Cystic Fibrosis Transmembrane Conductance Regulator Reduces Microtubule-Dependent *Campylobacter jejuni* Invasion

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Running title: Role of CFTR in *Campylobacter jejuni* infection

Abstract

Campylobacter jejuni (*C. jejuni*) is gastroenteritis inducible food-born pathogen. Invasion and adhesion process are essential for leading gastroenteritis in *C. jejuni* infection process. As against bacterial strategy for efficacy invasion and adhesion, mucosal layer play a key role in defense systems, which modulated by several ion channels and transporters mediated water flux on the intestine. Cystic fibrosis transmembrane conductance regulator (CFTR) play the main role in waterfulux in intestine, and it closely related with bacterial clearance. We previously reported that *C. jejuni* infection suppresses CFTR channel activity in intestinal epithelial cells, however the mechanism and importance of this suppression is unclear. This study seeks to elucidate the role of CFTR in *C. jejuni*-infection. Using HEK293 cells that stably express wild type and mutated CFTR, we found that CFTR attenuated *C. jejuni* invasion, it was not involved bacterial adhesion or intracellular survival but associated with microtubule-dependent cellular transport. Moreover we revealed that CFTR attenuated function of microtubule motor protein but not microtubule stability, which causes inhibition of *C. jejuni*-invasion. Meanwhile, the CFTR mutant

G551D-CFTR, which has defects in channel activity, suppressed *C. jejuni*-invasion, whereas ΔF508-CFTR, which has defects in maturation, did not suppress, suggesting that CFTR suppression of *C. jejuni*-invasion is related to CFTR maturation but not channel activity.

Taken together, mature CFTR inhibited *C. jejuni* invasion by regulating microtubule-mediated pathways. We suggest that CFTR plays a critical role in cellular defenses against *C. jejuni*-invasion, and CFTR suppression may be an initial step in promoting cellular invasion during *C. jejuni*-infection.

Introduction

Campylobacter jejuni (*C. jejuni*) is a spiral-shaped Gram-negative bacterium that is commonly found in the gut microflora of birds or domestic animals used for food. *C. jejuni* is the most common cause of bacterial food-borne illness worldwide and causes gastrointestinal symptoms such as diarrhea, fever, abdominal cramping, and gastroenteritis. Despite its frequency, the virulence factors that contribute to *C. jejuni*-induced gastroenteritis remain largely unknown. Genomic studies revealed that *C. jejuni* strains lack type III secretion systems that are essential for the virulence of many other Gram-negative enteric pathogens (1). Thus, *C. jejuni* pathogenesis likely involves multifactorial virulence processes, including motility, adherence, invasion, and intracellular survival (2,3). Indeed, a study that examined mutant *C. jejuni* strains that were defective in adhesion or invasion found that cultured cells infected with these strains had decreased secretion of the pro-inflammatory cytokine IL-8 (4,5), which suggests that adhesion and invasion are the main pathogenic processes in *C. jejuni* infection (6,7,8,9).

In the human gut, the mucosal layer represents the first defense against bacterial adhesion and invasion (10). The binding of intestinal bacteria to host epithelial cells is assumed to play a fundamental role in intestinal bacterial colonization and disease progression (11). A previous study reported that mutant mice with defective mucosal function had a high colonization rate in *C. jejuni*-inoculation models (12). The mucosal layer consists of mucins, which are high molecular mass oligomeric glycoproteins. This layer is also critical for maintaining gut homeostasis by regulating water flux through the activity of several ion transporters and ion channels in the intestine (13). Among these ion channels, the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated chloride channel, is one of the most important factors that governs water movement. CFTR is expressed in several tissues such as those in the lung, pancreas, liver, intestine, sweat ducts, and reproductive system (14,15). In the intestinal tract, CFTR is associated with intestinal tract hydration and clearance of intestinal contents, including bacteria (16).

CFTR activation can disrupt the water balance in the gut to lead to diarrhea that

79 is associated with intestinal infections caused by pathogens such as *Escherichia*
80 *coli* (17) and *Vibrio cholera* (18) as well as other infectious bacteria (19).
81 However, we previously reported that *C. jejuni* infection suppressed
82 CFTR-mediated Cl^- secretion in intestinal cells (20), which is in opposition to
83 clinical *C. jejuni* symptoms such as diarrhea. Thus, the relationship between
84 CFTR suppression and *C. jejuni* infection is unclear.
85 In order to investigate the relationship between CFTR suppression and *C. jejuni*
86 infection, we referred to respiratory infections that occur in the presence of
87 CFTR mutations that are also associated with cystic fibrosis (CF) (21). CF
88 patients frequently experience respiratory bacterial infection caused by
89 *Pseudomonas aeruginosa* (*P. aeruginosa*) or *Burkholderia cenocepacia* (22).
90 CFTR dysfunction disrupts CFTR-mediated water flux that in turn affects
91 mucosal layer function and attenuates bacterial clearance such that bacteria
92 accumulate in the respiratory tract. Epithelial cells in CF patients have increased
93 cell surface expression of Toll-like receptors (TLR5 and TLR2), contribution of
94 TLR2 has been questioned, and enhanced inflammatory responses (23).

Moreover, *P. aeruginosa* is reported to suppress CFTR channel activity by secreting proteins that promote severe inflammation (24,25). These reports indicated that CFTR plays a defensive role against respiratory infection, and dysfunctional CFTR may promote conditions that increase the likelihood of bacterial infection.

In this study, we studied the role of CFTR in *C. jejuni* infection. Our data showed that CFTR expression decreased *C. jejuni* invasion. In particular, CFTR expression inhibited microtubule-mediated transport processes during *C. jejuni* invasion, suggesting that CFTR might be a protective factor against *C. jejuni* infection.

106 **Material and Methods**

107 ***Bacterial strains and culture conditions.*** The *Campylobacter jejuni* strains
108 NCTC11168 (ATCC 700819) and 81-176 (ATCC BAA2151) were purchased
109 from American Type Culture Collection (ATCC). Bacteria were grown in Muller
110 Hinton (MH) broth (DIFCO 275730) at 37 ℃ under microaerobic conditions (5%
111 O_2, 10% CO_2 and 85% N_2) for 36 hours. Bacteria were then collected by
112 centrifugation of the media at 12,000 rpm for 3 minutes, concentrated, and
113 grown on selective supplemental media containing Campylobacter charcoal
114 differential agar (CCDA:OXOID) for 36 hours. Single colonies were picked and
115 grown in MH broth for 48 hours, after which the media was centrifuged at 12,000
116 rpm 3 minutes, diluted into 15% glycerol (Wako) and stored at -80 ℃.

117 For the experiments, samples from frozen bacterial strains were grown in MH
118 broth for 48 hours under microaerobic conditions. After centrifugation at 12,000
119 rpm for 3 min, the supernatant was removed and the pellet was diluted into fresh
120 MH broth and cultured for 36 hours. Bacteria were collected by centrifuging the
121 media at 3,000 rpm for 15 minutes, and the supernatant was removed. The

122 bacterial cells were washed with phosphate buffered saline (PBS(-): 137 mM
123 NaCl, 8.1 mM anhydrous Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4),
124 centrifuged and resuspended in PBS(-). Finally, bacterial cell numbers were
125 adjusted to an optical density of 600 nm for OD600=1.0.

126 ***Reagents and antibodies.*** Forskolin and VX-809 were purchased from
127 Calbiochem and AdooQ Bioscience, respectively. Methyl-β-cyclodextrin (mβ-cd),
128 chlorpromazine hydrochloride, paclitaxel and nocodazole were purchased from
129 Sigma-Aldrich. The following antibodies were diluted in 3% skim milk and used
130 for western blotting: CFTR (1:1,000, Millipore), α-tubulin (1:2,000, Wako), and
131 β-actin (1:2,000, Santa Cruz).

132 ***Cell culture.*** Human embryonic kidney cells (HEK293) were cultured for 3-4
133 days in Dulbecco’s modified eagle’s medium (DMEM) high glucose (Sigma)
134 supplemented with 10% FBS (Thermo-Fisher), and 50 μg/ml gentamicin (Sigma)
135 at 37℃ and 5% CO_2. The human intestinal epithelial cell line T-84 was cultured
136 for 7 days in Ham’s F-12 media (DMEM/F-12, 1:1, Sigma) supplemented with
137 10% FBS (Thermo), and 50 μg/ml gentamicin (Sigma) at 37 ℃ and 5% CO_2.

The culture medium was changed every 2 days.

Stable transfection of HEK293 cells. HEK293 cells were seeded at a density of 5×10^5 cells/35 mm dish and incubated for 24 hours. Media was changed to DMEM high glucose without FBS and 1 ml Gentamicin High Glucose free. HG free (200 µl) was mixed with 1 µg/well pcDNA-wt-CFTR vector and 4 µg/well Lipofectamine 2000 (Invitrogen) for 15 minutes before incubating at 37℃ for 3 hours. The cells were then incubated overnight at 37℃ in DMEM high glucose containing 20% FBS and 50 µg/ml gentamicin (1.2 ml total). The supernatant was removed, and the cells were washed with 1 ml 0.02% EDTA/ PBS (-) and then 1 ml 0.02%EDTA/PBS (-) was added and the cells were incubated at 37℃ for 5 minutes. The cells were collected by centrifugation at 800 rpm for 3 minutes, and incubated with DMEM high glucose supplemented with 1 mg/ml G418, 10% FBS, and gentamicin for 10 days to produce cells that stably express CFTR. Colonies were picked with paper filter, incubated in a 24-well plate for 2 days before the filter was removed and the cells were grown to confluence. CFTR protein expression was assessed by western blotting and cells with high

154 expression levels were selected. Cells stably expressing mutated CFTR were
155 produced using the same method.
156 ***Western blotting.*** Cultured cells were washed with PBS (-), diluted into RIPA
157 buffer (pH 7.4, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% sodium
158 deoxycholate, 0.1% SDS, 1% Triton-X) + 10% protease inhibitor mixture
159 (Nacalai Tesque), centrifuged at 15,000 rpm for 10 min at 4℃, and the
160 supernatant was collected. Protein levels were determined using a BCA Protein
161 assay kit (Thermo). Samples were added to 5× sample buffer (pH 6.8, 250 mM
162 Tris-HCl, 5% SDS, 25% 2-mercaptoethanol, 50% glycerol, BPB) and separated
163 on a SDS-polyacrylamide gel (7.5% or 10%). Proteins were transferred to
164 Immobilon-P Transfer Membranes (Millipore) that were blocked with Tris
165 Buffered Saline with Tween-20 (TBS-T: pH 7.6, 20 mM Tris, 150 mM NaCl,
166 0.02% Polyoxyethylene (20) sorbitan monolaurate) containing 3% skim milk for 1
167 hour at RT and incubated overnight at 4 ℃ with primary antibodies in TBS-T
168 containing 3% skim milk. After washing with TBS-T for 30 minutes, membranes
169 were exposed to horseradish peroxidase-conjugated secondary antibodies

(1:2,000, anti mouse Ig HRP, Biosource) for 2 hours at RT. Membranes were then washed with TBS-T for 30 minutes and detected with ECL (GE Healthcare). Hyperfilm ECL (GE Healthcare) and imaging with a Fuji medical film processor FPM 100 was used to detect CFTR expression. β-actin protein was detected used medical X-ray film or LASS-2000.

Efflux assay. Cells were seeded at a density of 5×10^5 cells/well in a 6-well plate and incubated for 4 days. The supernatants were removed, exchanged for HEPES buffer (10 mM HEPES, 145 mM NaCl, 10 mM glucose, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) containing $^{125}I^-$ (2 μCi/ml) and incubated at 37 ℃ for 1 hour. The media was removed and the cells were washed twice with isotope-free HEPES buffer before exchanging either for HEPES buffer or HEPES buffer containing 10 μM forskolin. After 5 minutes, supernatants were recovered for sample detection. Cells were incubated 0.1 M NaOH at 37 ℃ for 1 hour and samples were collected to detect intracellular $^{125}I^-$ using a gamma-counter. The total amount of $^{125}I^-$ in the supernatant and cells was calculated as: % Efflux = ($^{125}I^-$ secretion amount /($^{125}I^-$ cellular amount + $^{125}I^-$ secretion amount))×100

Transient transfection with shRNA. HEK-wt-CFTR or HEK-G551D-CFTR cells were seeded at a density of 1×10^6 cells/well in 6-well plates. Cells were then washed and the media was replaced with HG free before transfection with 1 μg pLKO-CFTR vector or pLKO.1-puro Non-Target shRNA Control vector using Lipofectamine 2000 reagent (Invitrogen) for 3 hours. DMEM-HG(+) containing 20 % FBS and 50 μg/ml gentamicin was then added to the cells.

T84 cells were cultured for 7 days and the cells were harvested with 0.05% trypsin-EDTA and diluted in HEPES buffer. pLKO-CFTR vector or pLKO.1-puro Non-Target shRNA Control vector (2.5-5ug) were mixed with T-84 cells suspension cultures (4×10^5 / 24well) for 20 minutes. Cells were electroporated with 50 voltage-20 ms for 3 times. After electroporation, cells were suspended culture medium for 48 hours.

Invasion and adhesion and degradation assay. For an invasion assay, HEK293 cells and HEK-wt-CFTR cells were seeded at a density of 4×10^5 cells/well in 6-well plates and incubated at 37 ℃ for 4 days. The supernatants were removed and replaced with HG-free media before the cells were infected

202 with *C. jejuni* at a multiplicity of infection (MOI) of 50 for 6 hours at 37 ℃ in 5%
203 CO_2. After infection, the supernatant was replaced with DMEM-HG(+) containing
204 100 μg/ml gentamicin for 2 hours. The supernatant was removed and the cells
205 were washed with PBS (-) and lysed with PBS containing 1% Triton-X. The
206 lysates were plated on MH agar plates and incubated for 48 hours under
207 microaerobic conditions. HEK-ΔF508-CFTR cells were treated with VX-809 or
208 DMSO at 27 ℃ for 24 hours before infection.
209 For adhesion assays, HEK293 cells and HEK-wt-CFTR cells were seeded at a
210 density of 1×10^6 cells/well in 60 mm well plates. After infection, the supernatants
211 were removed and the cells were washed with PBS (-) three times before lysing
212 at 37℃ for 5 minutes in PBS (-) containing 0.01% Triton-X. Cell lysates were
213 plated on MH agar plates and incubated for 48 hours under microaerobic
214 conditions.
215 In degradation assays, HEK293 cells and HEK-wt-CFTR cells were seeded at a
216 density of 4×10^5 cells/well in 6-well plates. After infection, the supernatants were
217 removed and replaced with DMEM-HG(+) containing 100 μg/ml gentamicin for 2,

4, 6, or 8 hours. The cells were then lysed with PBS (-) containing 1% Triton-X, plated on MH agar plates, and incubated for 48 hours under microaerobic conditions. All intracellular bacterial number normalized with 1×10^7 cell.

Separation of soluble and insoluble tubulin. HEK293 cells and HEK-wt-CFTR cells were seeded at a densitiy of 4×10^5 cells/well in 6-well plates. The supernatant was removed and the cells were washed with PBS (-) before adding microtubule buffer (0.1 M PIPES pH 7.6, 2 M glycerol, 5 mM $MgCl_2$, 2 mM EGTA) containing 0.1% Triton-X, 1 mM PMSF, 5 μg/ml aprotinin and leupeptin and incubating for 10 min RT. Cells were collected and centrifuged for 10 min at 300 g at room temperature. The resulting supernatant represented the soluble fraction. Meanwhile, pellets containing the insoluble fraction were washed again with microtubule buffer and treated with lysis buffer (25 mM Na_2HPO_4 pH 7.2, 400 mM NaCl, 0.5% SDS), and centrifuged for 10 min at 20,000 g. Protein concentrations were measured with a BCA protein assay kit and equal amounts of protein were separated by SDS-PAGE. Anti-α-tubulin and anti-β-actin antibodies were used for western blotting.

RNA isolation and RT-PCR

Total RNA was extracted from T-84 cells using RNeasy Mini Kit (QIAGEN 74104) according to the manufacture's instruction. Reverse transcription were preformed with 1 μg of total RNA using the PrimeScript RT-Reagent Kit (TaKaRa RR037A). Quantitative real-time reverse-transcription PCR was performed in the LightCycler Real Time PCR System (Roche Applied Science) with SYBER Premix ExTaq (TaKaRa RR820). 18S ribosomal RNA housekeeping gene (as internal control) and CFTR primers are as follow: forward: 5'-AAACGGCTACCACATCCAAG-3' and reverse: 5'-GGCCTCGAAAGAGTCCTGTA-3', forward: 5'GCAGTTGATGTGCTTGGCTAG-3' and reverse: GAATCGTACTGCCGCACTTTG-3', respectively. Fold change was calculated relatively to 18S.

Statistical Analysis

All data were performed statistical analysis by using Student's t-test for paired data. Data were done 3 independent experiments. All tests were one-tailed; *$p<0.05$; ** $p<0.01$, NS.: not significant.

Results

Exogenous CFTR expressed cells were useful for the analyzing of CFTR effect on *C. jejuni* infection

In our previous study, CFTR channel activity was suppressed by *C. jejuni* infection (Figure 1A) in T-84 cells, endogenous CFTR expressing cells. To examine whether *C. jejuni* infection affect exogenous CFTR, we established CFTR-expressing HEK293 (HEK-wt-CFTR cells). CFTR expression and channel activity were confirmed by western blot (supplementary Figure 1A) and efflux assay (supplementary Figure 1B), respectively. Interestingly, in contrast to the T-84 cells, CFTR ion channel activity was not suppressed by *C. jejuni* infection in HEK-wt-CFTR cells (Figure 1B). Together, these results suggest that *C. jejuni* infection did not directly affect CFTR function. Thus, we used HEK-wt-CFTR cells, in this study, to assess the CFTR contribution on the *C. jejuni*-infection.

Numbers of intracellular *C. jejuni* bacteria were decreased by CFTR expression

Adhesion and invasion processes are thought to be essential for *C. jejuni*

virulence and infection (26) and decreased amounts of CFTR expression induced by *C. jejuni* may provide increased opportunities for bacterial adhesion or invasion. To test whether CFTR expression levels were related to *C. jejuni* invasion, we investigated the numbers of intracellular bacteria in CFTR-expressing cells infected with *C. jejuni*. Relative to HEK293 cells, HEK-wt-CFTR cells infected by *C. jejuni* for 6 hours showed reduced amounts of *C. jejuni* invasion (Figure 1C). Next, HEK-wt-CFTR cells were transfected with a CFTR knockdown vector expressing shRNA specific for CFTR, pLKO-CFTR (shCFTR). Cells transfected with pLKO.1-puro Non-Target shRNA control vector (shControl) were used as control and knockdown efficiency was confirmed by western blotting (Figure 1D). CFTR knockdown increased intracellular bacterial cellular numbers were assessed with a gentamicin protection assay (Figure 1E). The results indicated that *C. jejuni* invasion was prevented by CFTR expression. Another *C. jejuni* strain, 81-176, which was isolated from a patient during an outbreak of *Campylobacter* enteritis (27) gave the same results as the NCTC 11168 strain (Supplementary Figure 2A and B). Therefore CFTR attenuated

invasion by another *C. jejuni* strain infection.

CFTR attenuated microtubule-mediated *C. jejuni* invasion processes

Since the number of intracellular bacterial cells in host cells can be influenced at several steps, including adhesion, invasion, and survival, we next assessed the relationship between CFTR and *C. jejuni* in the context of these processes. In terms of *C. jejuni* cell numbers, adhesion and survival, there were no differences between control cells and CFTR-expressing cells (Figure 2A and C). In contrast, *C. jejuni* invasion was decreased by CFTR expression during the early stage of infection (Figure 2B). Thus, we focused on *C. jejuni* invasion pathways, which have two main parts: the endocytosis-like uptake pathway (26) and the microtubule-dependent cellular transport pathway (28). Endocytosis-like uptake pathways include those involving lipid rafts or clathrin-mediated processes (29). Lipid raft domains are rich in cholesterol that promotes bacterial adhesion and produces bacteria-containing vacuoles for cellular uptake (30). Meanwhile, the scaffold protein clathrin binds to adaptor proteins to generate

clathrin-coated vesicles and promote endocytosis (31). Mβ-cd can affect lipid raft components such as cholesterol to inhibit lipid raft-mediated endocytosis (32). Chlorpromazine prevents clathrin formation at the cell membrane to decrease clathrin-mediated endocytosis activity (33). Here, treatment of cells with either mβ-cd or chlorpromazine dramatically decreased the extent of *C. jejuni* invasion in control cells. However, CFTR-mediated suppression of *C. jejuni* invasion was sustained (Figure 3A and B). These data indicated that the effect of CFTR on *C. jejuni* invasion was not related to the activity of endocytosis pathways.

Microtubules are dynamic structures that undergo polymerization and depolymerization of tubulin to regulate both cell skeleton integrity and cellular transport (34). After entry into host cells, *C. jejuni* invasion proceeds along microtubules that act as intracellular tracks (28). Here, treatment with the microtubule polymerization inhibitors nocodazole or colchicine (35,36) suppressed *C. jejuni* invasion, but there was no difference in the number of intracellular bacterial in control cells or cells CFTR-expressing cells (Figure 3C). These data implied that CFTR negatively affected intracellular transport of *C.*

316 *jejuni* via the microtubule track, which in turn inhibited invasion.

317

318 **CFTR expression affect microtubule motor protein**

319 Microtubule polymerization promotes bacterial uptake and vacuole motility (37).
320 For *C. jejuni,* microtubules provide a means for invasion (28). To investigate the
321 participation of CFTR in microtubule-mediated *C. jejuni* invasion, we focused
322 microtubule tract and motor protein.

323 First, we investigated CFTR effect on microtubule transport tract. *C. jejuni*
324 infection did not change the amount of polymerized microtubules
325 (supplementary Figure 3A and B). The amount of polymerized microtubules was
326 estimated by western blot in CFTR-expressing cells (Figure 4A). But there were
327 no differences between HEK293 and HEK-wt-CFTR cells. And we confirmed
328 microtubules stability in CFTR-expressing cells by using the microtubule
329 depolymerization inhibitor, paclitaxel. Microtubule polymerization was increased
330 by paclitaxel treatment in CFTR-expressing cells (Figure 4B). Also, according th
331 the microtubule polymerization, the number of intracellular bacteria was

increased by paclitaxel treatment in HEK293 cells (Figure 4C). However, in HEK-wt-CFTR cells, the intracellular bacterial number did not increase significantly similar to HEK293 cells. These data indicated that CFTR did not affect microtubule tract stability in microtubule-mediated transport.

Next, we checked that CFTR effect on microtubule motor protein, dynein. In the previous study reported tht dynein inhibitor Na3O4 decreased *C. jejuni* invasion via microtubule-mediated transport (38). Thus we used dynein inhibitor, Na_3VO_4, on CFTR expressing cells, to stop the delivery in microtubule tract. According to the Na_3VO_4 content, intracellular bacteria were decreased, and the differences of bacterial number between HEK-wt-CFTR shControl and shCFTR cells were abolished by Na_3VO_4 treatment (Figure 4D). Another strains, *C. jejuni* 81-176, also result in similar to 11168 (supplementary Figure 4).

These results suggest that CFTR suppressed microtubule motor protein, which functions interfere with microtubule mediated *C. jejuni* invasion.

CFTR maturation is essential to inhibit *C. jejuni* invasion

348 Cellular surface localization and appropriate folding are necessary for CFTR
349 channel activity (14,15). To examine how CFTR inhibited *C. jejuni* invasion, we
350 established cell lines that stably expressed two different CFTR mutants. The
351 G551D-CFTR mutant has a substitution of aspartic acid for glycine at amino acid
352 position 551. This mutant has appropriate folding trafficking to the cell surface
353 membrane, but does not have channel activity (39). Meanwhile, the
354 ΔF508-CFTR mutant has a single deletion of a phenylalanine residue at position
355 508. This mutant does not have appropriate glycosylation, folding, or trafficking
356 to the cell surface membrane (40). The expression of the mutant CFTR proteins
357 was confirmed by western blot (supplementary Figure 5A). Band C, indicative of
358 the mature complex-glycosylated form of CFTR, was detected for both wt-CFTR
359 and G551D-CFTR. Bands A and B indicate the non-glycosylated form and
360 immature core-glycosylated form, respectively, of CFTR (41). Band A and B
361 were both detected for cells expressing ΔF508-CFTR. The ion channel activity
362 as measured by efflux assay showed that active ion channels were detected
363 only for wt-CFTR cells (supplementary Figure 5B). Cells expressing the G551D

364 CFTR mutant, HEK-G551D-CFTR, had inhibition of *C. jejuni* invasion that was
365 similar to that of wt-CFTR cells (Figure 5A). G551D-CFTR knockdown also had
366 increases in intracellular bacterial cell numbers (Figure 5B and C). These results
367 indicated that suppression of *C. jejuni* invasion by CFTR expression was not
368 associated with CFTR ion channel activity. In contrast, expression of
369 ΔF508-CFTR did not suppress *C. jejuni* invasion (Figure 5A). A previous study
370 showed that defects in folding and trafficking of ΔF508-CFTR could be rescued
371 by treatment of cells with pharmacological folding correctors such as VX-809 at
372 low temperature conditions (27 ℃) (42). Here we showed that treatment of cells
373 with VX-809 at low temperatures facilitated CFTR maturation and increased the
374 amount of band C, which indicates mature complex-glycosylated CFTR (Figure
375 5D). During CFTR maturation, *C. jejuni* invasion was suppressed by expressing
376 mature ΔF508-CFTR in the presence of VX-809 (Figure 5E). These results
377 suggest that CFTR expression, especially expression of mature and cell
378 surface-localized CFTR, was important for *C. jejuni* invasion.
379

Endogenous CFTR attenuated *C. jejuni* invasion

Next we confirmed CFTR-mediated suppression of *C. jejuni* invasion in endogenously CFTR expressed T-84 cells. The shControl and shCFTR vector, were transfected into T-84 cells by electroporation to assesse whether endogenous CFTR also inhibits *C. jejuni* invasion. We checked CFTR knockdown efficiency by western blotting (Figure 6A). Similar to the HEK-wt-CFTR experiment, intracellular bacterial number was increased by endogenous CFTR knockdown in 3 hours *C. jejuni* infection (Figure 6B). Finally, we checked endogenous CFTR express ion level in *C. jejuni* infected T-84 cells. In order to examine the mechanism how *C. jejuni* suppresses CFTR channel activity in T-84 cells (Figure 1A). And we revealed that *C. jejuni* attenuated CFTR gene expression and protein level in long term, 12 hours, infection (Figure 6C and D). Taken together, these results suggest that, in intestinal cells, *C. jejuni* suppressed CFTR to effectively into host cells.

Discussion

In this study, we investigated the role of CFTR in *C. jejuni* infection. We found that overexpression of CFTR specifically inhibited invasion of *C. jejuni* (Figures 2 and 3) and that this inhibition appeared to depend on microtubules for transport of *C. jejuni* vacuoles needed for bacterial invasion (Figures 4 and 5). *C. jejuni* invasion was not suppressed in cells expressing an immature mutant CFTR that is defective in glycosylation and trafficking, whereas cells expressing a CFTR mutant that lacked ion channel activity had *C. jejuni* invasion that was similar to that seen for HEK cells expressing wild type CFTR (Figure 6). Thus, we conclude that CFTR glycosylation and localization to the cell surface might affect bacterial interactions with cellular microtubules to suppress bacterial invasion.

The specific functions of intestinal epithelial cells, such as mucus production or cellular polarization, might affect invasion processes of *C. jejuni*. Because we focused on the relationship between CFTR and *C. jejuni* infection, we overexpressed CFTR in HEK293 cells, a fibroblast line.

CFTR at the cell surface has several functional domains that have specific

411 structures, including two membrane-spanning domains, two nucleotide-binding
412 domains, and a regulatory region (14,15). The carboxyl terminal domain of
413 CFTR includes a PDZ domain that interacts with the cellular cytoskeleton by
414 binding scaffolding proteins such as NHERF1, N-WASP, EZRIN, and F-actin.
415 Localization of CFTR on the cell surface helps regulate clathrin and
416 N-WASP-mediated endocytosis pathways as well as Rab11 and
417 Rme-1-mediated recycling pathways. The ΔF508-CFTR mutant is thought to
418 have impaired interactions with NHERF1 and attenuated stabilization at the cell
419 surface (43). We considered that expression of wild type CFTR would result in
420 stable cell surface CFTR and increased interactions with scaffolding proteins
421 that in turn inhibit *C. jejuni* invasion. However, future studies should include
422 quantification of the amount of cell surface CFTR, which was examined only at a
423 qualitative level in this study.

424 During *C. jejuni*-invasion, microtubule tracts were associated with
425 CFTR-dependent inhibition of *C. jejuni* invasion (Figures. 4 and 5). Motor
426 proteins, such as members of the dynein and kinesin families, slide along

427 microtubules and are important for microtubule-mediated cellular transport (44).
428 Dynein and kinesin transports cargo toward the perinuclear region and from the
429 perinuclear region to the cell surface, respectively. CFTR trafficking is
430 maintained by microtubule-dependent transport involving dynein. Thus, CFTR
431 expression could regulate microtubule motor protein function, which might affect
432 delivery of *C. jejuni* vacuoles along microtubule tracts from the cell surface.
433 Further studies will also be needed to characterize the relationship between
434 CFTR and motor protein function.
435 Recently, other intestinal infection bacteria, including enteropathogenic
436 *Escherichia coli* (EPEC) and *Salmonella* typhimurium (*S*. typhimurium), were
437 reported to suppress CFTR ion channel activity (45,46). Moreover, in the
438 respiratory tract *P. aeruginosa* was shown to invade host cells via a
439 microtubule-dependent pathway that is regulated by CFTR (47). These findings
440 suggest that regulation of microtubule function by CFTR might be essential for
441 protection against invasive bacterial infection in a variety of tissues.
442

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Figure Legends

Figure 1. Changing of intracellular *C. jejuni* numbers in the presence of CFTR expression Cl^- efflux rates were estimated by 12 hours C. jejuni infected cells in (A) T-84 cells and (B) HEK-wt-CFTR cells. After the stimulation, $125I^-$ was incorporated into cells for 6 minutes and the levels were assessed using a gamma counter. (C) HEK293 cells and HEK-wt-CFTR cells were infected by *C. jejuni* for 6 hours. The number of intracellular bacteria was estimated with a gentamicin protection assay. (D) HEK-wt-CFTR cells were transfected with pLKO-CFTR vector or pLKO.1-puro Non-Target shRNA control vector and the protein expression of CFTR was detected by western blot. (E) HEK-wt-CFTR shControl cells and HEK-wt-CFTR shCFTR cells were infected by *C. jejuni* for 6 hours and intracellular bacteria numbers were estimated with a gentamicin protection assay. All data are means ± SD of 3 independent experiment. Statistical significance: **, $p<0.01$. NS.,No Significance. n=3-6.

Figure 2. CFTR affects *C. jejuni* invasion during the early phase of infection time, but does not affect bacterial adhesion and cellular survival.

617 HEK-wt-CFTR cells were transfected with shControl or shCFTR vectors. The
618 cells were then infected by *C. jejuni* and (A) bacterial adhesion, (B) invasion
619 and (C) intracellular survival were assessed after 0, 1, 3 hours. All data are
620 means ±SD of 3 independent experiment. Statistical significance: **, p<0.01.
621 NS.,No Significance. n=3-6.
622
623 **Figure 3. CFTR affected microtubule tracks in *C. jejuni* invasion.** HEK293
624 cells and HEK-wt-CFTR cells were treated with (A) methyl-β-cyclodextrin; mβ-cd
625 (7.5 mM), (B) chlorpromazine (47 μM), (C) nocodazole (33 μM), or colchicine (10
626 μM) for 1 hour, and infected by *C. jejuni* for 3 hours. In all experiments, the
627 number of intracellular bacteria was estimated using a gentamicin protection
628 assay. All data are means ± SD of 3 independent experiment. Statistical
629 significance: **, p<0.01., *, p<0.05 NS. , No Significance. n=3-6.
630
631 **Figure 4. CFTR expression attenuated affect microtubule motor protein in**
632 ***C. jejuni* invasion.** (A) The polymerized tubulin content in HEK293 and

633 HEK-wt-CFTR cells were assessed by western blotting, treatment with paclitaxel
634 (5, 10 μM) or DMSO for 1 hour. (B) The changing of microtubule
635 polymerization were quantified by measurement of polymerized α-tubulin and
636 normalized with respect to β-actin that was used as a loading control. (C)
637 HEK293 and HEK-wt-CFTR cells were treated with paclitaxel (5 or 10 μM) or
638 DMSO for 1 hour prior to *C. jejuni* infection. And Intracellular bacterial numbers
639 were assessed by gentamicin protection assay. (D) HEK-wt-CFTR cells
640 shControl and shCFTR cells were treated by Na_3VO_4 (1 or 2 mM) for 1 hour prior
641 to *C. jejuni* infection. Intracellular bacterial numbers were assessed with
642 gentamicin protection assay. . All data are means ±SD of 3 independent
643 experiment. Statistical significance: **, p<0.01., *, p<0.05 NS. , No Significance.
644 n=3-6.
645
646 **Figure 5. Immature CFTR did not inhibit *C. jejuni* invasion.** (A) HEK293,
647 HEK-wt-CFTR, HEK-G551D-CFTR, or HEK-ΔF508-CFTR cells were infected
648 with *C. jejuni* for 0, 1, and 3 hours. Numbers of intracellular bacteria were

649 estimated using a gentamicin protection assay. (B) HEK-G551D cells were
650 transiently transfected with shControl or shCFTR, and CFTR levels were
651 detected by western blot. (C) HEK-G551D shControl and shCFTR cells were
652 infected with *C. jejuni* for 6 hours. Numbers of intracellular bacteria were
653 estimated with a gentamicin protection assay. (D) HEK-ΔF508-CFTR cells were
654 treated with VX-809 (40 μM) or DMSO at 27 °C for 24 hr. CFTR expression was
655 detected by western blot. Bands A, B, and C indicate non-glycosylated,
656 immature core-glycosylated, and mature complex-glycosylated CFTR,
657 respectively. (E) HEK-ΔF508 and HEK-wt-CFTR cells were treated with VX-809
658 or DMSO at 27 ℃ for 24 hours and infected by *C. jejuni* for 3 hours. The
659 number of intracellular bacteria was estimated using a gentamicin protection
660 assay. All data are means ±SD of 3 independent experiment. Statistical
661 significance: **, p<0.01., *, p<0.05., NS. , No Significance., n=3-6.
662
663 **Figure 6. Endogenous CFTR inhibited *C. jejuni* invasion in intestinel cells**
664 (A) CFTR knockdown level in, T-84 shControl and shCFTR cells, were detected

by western blotting. (B) The T-84, induced shControl and shCFTR plasmid, cells were infected by *C. jejuni* 3 hours infection and estimated intracellular bacterial number by gentamicin protection assay. During the 12 hours *C. jejuni*-infection, CFTR (C) mRNA and (B) protein expression level were estimated in T-84 cells. All data are means ±SD of 3 independent experiment. Statistical significance: **, $p<0.01$., *, $p<0.05$., NS. , No Significance. n=3-6.

Figure 1.

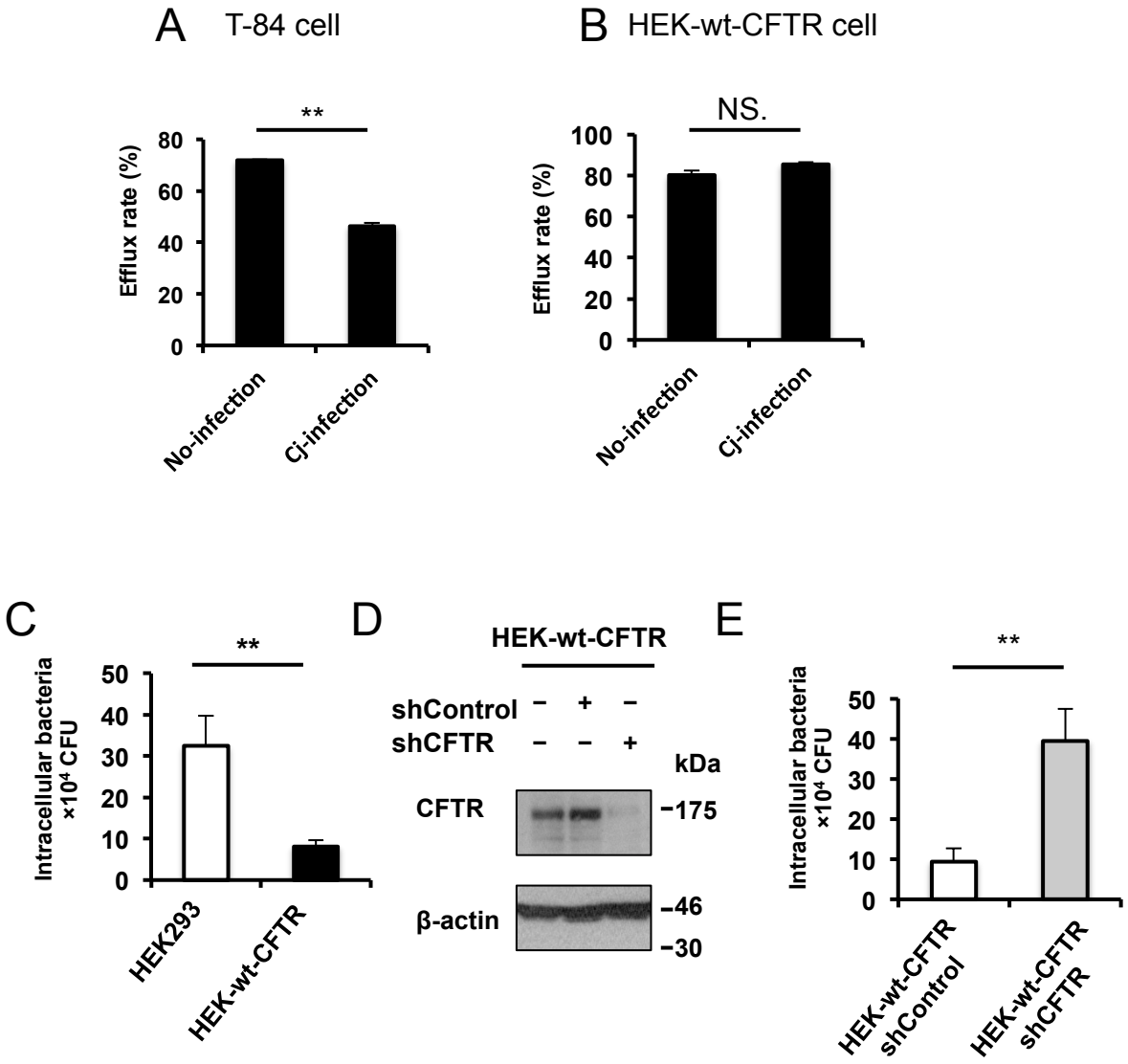

A
T-84 cell
**
Efflux rate (%)
80
60
40
20
0
No-infection
Cj-infection
B
HEK-wt-CFTR cell
NS.
Efflux rate (%)
100
80
60
40
20
0
No-infection
Cj-infection
C
**
Intracellular bacteria
×10⁴ CFU
50
40
30
20
10
0
HEK293
HEK-wt-CFTR
D
HEK-wt-CFTR
shControl − + −
shCFTR − − +
kDa
CFTR
175
β-actin
46
30
E
**
Intracellular bacteria
×10⁴ CFU
50
40
30
20
10
0
HEK-wt-CFTR shControl
HEK-wt-CFTR shCFTR

Figure 2.

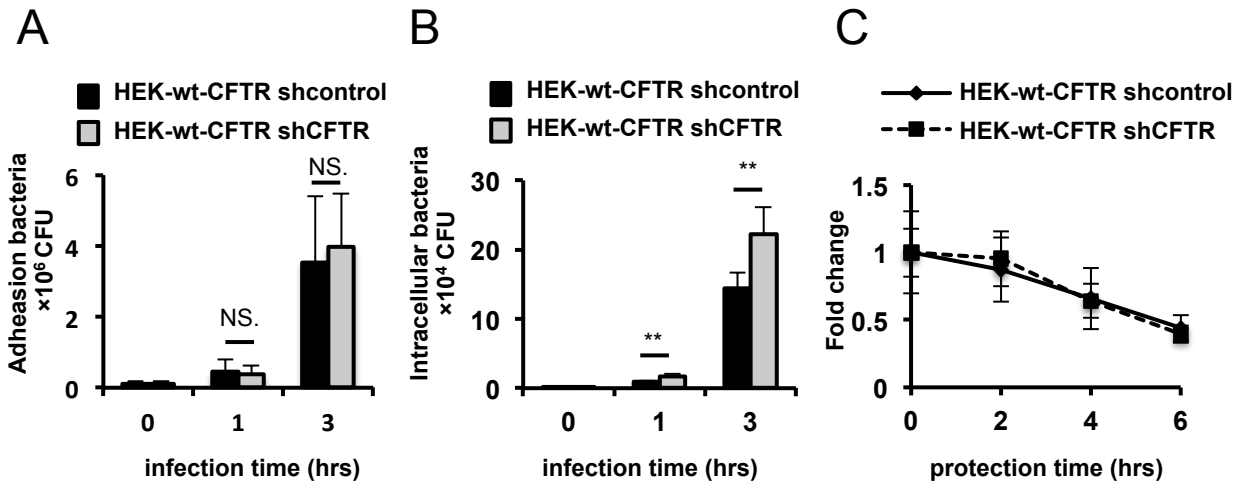

A
HEK-wt-CFTR shcontrol
HEK-wt-CFTR shCFTR
Adheasion bacteria
×10⁶ CFU
6
4
2
0
NS.
NS.
0
1
3
infection time (hrs)
B
HEK-wt-CFTR shcontrol
HEK-wt-CFTR shCFTR
Intracellular bacteria
×10⁴ CFU
30
20
10
0
**
**
0
1
3
infection time (hrs)
C
HEK-wt-CFTR shcontrol
HEK-wt-CFTR shCFTR
Fold change
1.5
1
0.5
0
0
2
4
6
protection time (hrs)

Figure 3.

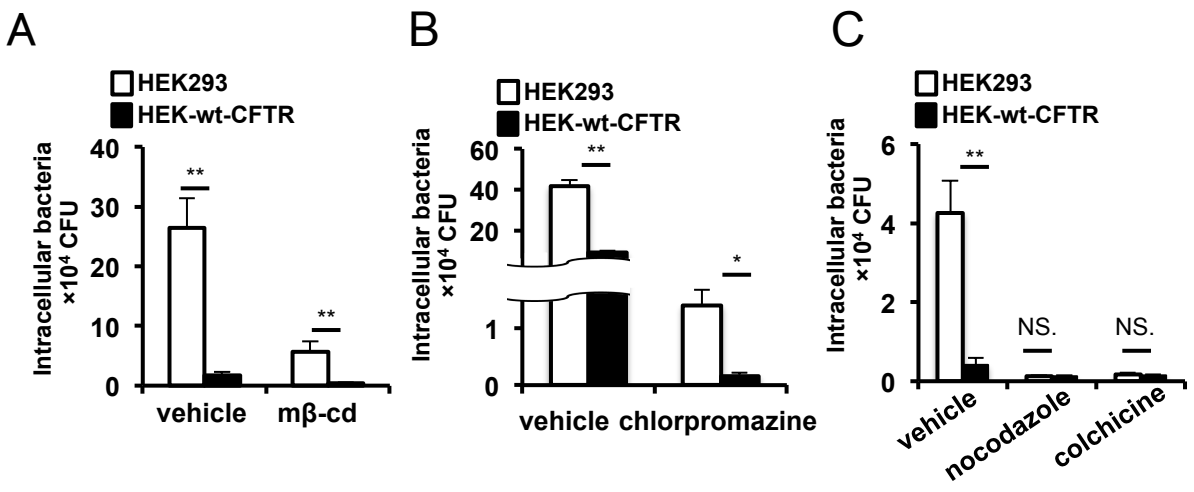

A
HEK293
HEK-wt-CFTR
Intracellular bacteria
×10⁴ CFU
40
30
20
10
0
**
**
vehicle
mβ-cd
B
HEK293
HEK-wt-CFTR
Intracellular bacteria
×10⁴ CFU
60
40
20
1
0
**
*
vehicle
chlorpromazine
C
HEK293
HEK-wt-CFTR
Intracellular bacteria
×10⁴ CFU
6
4
2
0
**
NS.
NS.
vehicle
nocodazole
colchicine

Figure 4.

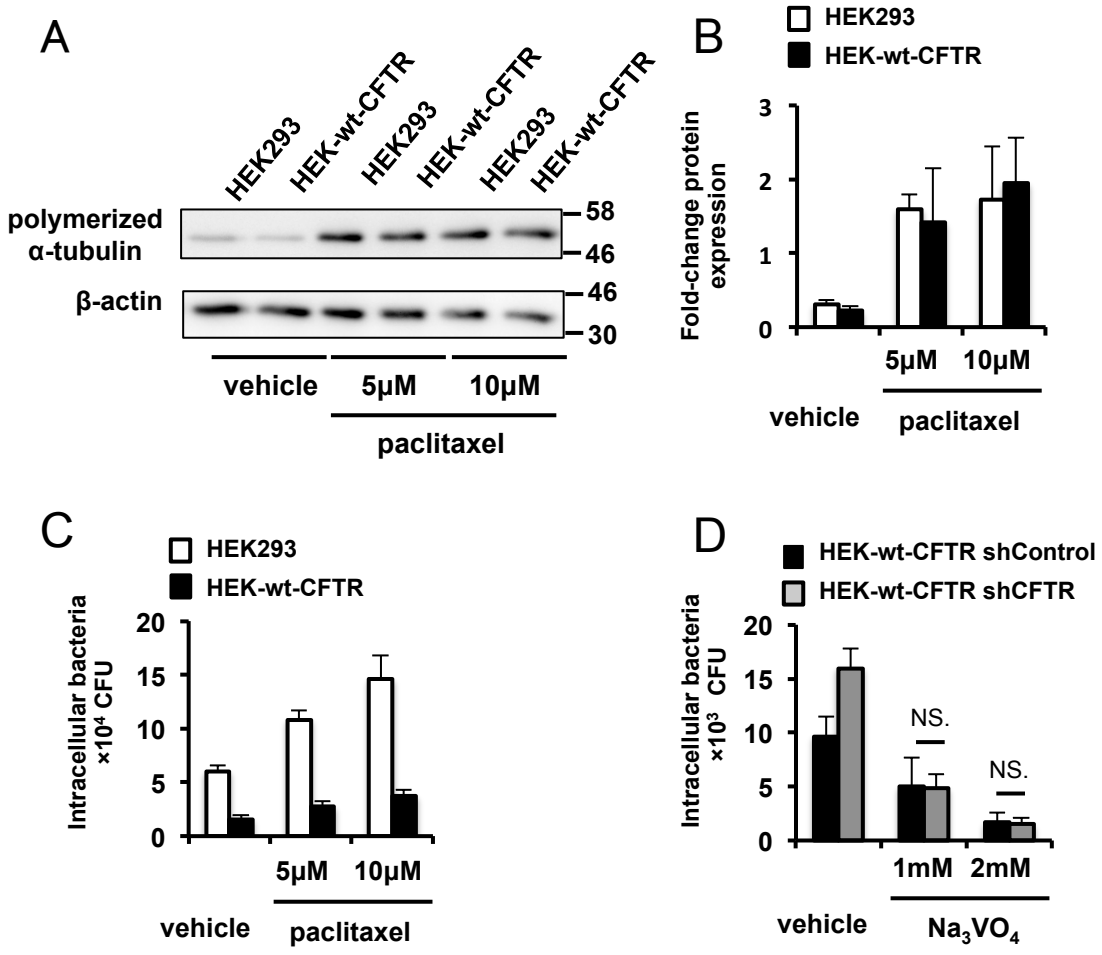

A
HEK293
HEK-wt-CFTR
HEK293
HEK-wt-CFTR
HEK293
HEK-wt-CFTR
polymerized α-tubulin
β-actin
58
46
46
30
vehicle
5μM
10μM
paclitaxel
B
HEK293
HEK-wt-CFTR
Fold-change protein expression
3
2
1
0
5μM
10μM
vehicle
paclitaxel
C
HEK293
HEK-wt-CFTR
Intracellular bacteria ×10⁴ CFU
20
15
10
5
0
5μM
10μM
vehicle
paclitaxel
D
HEK-wt-CFTR shControl
HEK-wt-CFTR shCFTR
Intracellular bacteria ×10³ CFU
20
15
10
5
0
NS.
NS.
1mM
2mM
vehicle
Na3VO4

Figure 5.

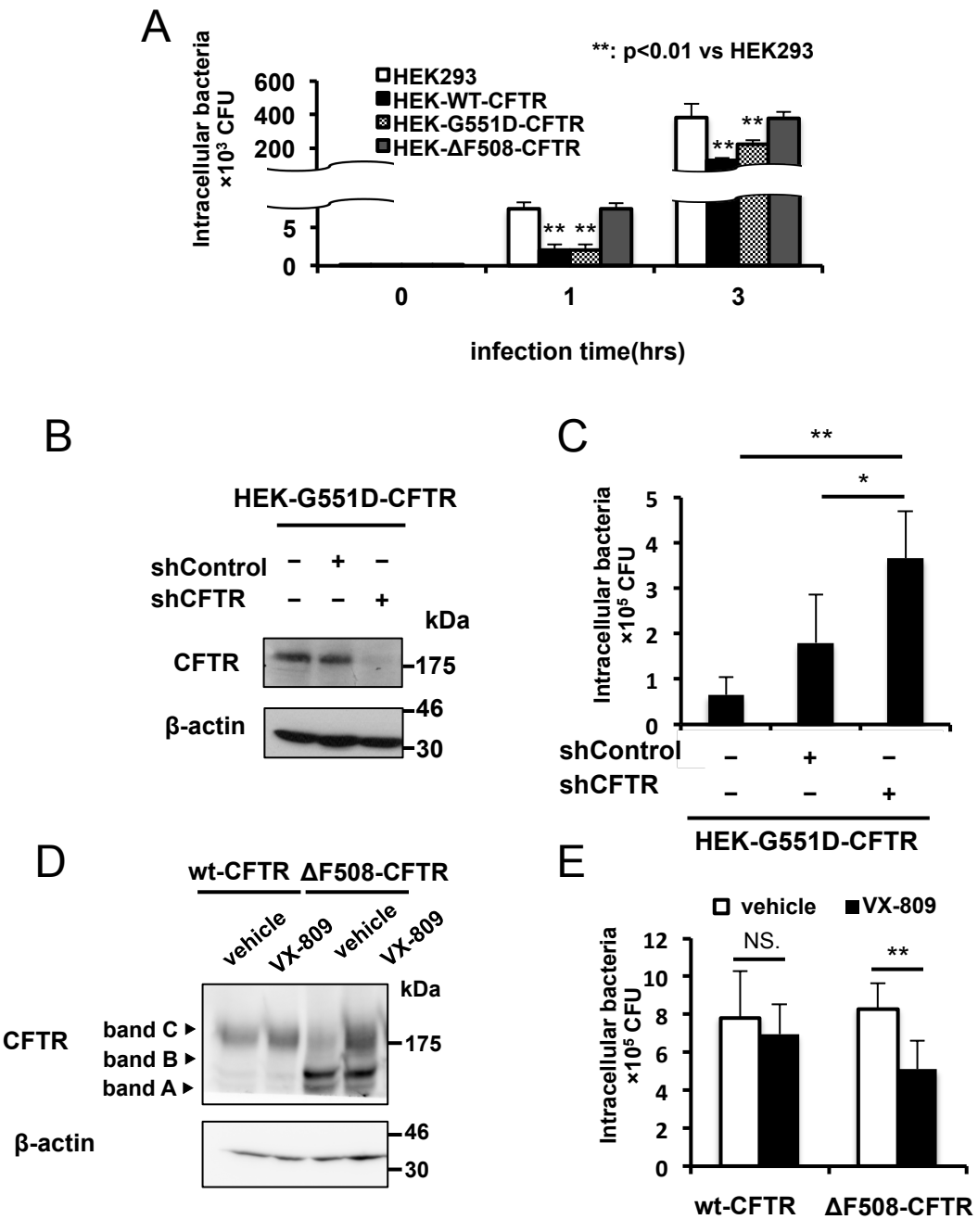

A
**: p<0.01 vs HEK293
HEK293
HEK-WT-CFTR
HEK-G551D-CFTR
HEK-ΔF508-CFTR
Intracellular bacteria ×10³ CFU
600
400
200
5
0
**
**
**
**
0
1
3
infection time(hrs)
B
HEK-G551D-CFTR
shControl – + –
shCFTR – – +
kDa
CFTR
175
β-actin
46
30
C
**
*
Intracellular bacteria ×10⁵ CFU
5
4
3
2
1
0
shControl – + –
shCFTR – – +
HEK-G551D-CFTR
D
wt-CFTR ΔF508-CFTR
vehicle
VX-809
vehicle
VX-809
kDa
CFTR
band C
band B
band A
175
β-actin
46
30
E
vehicle
VX-809
Intracellular bacteria ×10⁵ CFU
12
10
8
6
4
2
0
NS.
**
wt-CFTR
ΔF508-CFTR

Figure 6.

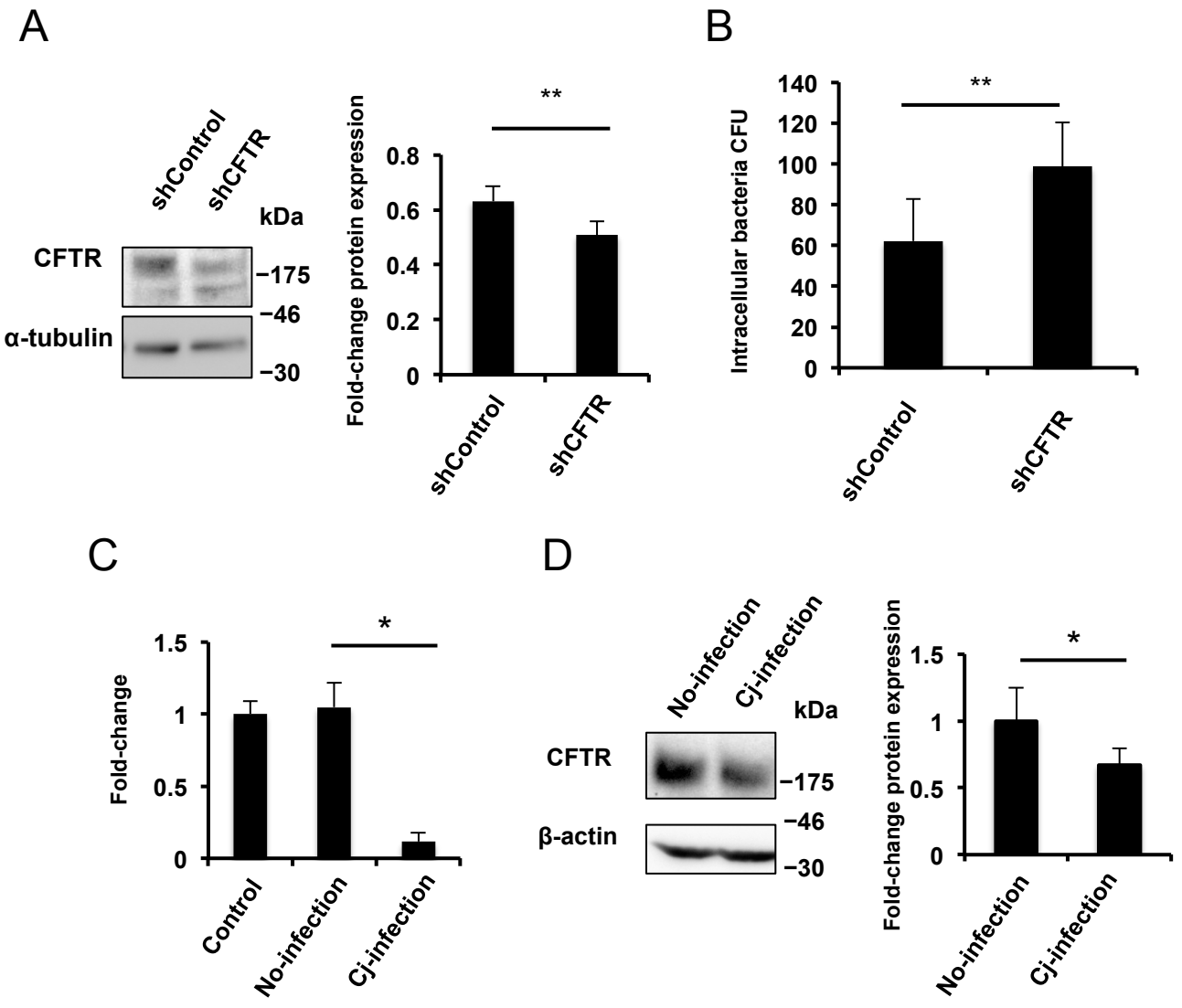
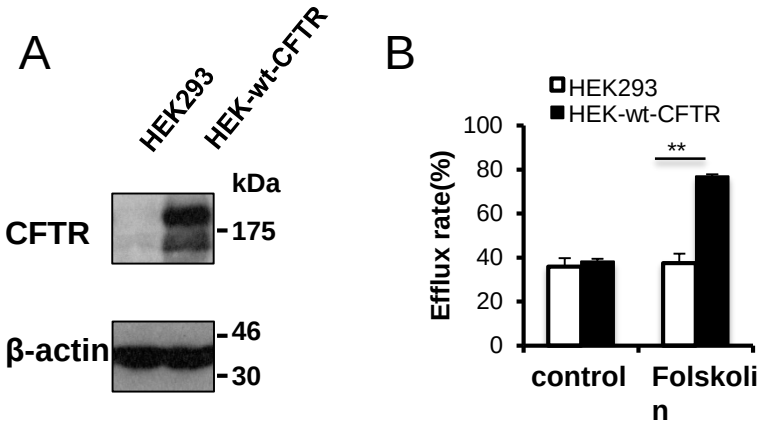
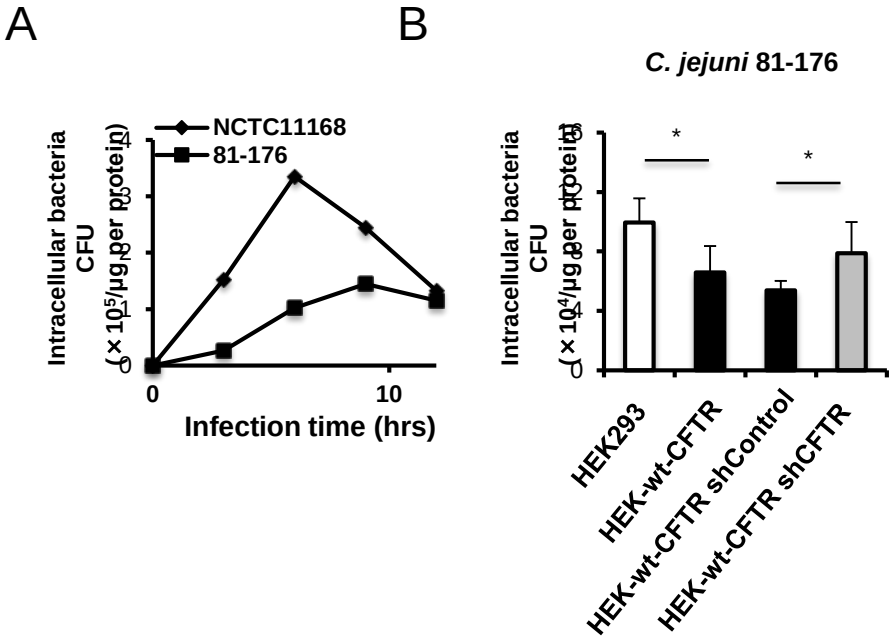

A
shControl
shCFTR
kDa
CFTR
α-tubulin
175
46
30
Fold-change protein expression
**
0.8
0.6
0.4
0.2
0
shControl
shCFTR
B
Intracellular bacteria CFU
**
140
120
100
80
60
40
20
0
shControl
shCFTR
C
Fold-change
*
1.5
1
0.5
0
Control
No-infection
Cj-infection
D
No-infection
Cj-infection
kDa
CFTR
β-actin
175
46
30
Fold-change protein expression
*
1.5
1
0.5
0
No-infection
Cj-infection

FIG S1.



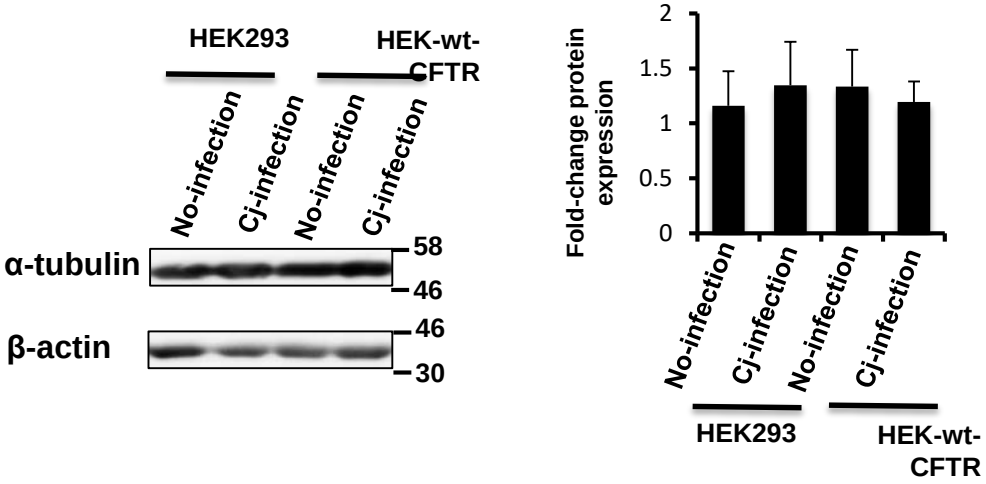
supplementary Figure 1. HEK-wt-CFTR cells stably and functionally express wt-CFTR. (A) HEK293 cells transfected with pcDNA-wt-CFTR vector stably expressed CFTR (HEK-wt-CFTR) as detected by western blot. (B) HEK293 cell and HEK-wt-CFTR cells were treated with forskolin or DMSO and CFTR channel activity was measured by assessing intracellular levels of 125I- isotope incorporated into cells for 5 minutes prior to measurement with a gamma counter system. All data are means ±SD of 3 independent experiment. Statistical significance: **, $p<0.01$

FIG S2.



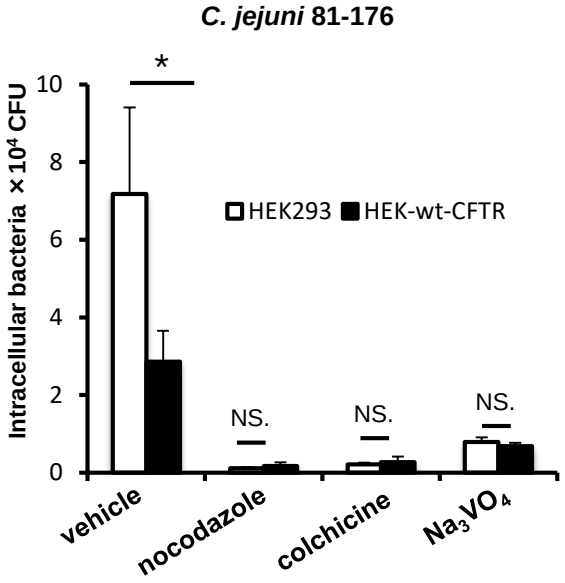
supplementary Figure 2. CFTR expression inhibited invasion by other *jejuni* strains (NCTC 11168 and 81-176). (A) HEK293 cells were infected by NCTC 11168 or 81-176 *C. jejuni* strains for 0, 3, 6, 9, or 12 hours. The number of intracellular bacteria were estimated using a gentamicin protection assay. (B) HEK293, HEK-wt-CFTR, HEK-wt-CFTR shControl and HEK-wt-CFTR shCFTR cells were infected by *C. jejuni* (81-176) for 6 hours. The numbers of intracellular bacteria were estimated with a gentamicin protection assay. All data are means ±SD of 3 independent experiment. Statistical significance: *, $p<0.05$

FIG S3.



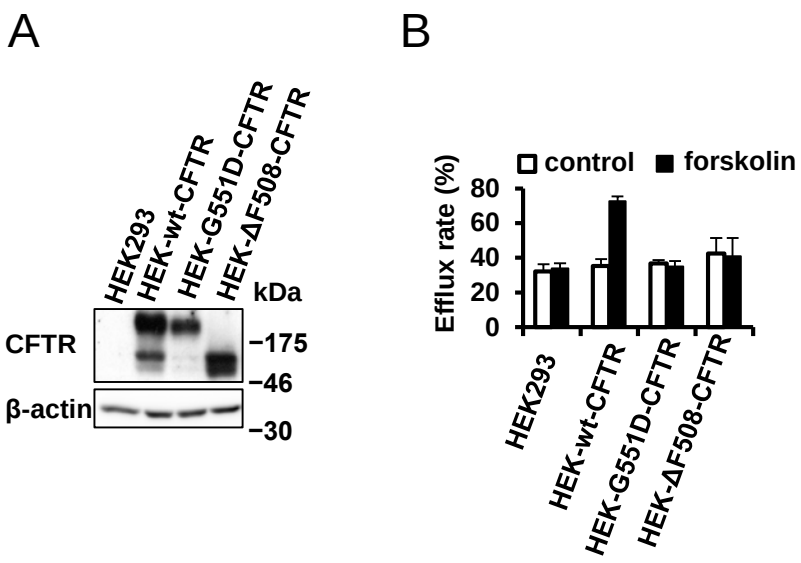
supplementary Figure 3. *C. jejuni* infection did not affect tubulin polymerization. HEK293 cells and HEK-wt-CFTR cells were infected with *C. jejuni* for 3 hours. The level of polymerized tubulin was assessed with a western blot using an α-tubulin antibody and normalized relative to b-actin .

FIG S4.



supplementary Figure 4. CFTR expression inhibited microtubule-dependent invasion with other *jejuni* strains (81-176). (A) HEK293 cells and HEK-wt- CFTR cells were treated with nocodazole, colchicine, and Na3VO4 before 1 hour infection. The number of intracellular bacteria were estimated using a gentamicin protection assay. All data are means ±SD of 3 independent experiment. Statistical significance: *, $p<0.05$. NS.,No Significance. n=6.

FIG S5.



supplementary Figure 5. Stable expression of mutant CFTR in transfected HEK293 cells. (A) HEK293 cells were transfected with pCDNA-G551D-CFTR and pCDNA-Δ-F508-CFTR. CFTR expression was detected by western blot and (B) CFTR channel activity was measured with an efflux assay.