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Original Article

# *Salmonella* fimbrial protein StcD induces cyclooxygenase-2 expression via Toll-like receptor 4

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**KEYWORDS**
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StcD;
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**Abstract** *Introduction:* The genome of *Salmonella enterica* serovar Typhimurium contains 13 operons with homology to fimbrial genes.
*Methods:* To investigate the involvement of these fimbrial gene clusters in the expression of cyclooxygenase-2 (COX-2), which is an inducible enzyme involved in the synthesis of prostanoids, in J774 macrophages infected with *S. enterica* serovar Typhimurium, we constructed strains carrying a mutation in genes encoding the putative subunit proteins in 12 fimbrial operons.
*Results:* The level of COX-2 expression was lower in macrophages infected with *fimA* or *stcA* mutant *Salmonella* than in those infected with wild-type *Salmonella*. Therefore, we focused on putative subunit protein StcA and adhesive like protein StcD encoded in the *stc* operon. Treatment of macrophages with purified recombinant StcD protein, but not StcA, resulted in the activation of the mitogen-activated protein kinase and nuclear factor kappa B signaling pathways, leading to the expression of not only COX-2 but also of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha. The expression of StcD-induced COX-2 was inhibited by treatment with the Toll-like receptor 4 (TLR4) inhibitor TAK-242, but not by treatment with the lipopolysaccharide (LPS) antagonist polymyxin B. Furthermore, StcD treatment stimulated HEK293 cells expressing TLR4 in the presence of CD14 and MD-2.
*Conclusion:* StcD is a pathogen-associated molecular pattern of *S. enterica* serovar Typhimurium that is recognized by TLR4 and plays a significant role in the induction of COX-2 expression in macrophages.


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## Introduction

Salmonellae are invasive gastrointestinal pathogens that infect both humans and animals, and cause a variety of diseases. Salmonellae invade and destroy specialized epithelial cells in the host's intestine and migrate to the mesenteric lymph nodes, where they encounter macrophages that play an important role in host defense.[1] The ability of salmonellae to survive within macrophages is required for systemic disease.[2] Specific virulence factors encoded within the *Salmonella* pathogenicity islands (SPIs) are required at various stages of *Salmonella* infection.[3] Among these virulence factors, SPI-2 is required for survival and replication in the intracellular environment of macrophages.[4,5]

Several cytokines and eicosanoids, such as prostaglandins (PGs) and leukotrienes, are known to affect the function of macrophages.[6] The rate-limiting step in PG synthesis is catalyzed by cyclooxygenase (COX). COX converts arachidonic acid to $PGH_2$, the common precursor to all PGs and thromboxanes.[7] There are two isoforms of COX enzyme that are encoded by distinct genes.[8] Whereas COX-1 is constitutively expressed in most cell types and plays a role in gastrointestinal and reproductive function, COX-2 is normally expressed at very low levels but is strongly induced by various stimuli, including mitogens, cytokines, hormones, and oncogenes.[9]

*Salmonella* species encode different fimbrial gene clusters.[10] *Salmonella enterica* serovar Typhimurium carries 13 putative fimbrial operons termed *bcf*, *csg*, *fim*, *lpf*, *pef*, *saf*, *stb*, *stc*, *std*, *stf*, *sth*, *sti*, and *stj*.[11] Of these operons, *fim* and *csg* have been shown by electron microscopy to mediate the expression of fimbrial filaments on the surface of bacterial cells.[12] However, little is known about the structure and function of *Salmonella* fimbriae, except for type 1 fimbriae encoded in the *fim* operon. Type 1 fimbriae are mainly composed of major subunit protein FimA and adhesive protein FimH. The adhesive properties of type 1 fimbriae depend on the adhesion of lectin-like FimH, which is located at the tip of the fimbrial shaft. The interactions between type 1 fimbriae and host cell receptors play a critical role in the initiation and modulation of innate and adaptive immune responses.[13–15] Our previous study showed that type 1 fimbriae are involved in the expression of pro-inflammatory cytokines in *Salmonella*-infected macrophages.[16]

Pathogen-associated molecular patterns (PAMPs), which are components of bacteria and viruses, induce the expression of mediators by several cell types including macrophages and influence the host immune system.[17] PAMPs include cell wall components derived from Gram-positive bacteria, lipopolysaccharides (LPS) from Gram-negative bacteria, lipoteichoic acid, flagella, and fimbriae, all of which are recognized by pattern-recognition receptors such as Toll-like receptors (TLRs). For example, bacterial cell wall components and lipoteichoic acid are recognized by TLR2,[18] and LPS and the flagellar filament protein FliC bind TLR4 and TLR5, respectively.[19,20] In our previous study, *Salmonella* FimH induced the expression of pro-inflammatory cytokines via TLR4 in macrophages.[16]

In this study, we investigated the involvement of 12 fimbrial operons in the expression of COX-2 in macrophages infected with *S. enterica* serovar Typhimurium. We found that adhesive like protein StcD encoded in the *stc* operon is a PAMP that is recognized by TLR4 and plays a significant role in the induction of COX-2 expression in macrophages.

## Methods

### Reagents

Reagents for cell culture, LPS (*Escherichia coli*, 0111:B4), and polymyxin B were purchased from Sigma–Aldrich (St. Louis, MO). SB203580, SP600125, PD98059, MG-132, and TAK-242 were obtained from Millipore (Billerica, MA).

### Bacterial strains, plasmid, and growth conditions

The bacterial strains used in this study were derived from the wild-type *S. enterica* serovar Typhimurium strain 14028s. Strains carrying a mutation in *bcfA*, *csgA*, *fimA*, *lpfA*, *pefA*, *safA*, *sthA*, *stiA*, *stbA*, *stcA*, *stdA*, and *stfA* genes encoding the putative fimbrial subunit proteins located in 12 operons were constructed using the Red recombination system,[21] as described previously.[16] The primers used in this experiment are shown in Table S1. Bacteria were grown at 37 °C in Luria broth (LB).

### Purification of recombinant proteins

The *S. enterica* serovar Typhimurium strain 14028s *stcA* gene (465-bp DNA fragment, except for 66-bp signal sequence) and *stcD* gene (954-bp DNA fragment, except for 54-bp signal sequence) were each cloned into the pET151/D-TOPO vector using Champion™ pET Directional TOPO® Expression Kits (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The following DNA fragments were amplified by PCR using the corresponding primers: *stcA*, 5′-CACCGTTGATGAGTATGATTCAGGC-3′ and 5′-TTAGTCAGTAACAACCGTTAAAGT-3′; and *stcD*, 5′-CACCG CCTGTACGGGAGAAATTACT-3′ and 5′-TTAATCGTATTTCACA-TAGATCAGC-3′. The resulting constructs were verified by direct sequencing. The recombinant proteins StcA and StcD were made and purified as described previously.[16] The concentrations of LPS contained in purified StcA and StcD proteins were determined using Limulus ES-IISingle Test *Wako* Kit (FUJIFILM WAKO, Osaka, Japan) according to the manufacturer's instructions.

### Cell culture and bacterial infection

The mannose receptor-positive murine macrophage cell line J774 E, and Human embryonic kidney (HEK) 293 cells were maintained at 37 °C in an incubator with a 5% $CO_2$ atmosphere. Cells were cultured in Dulbecco's modified Eagle's medium (Sigma–Aldrich) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), 100 U/mL penicillin, and 100 μg/mL streptomycin. Bacterial infection experiments using macrophages were performed as described previously.[16]

### RNA preparation and quantitative real-time polymerase chain reaction

Total RNA preparation and qRT-PCR analysis were performed as described previously.[16] Each sample was analyzed in triplicate. The sequences of primers were as follows: for *COX2*, 5′-TTTGTTGAGTCATTCACCAGACAGAT-3′ and 5′-GCGCTACTATTGAGAGACTGGTATT-3′; for interleukin (IL)-1β (*IL1B*), 5′-TGTCTTGGCCGAGGACTAAGG-3′ and 5′-TGGGCTGGACTGTTTCTAATGC-3′; for *IL6*, 5′-CCAAACTGGATATAATCAGGAAAT-3′ and 5′-CTAGGTTTGCCGAGTAGATCTC-3′; for tumor necrosis factor alpha (*TNFA*), 5′-AGAAACACAAGATGCTGGGACAGT-3′ and 5′-CCTTTGCAGAACTCAGGAATGG-3′; and for *GAPDH*, 5′-TGGCCAAGGTCATCCATGACAAC-3′ and 5′-TCCAGAGGGGCCATCCACAGTCTTCTG-3′.

### Western blot analysis

Western blot analyses were performed essentially as described previously.[22] Cell lysates from macrophages were prepared, and the expression of COX-2, IκB-α, and three MAPKs (ERK, p38, and JNK) was evaluated as described previously.[22,16] Bands were analyzed using a FUSION Chemiluminescence Imaging System (M&S, Tokyo, Japan).

### Luciferase assay

HEK293 cells, HEK293 cells expressing mouse TLR4 (HEK293/mTLR4), and HEK293 cells expressing mouse TLR4 and MD-2/CD14 (HEK293/mTLR4/MD-2/CD14) were purchased from InvivoGen (San Diego, CA). These cells were transiently transfected with the pNiFty-Luc NF-κB firefly luciferase reporter construct (InvivoGen) and the pRL-SV40 *Renilla* luciferase reporter vector (Promega, Madison, WI) as a control for transfection efficiency using Lipofectamine 3000 reagent (Life Technologies). Luciferase assay was performed as described previously.[16]

### Statistical analysis

Each experiment was performed at least three times. The results are expressed as the mean ± standard deviation. Data were analyzed by one-way analysis of variance followed by Tukey's multiple-comparison test. Differences at $p < 0.05$ were considered statistically significant.

## Results

### Involvement of the *fimA* and *stcA* genes in the expression of COX-2 in *Salmonella*-infected macrophages

We constructed strains carrying a mutation in genes encoding the putative fimbrial subunit proteins located in 12 operons including *bcfA*, *csgA*, *fimA*, *lpfA*, *pefA*, *safA*, *sthA*, *stiA*, *stbA*, *stcA*, *stdA*, and *stfA*, and examined whether these fimbrial genes participate in COX-2 expression in J774 macrophages infected with *S. enterica* serovar Typhimurium. COX-2 expression in cytosolic extracts from macrophages at 2.5 h post-infection was analyzed by western blotting using an anti-COX-2 antibody. To reduce disparities in bacterial adhesion, we promoted adhesion of the bacteria to the macrophages by centrifugation, as described in the Materials and Methods. Indeed, almost no difference was observed between the uptake of bacteria by macrophages infected with the wild-type strain and macrophages infected with mutant *Salmonella* strains (Fig. S1). As shown in Fig. 1, the level of COX-2 expression in macrophages was lower when infected with the *fimA* or *stcA* mutant than with wild-type *Salmonella*. These results indicated that type 1 fimbriae and *stc* fimbriae are involved in COX-2 expression in *Salmonella*-infected macrophages.

### StcD induces the expression of COX-2 and pro-inflammatory cytokines in macrophages

StcD encoded in the *stc* operon may exhibit adhesive function similar to FimH due to its amino acid sequence homology. Therefore, we constructed a strain carrying a mutation in *stcD* to examine whether this fimbrial gene participates in COX-2 expression in *Salmonella*-infected macrophages. As shown in Fig. 2A, the level of COX-2 expression in macrophages was significantly reduced when infected with the *stcD* mutant or the *stcA* mutant compared with wild-type *Salmonella*.

Next, to examine the direct contribution of StcA and StcD to COX-2 expression, both proteins were purified as described in the Materials and Methods. The purified recombinant proteins appeared as single bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of approximately 19.8 kDa for StcA and 36.9 kDa for StcD (Fig. 2B). After macrophages were treated for 2.5 h with StcA (5 μg/mL) or StcD (1 and 5 μg/mL), cytosolic extracts were prepared and the expression of COX-2 was assessed by western blotting analysis. Treatment of macrophages with StcD induced dose-dependent expression of

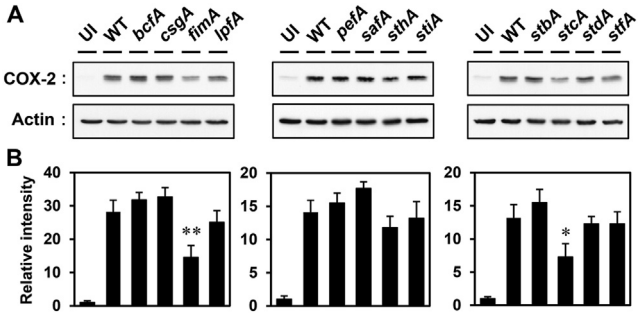


**Figure 1.** Involvement of fimbrial genes in the expression of COX-2 in *Salmonella*-infected macrophages. Macrophages were infected with wild-type (WT) or each mutant *Salmonella*. At 2.5 h post-infection, cytosolic extracts were prepared and analyzed by western blotting for COX-2. (A) Images of the original blots. (B) COX-2 quantities were normalized to those of actin. Graphs show values as relative intensity compared with uninfected macrophages. The levels of COX-2 in the macrophages infected with the *fimA* or *stcA*-mutant *Salmonella* were decreased compared with that of wild-type *Salmonella*-infected cells. *, $p < 0.05$; **, $p < 0.01$, significantly different from macrophages infected with wild-type *Salmonella*. UI, uninfected.

COX-2 (Fig. 2C). On the other hand, StcA did not induce COX-2 expression, indicating the involvement of StcD, but not StcA, in the induction of COX-2 expression in macrophages.

We further examined whether the expression of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α are induced in StcD-treated macrophages. After treatment, the expression of *COX2*, *IL1B*, *IL6* and *TNFA* mRNA was assessed by qRT-PCR. As shown in Fig. 3, the expression of these genes increased, indicating that StcD can induce the expression of pro-inflammatory cytokines as well as COX-2.

## StcD participates in COX-2 expression via activation of the NF-κB and p38 MAPK signaling pathways in macrophages

We examined the involvement of the nuclear factor kappa B (NF-κB) signal transduction pathway in the expression of COX-2. As shown in Fig. 4A, COX-2 expression was decreased in a dose-dependent manner upon addition of the NF-κB inhibitor MG-132. This result indicates that the NF-κB pathway participates in StcD-induced expression of COX-2. To confirm whether the NF-κB pathway participates in COX-2 expression, we estimated NF-κB activation by measuring the level of IκB-α degradation in StcD-treated macrophages. To assess NF-κB activation by measuring IκB-α degradation, experiments were performed in the presence of cycloheximide (0.1 μg/mL) to block the synthesis of new IκB-α. LPS, an activator of NF-κB, was used as a positive control. As shown in Fig. 4B, IκB-α protein quantities in StcD-treated macrophages at 2.5 h post-treatment decreased compared with those in untreated macrophages. These findings demonstrate that the NF-κB pathway is involved in StcD-induced COX-2 expression.

We next examined the involvement of the mitogen-activated protein kinase (MAPK) signal transduction pathways on the expression of COX-2. Fig. 5 shows that COX-2 expression in StcD-treated macrophages was reduced by SB203580, which inhibits p38 MAPK, in a concentration-dependent manner, but not by the extracellular signal-regulated kinase (ERK) inhibitor PD98059 and the c-Jun amino-terminal kinase (JNK) inhibitor SP600125. To confirm the participation of the MAPK pathways, we assessed MAPK activation by quantifying phosphorylated p38, ERK, and JNK MAPKs in StcD-treated macrophages. As shown in Fig. 6, treatment of macrophages with StcD increased p38, ERK, and JNK MAPK phosphorylation in a dose-dependent manner when measured 2.5 h post-treatment. Together with the results from Fig. 4, these findings demonstrate that the NF-κB and p38 MAPK pathways play significant roles in StcD-induced expression of COX-2.

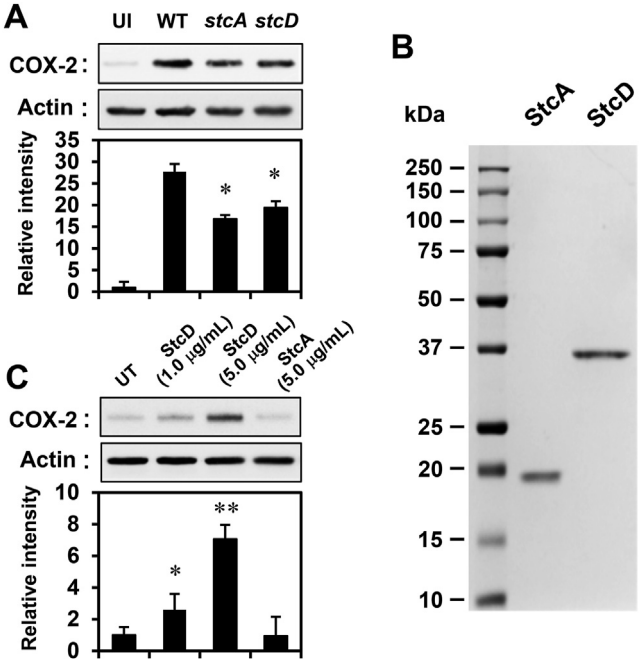


**Figure 2.** StcD induces COX-2 expression in macrophages. (A) Involvement of the *stcA* and *stcD* genes in the induction of COX-2 expression in *Salmonella*-infected macrophages. Macrophages were infected with wild-type (WT), *stcA*, or *stcD*-mutant *Salmonella*. At 2.5 h post-infection, cytosolic extracts were prepared and analyzed by western blotting for COX-2. COX-2 quantities were normalized to those of actin. *, $p < 0.05$, significantly different from macrophages infected with wild-type. (B) SDS-PAGE of purified recombinant StcA and StcD proteins stained with Coomassie Brilliant Blue. Molecular masses of standard proteins are indicated on the left. (C) Involvement of StcD in the induction of COX-2 expression in macrophages. Macrophages were treated with StcA or StcD at the indicated concentrations. After 2.5 h treatment, cytosolic extracts were prepared and analyzed by western blotting for COX-2. COX-2 quantities were normalized to those of actin. *, $p < 0.05$; **, $p < 0.001$, significantly different from untreated macrophages. UI, uninfected. UT, untreated.

## Involvement of TLR4 in the expression of StcD-induced *COX2* mRNA

We next explored the mechanism by which StcD induces *COX2* expression in macrophages. Treatment of macrophages with the TLR4 agonist LPS (1 μg/mL) resulted in an increase in *COX2* expression at 2.5 h post-treatment, and this increase was inhibited by treatment with the TLR4 inhibitor TAK-242 (Fig. 7A), indicating that stimulation of TLR4 by LPS can induce *COX2* expression in macrophages. Blocking of TLR4 with TAK-242 also showed that TLR4 is necessary for StcD-induced *COX2* expression (Fig. 7A).

We also investigated the possibility that TLR4-mediated induction of *COX2* expression by StcD could be an artifact of LPS contamination in the purified StcD protein solution in the following ways. Firstly, we determined the LPS levels in the purified StcD and StcA solutions using the *Limulus* amebocyte lysate assay. The samples were contaminated with 115.8 pg and 89.4 pg of LPS per μg of StcD and StcA protein, respectively. Treatment of macrophages with StcD at 1 μg/mL induced 2- to 3-fold increased expression of *COX2* compared to untreated cells, whereas treatment with 10-fold more StcA (10 μg/mL) did not increase *COX2* expression (Fig. 3A). Secondly, we investigated the effects of the LPS neutralizer polymyxin B[23] on StcD-induced *COX2* expression in macrophages. As shown in Fig. 7B, polymyxin B (100 μg/mL) completely blocked LPS-induced expression

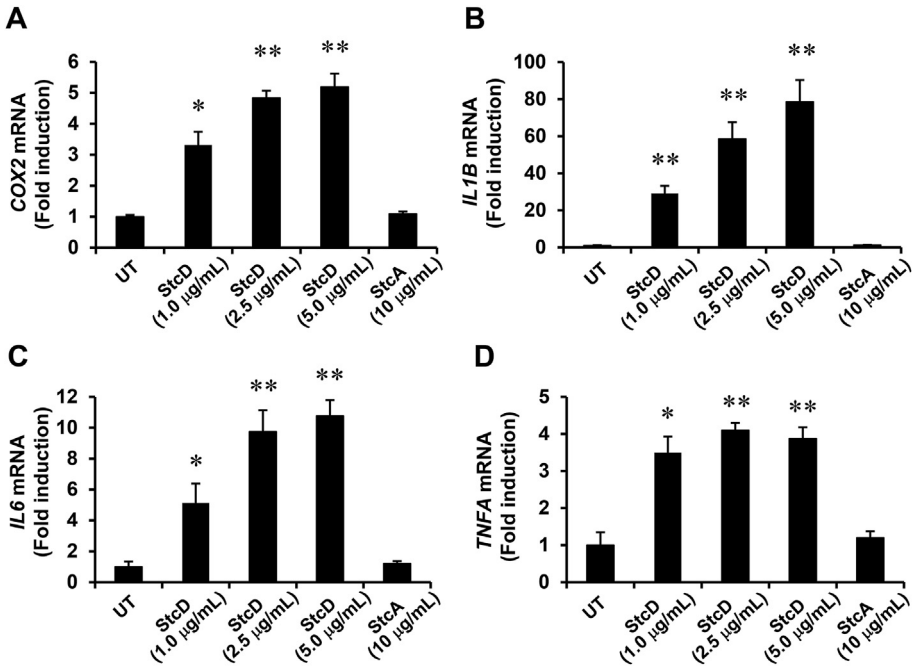


**Figure 3.** Expression of *COX2*, *IL1B*, *IL6*, and *TNFA* mRNA in macrophages treated with StcD and StcA. Macrophages were treated with StcD or StcA at the indicated concentrations. After 2.5 h treatment, total RNA was prepared and analyzed by qRT-PCR. (A) Quantitative analysis of *COX2* mRNA expression. (B) Quantitative analysis of *IL1B* mRNA expression. (C) Quantitative analysis of *IL6* mRNA expression. (D) Quantitative analysis of *TNFA* mRNA expression. Abundances of *COX2*, *IL1B*, *IL6*, and *TNFA* mRNA were normalized to those of *GAPDH* mRNA. *, $p < 0.01$; **, $p < 0.001$, significantly different from untreated macrophages. UT, untreated.

but did not affect StcD-induced *COX2* expression. Thirdly, we examined the effect of heat treatment on the ability of StcD to induce *COX2* expression in macrophages. As shown in Fig. 7C, LPS treatment increased *COX2* expression even after a 30 min heat treatment at 100 °C, whereas heat treatment largely abolished StcD-induced *COX2* expression. This result shows that the ability of StcD to induce *COX2* expression is not thermostable, unlike that of LPS. Together, these results strongly suggest that the induction of TLR4-mediated *COX2* expression by StcD is not an artifact due to LPS contamination.

### StcD is recognized by TLR4

To confirm the participation of TLR4 in the induction of *COX2* expression by StcD, we examined the ability of StcD to stimulate HEK293 cells, HEK293 cells expressing mouse TLR4 (HEK293/mTLR4), and HEK293 cells expressing mouse TLR4 and MD-2/CD14 (HEK293/mTLR4/MD-2/CD14). The above cells were transfected with the pNiFty-Luc NF-κB firefly luciferase reporter construct and the pRL-SV40 *Renilla* luciferase reporter vector as a control for transfection efficiency. The transfected cells were then stimulated with StcD (0.5 μg/mL) or LPS (10 ng/mL) for 6 h and luciferase activity was measured as described in the Materials and Methods. As shown in Fig. 7D, the HEK293/mTLR4/MD-2/CD4 cells responded to StcD or LPS with a significant increase in luciferase activity compared with HEK293 cells transfected with the empty vector (HEK293/vector). In contrast, luciferase activity after LPS treatment in HEK293/mTLR4 cells was approximately one-third of that in HEK293/mTLR4/MD-2/CD4 cells, and luciferase activity after StcD treatment was comparable to that of untreated cells. Together with Fig. 7A, these results confirmed that StcD stimulates luciferase activity through TLR4, and that MD-2 and CD14 are essential components in TLR4-mediated stimulation of luciferase activity by StcD.

## Discussion

*Salmonella* fimbriae can be produced via three types of biogenesis: the classical chaperone-usher system, precipitation-nucleation, and type IV fimbriae. Most *Salmonella* fimbriae belong to the chaperone-usher group of fimbriae.[24] These fimbriae have structural subunits that are exported and assembled in an ordered manner on the bacterial surface by cognate periplasmic chaperone protein and an outer membrane usher protein. *Stc* fimbriae are encoded by the *stcABCD* genes. The *stcA* gene encodes putative subunit protein and the *stcD* encodes adhesive-like protein. However, little is known about the structure and function of *stc* fimbriae. This study showed that in addition to type 1 fimbriae, *stc* fimbriae affect the expression of COX-2 in *Salmonella*-infected macrophages.

There are many reports that *Porphyromonas gingivalis* fimbriae are a potent stimulator of immune and pro-inflammatory responses.[25—27] Detection of *P. gingivalis* FimA, a major fimbrial protein, by TLR2 results in activation of macrophages or epithelial cells.[28,29] Tükel et al. reported that *Salmonella* CsgA, a major subunit of thin curled fimbriae encoded in the *csg* operon, is recognized by TLR2 and involved in IL-8 production in macrophages.[30] However,

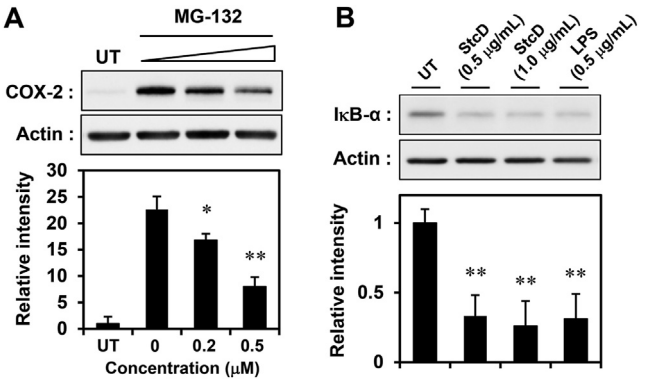


**Figure 4.** Involvement of the NF-κB signaling pathway in StcD-induced COX-2 expression in macrophages. (A) Effect of the NF-κB inhibitor MG-132 on StcD-induced expression of COX-2. Macrophages were treated with StcD (2.5 μg/mL) in the presence of the indicated inhibitor concentrations or 0.1% dimethyl sulfoxide alone (solvent control). After 2.5 h treatment, cytosolic extracts were prepared, and analyzed by western blotting for COX-2. COX-2 quantities were normalized to those of actin. *, $p < 0.01$; **, $p < 0.001$, significantly different from macrophages untreated with inhibitor. (B) Degradation of IκB-α in macrophages treated with StcD or LPS. Macrophages were treated with StcD (0.5 or 1 μg/mL) or LPS (0.5 μg/mL) as a positive control in the presence of cycloheximide (0.1 μg/mL). After 2.5 h treatment, cytosolic extracts were prepared, and IκB-α degradation was analyzed by western blotting for IκB-α. IκB-α quantities were normalized to those of actin. Graphs show values as relative intensity compared with untreated macrophages. **, $p < 0.001$, significantly different from untreated macrophages. UT, untreated with StcD or LPS.

this study showed that the *csgA* gene does not affect the expression of COX-2 in *Salmonella*-infected macrophages (Fig. 1). In our previous study, *Salmonella* FimH induced the expression of pro-inflammatory cytokines via TLR4.[16] As StcD may exhibit adhesive function similar to FimH due to its homology, we examined whether this protein participates in COX-2 expression in macrophages. This study showed that *Salmonella* StcD is a PAMP that is recognized by TLR4 and plays a significant role in the expression of COX-2 in macrophages. TLR4 plays an important role as an LPS receptor.[19,31] Effective recognition of LPS by TLR4 requires the assembly of a signaling complex comprised of LPS-binding protein CD14 and activating protein MD-2. These accessory components act in concert to bring LPS to TLR4, thereby inducing the subsequent signaling response.[32–34] Similarly, *Salmonella* StcD required MD-2 and CD14 for expression of its TLR4-mediated activity.

In subsequent experiments, we focused on the signal transduction pathways governing StcD-induced expression of COX-2 in macrophages. COX-2 is normally expressed at very low levels in most cell types and is strongly induced by various stimuli, including mitogens, cytokines, hormones, and oncogenes.[9,35] The cellular processes that lead to the expression of COX-2 are mainly regulated by the MAPK and NF-κB signaling pathways.[36–38] In this study, we showed that, in addition to the activation of the NF-κB pathway, StcD induces the phosphorylation of ERK, JNK, and p38 MAPK in macrophages. Furthermore, our results showed that the inhibition of NF-κB and p38 MAPK blocked StcD-induced COX-2 expression, indicating the involvement of both NF-κB and p38 MAPK signaling pathways in this process.

We previously reported that *Salmonella* causes the induction of COX-2 expression in a SPI-2-dependent manner,

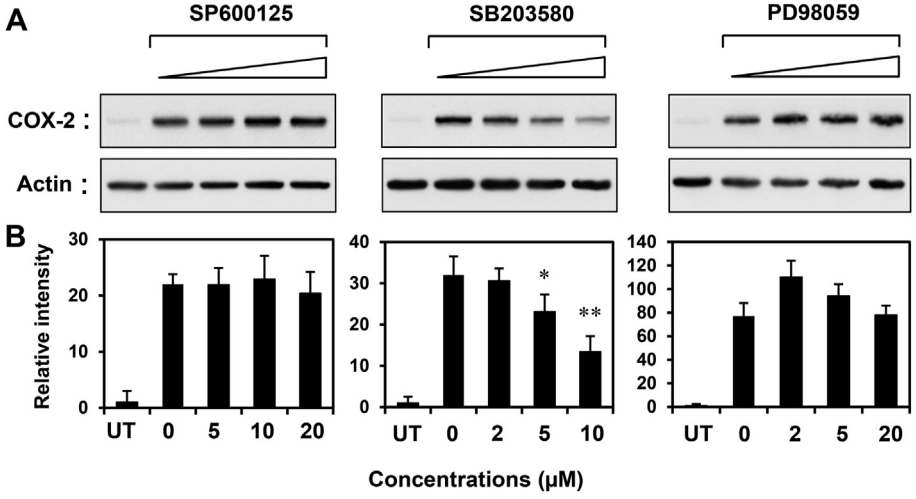


**Figure 5.** Effects of inhibitors of JNK (SP600125), p38 (SB203580), and ERK (PD98059) on StcD-induced COX-2 expression in macrophages. Macrophages were treated with StcD (2.5 μg/mL) in the presence of the indicated concentrations of inhibitors or 0.1% dimethyl sulfoxide (solvent control). After 2.5 h treatment, cytosolic extracts were prepared, and analyzed by western blotting for COX-2. (A) Images of the original blots. (B) COX-2 quantities were normalized to those of actin. Graphs show values as relative intensity compared with uninfected macrophages. *, $p < 0.01$; **, $p < 0.001$, significantly different from untreated macrophages. UT, untreated.

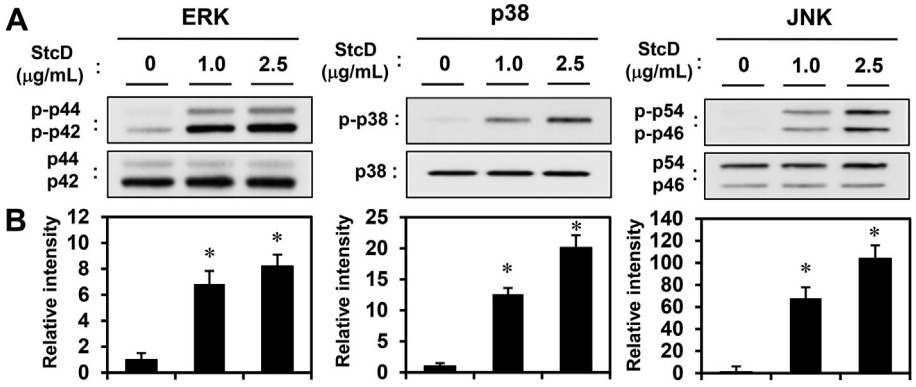


**Figure 6.** Western blot analysis of phospho-ERK, phospho-p38, and phospho-JNK in macrophages treated with StcD. Cytosolic extracts were prepared 2.5 h after treatment of cells with StcD (1 or 2.5 μg/mL) and were analyzed by western blotting with the indicated antibodies. (A) Image of the original blots. After analysis with anti-phospho-ERK, phospho-p38, and phospho-JNK (top), the membranes were stripped and re-probed with antibodies to ERK, p38, or JNK (bottom). (B) Densitometric analysis of the amounts of phospho-ERK, phospho-p38, and phospho-JNK normalized to the amounts of ERK (p44), p38, and JNK (p54) in the same samples. Graphs show values as relative intensity compared with untreated macrophages. *, $p < 0.001$, significantly different from untreated macrophages.

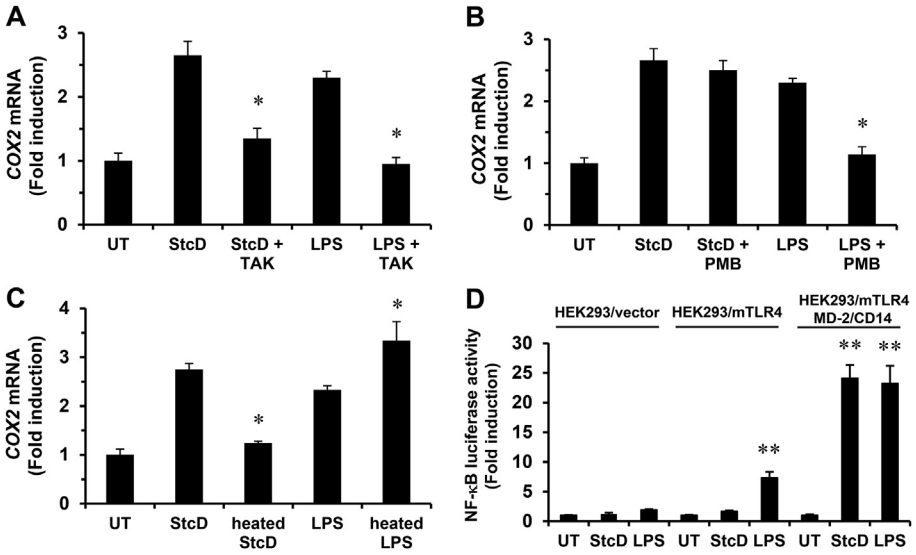


**Figure 7.** TLR4 is necessary for StcD-induced *COX2* expression. (A) Inhibition of StcD-induced *COX2* expression in macrophages by TAK-242. Macrophages were pretreated for 1 h at 37 °C with TAK-242 (40 μM) and then stimulated with StcD (1 μg/mL) or LPS (1 μg/mL) for 2.5 h *, $p < 0.01$, significantly different from untreated macrophages. (B) Effect of polymyxin B (PMB) on StcD-induced *COX2* expression. Macrophages were pretreated for 1 h at 37 °C with or without PMB (100 μg/mL) and then stimulated for 2.5 h with StcD (1 μg/mL) or LPS (1 μg/mL). *, $p < 0.01$, significantly different from untreated macrophages. (C) Macrophages were stimulated for 2.5 h with StcD (1 μg/mL) or LPS (1 μg/mL) that had been pre-treated at 100 °C for 30 min *, $p < 0.01$, significantly different from macrophages treated with unheated StcD or LPS. (D) StcD stimulates HEK293 cells expressing TLR4 in the presence of MD-2 and CD14. HEK293 cells, HEK293 cells expressing mouse TLR4 (HEK293/mTLR4), and HEK293 cells expressing mouse TLR4 and MD-2/CD14 (HEK293/mTLR4/MD-2/CD14) were transiently transfected with pNiFty-Luc and pRL-SV40 and then stimulated with StcD (0.5 μg/mL) or LPS (10 ng/mL). Six hours later, cell lysates were harvested and luciferase activity was measured. Firefly luciferase signals associated with NF-κB were normalized to internal *Renilla* luciferase levels as a transfection control. **, $p < 0.001$, significantly different from untreated cells. UT, untreated.

resulting in the up-regulation of $PGE_2$ production in macrophages.[22] $PGE_2$ has been shown to have anti-inflammatory effects on macrophages through activation of the protein kinase A (PKA) signaling pathway. In fact, it has been demonstrated that $PGE_2$ suppresses macrophage production of pro-inflammatory cytokines[39] and nitric oxide radicals and enhances the synthesis of anti-inflammatory cytokines.[40] These observations lead to the conclusion that $PGE_2$ may participate in the inhibition of the host defense by deactivating macrophage responses against many types of infection. Thus, *Salmonella* appears to utilize the COX-2 pathway to survive within macrophages. Therefore,

the expression of StcD-induced COX-2 via TLR4 might also play a significant role in the pathogenicity of *Salmonella*.

In conclusion, we showed that among 12 putative fimbriae, type 1 fimbriae and *stc* fimbriae are involved in the expression of COX-2 in macrophages infected with *S. enterica* serovar Typhimurium. Furthermore, adhesive like protein StcD encoded in the *stc* operon is a PAMP that is recognized by TLR4 in the presence of MD-2 and CD14 and plays a significant role in the induction of COX-2 expression in macrophages. Thus, the effect of StcD function on the pathogenicity of *Salmonella* requires further investigation in the future.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2021.11.001.