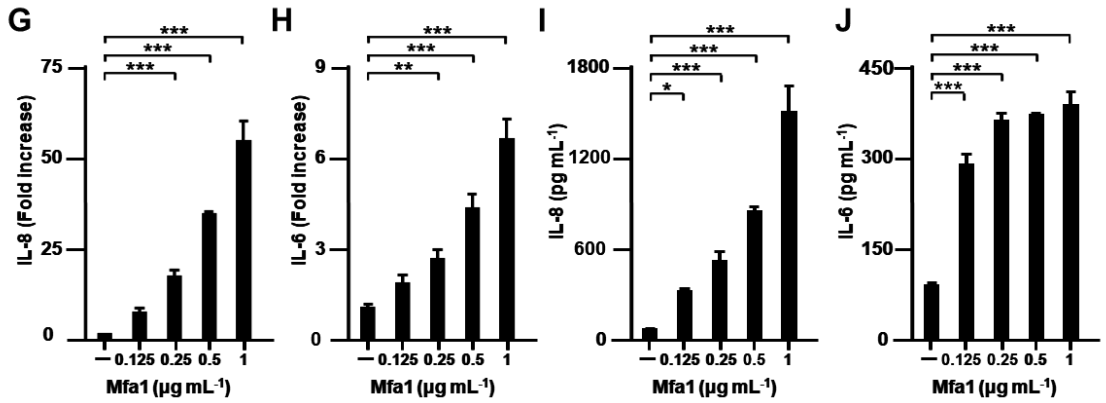

Figure 4. *Porphyromonas gingivalis* Mfa1 putatively triggers both IL-8 and IL-6 proinflammatory cytokine responses. Induction comparison of secreted (A) IL-8 and (B) IL-6 levels from BEAS-2B cells treated with *P. gingivalis* (Pg) Mfa1, Pg LPS, and FimA are shown. Time-dependent comparison of both (C) IL-8 and (D) IL-6 gene expression and both (E) IL-8 and (F) IL-6 protein levels are shown. Dose-dependent comparison of both (G) IL-8 and (H) IL-6 gene expression and both (I) IL-8 and (J) IL-6 protein levels are presented. Increasing *P. gingivalis* Mfa1 levels are indicated. These experiments were conducted in triplicate, and data are presented as the mean ± SD; n = 3 (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).