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Diffusely adherent *Escherichia coli* strains isolated from healthy carriers suppress cytokine secretions of epithelial cells stimulated by inflammatory substances

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Running Head: Suppression of inflammatory response by DAEC

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ABSTRACT Diarrheagenicity of diffusely adherent *Escherichia coli* (DAEC) remains controversial. Previously, we found that motile DAEC strains isolated from diarrheal patients induced high levels of interleukin 8 (IL-8) secretion via Toll-like receptor 5 (TLR5). However, DAEC strains from healthy carriers hardly induced IL-8 secretion, irrespective of their possessing flagella. In this study, we demonstrated that SK1144, a DAEC strain from a healthy carrier, suppressed IL-8 and IL-6 secretion from human epithelial cell lines. Suppression of IL-8 in human embryonic kidney (HEK293) cells that were transformed to express TLR5 was observed not only upon inflammatory stimulation by flagellin but also in response to tumor necrosis factor-alpha (TNF-α) and phorbol myristate acetate (PMA), despite the fact that the TNF-α- and PMA-induced inflammatory pathways reportedly are not TLR5-mediated. SK1144 neither decreased IL-8 transcript accumulation nor increased intracellular retention of IL-8. No suppression was observed when the bacteria were cultured in Transwell cups above the epithelial cells; however, a non-adherent bacterial mutant (lacking the afimbrial adhesin gene) still inhibited IL-8 secretion. Direct contact between the bacteria and epithelial cells was necessary, but diffuse adhesion was dispensable for the inhibitory effects. Infection in the presence of chloramphenicol did not suppress cytokine release by the epithelial cells, suggesting that suppression depended on effectors synthesized *de novo*. Inflammatory suppression was attenuated with infection by a bacterial mutant deleted for *hcp* (encoding a component of a type-VI secretion system). In conclusion, DAEC strains from healthy carriers impede epithelial cell cytokine secretion, possibly by interfering with translation via the type-VI secretion system.

INTRODUCTION

Diarrheagenic *Escherichia coli* (DEC) is a primary pathogen associated with enteric disease. DEC has been classified into several subgroups according to pathogenicity, including enteropathogenic *E. coli*, enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enterohemorrhagic *E. coli*, and enteroaggregative *E. coli* (EAEC). Colonization of the human intestine is an essential step in infection by DEC. Diffusely adherent *E. coli* (DAEC) shows diffuse adhesion to HEp-2 cells via bacterial afimbrial adhesive sheaths (Afa) and has been proposed to constitute a sixth DEC class. In Servin's comprehensive review of DAEC (1), the Afa/Dr adhesin family includes at least 14 adhesins, including both fimbrial and afimbrial proteins. Two classes of Afa/Dr were proposed: Afa/Dr_{DAF}, which recognize Dr antigens with an exposed decay-accelerating factor (DAF) domain of the Cromer blood-group system; and Afa/Dr^{-}, which do not bind to human DAF. In addition to the adhesin, the secreted autotransporter toxin gene (*sat*) seemed to correlate with diarrhea-associated Afa/Dr DAEC strains, since *sat* was not present in any of the non-diarrhea-associated strains (2). However, the etiological role of DAEC in diarrheal disease still has remained controversial, although the relationship between Afa/Dr DAEC and diarrhea in children was demonstrated in age-stratified studies that showed an increased incidence in children <1 to 5 years of age (1).

DAEC likely comprise a heterogeneous group of organisms with variable enteropathogenicity (3). Measuring diffuse adhesion activity alone is insufficient to evaluate the diarrheagenicity of these strains, and therefore, other distinguishing characteristics have been pivotal for analysis by clinical microbiology. Other work has indicated that diarrheagenic EAEC causes the release of a considerable amount of the proinflammatory chemokine interleukin 8 (IL-8) from human intestinal epithelial cells; IL-8 elevation in the feces of patients correlates

with the severity of clinical symptoms (4). Subsequently, we have found that Afa/Dr DAEC strains that induce high-level IL-8 secretion are prevalent among the isolates obtained from diarrheal patients but not among the isolates recovered from healthy carriers (5–8).

The role of flagella and Toll-like receptor 5 (TLR5) in IL-8 production is apparent; notably, motile Afa/Dr DAEC strains cause prominent IL-8 induction, but non-motile strains show weaker induction of IL-8 (5). However, 9 of 15 motile strains from healthy carriers did not induce high-level IL-8 secretion, and these isolates therefore were designated motile-but-low-inducer (MBLI) strains (8, 9). We hypothesized that motile DAEC organisms recovered from patients had virulence factors that acted to loosen tight junctions (TJs), permitting flagellin to reach the innate receptor (TLR5), which is displayed on the basolateral side of epithelial cells, thereby triggering signaling that results in the induction of IL-8 at high levels (10). However, contrary to our expectation, MBLI strains also disrupted TJs as much as DAEC strains recovered from patients (9).

In the present study, we investigated why MBLI from healthy carriers induced only low-level IL-8 secretion from cultured human epithelial cells, regardless of whether the bacteria possessed flagella. In our previous reports (5–10), we used several different cell culture models, including: Caco-2 cells, a well-established model of small intestinal epithelium; HEp-2 (human laryngeal epithelial) cells, another standard model, to study adhesion of DEC; and T84 cells. However, in the present work, we primarily used human embryonic kidney (HEK293) cells transformed with a construct encoding TLR5 and a nuclear factor kappa B (NF-κB) / secreted alkaline phosphatase (SEAP) reporter system to clarify the relationship between TLR5 expression and responsivity to the flagellin of DAEC strains, excluding effects of other TLRs and ligands. Our analysis focused on bacterial strain SK1144, an Afa/Dr DAEC isolate that has been shown to cause the lowest

85 level of IL-8 induction among the MBLI (9). We found that the lower IL-8 production observed
86 following infection with MBLI from healthy carriers reflected active suppression of the
87 inflammatory response in the epithelial cells, rather than the inability to stimulate the host cells.
88
89
90 **RESULTS**
91 **Inhibitory effects of SK1144 on secretion of inflammatory cytokines.** Strain SK1144
92 induced less IL-8 than did motile DAEC strains isolated from diarrheal patients (9). It is possible
93 that SK1144 did not induce IL-8 secretion due to a defect in flagellin. To test this possibility,
94 recombinant flagellin of SK1144 was used to inoculate TLR5 NF-κB/SEAP-transfected HEK293
95 cells; 16 h later, the culture supernatant was assessed by ELISA for cytokines. The amount of
96 IL-8 induced by the recombinant SK1144 flagellin matched that obtained with purified
97 *Salmonella* flagellin used as a positive control (Fig. 1A, Fig. S1). Previous work failed to detect a
98 correlation (a) between the motility of motile DAEC strains and the induced level of IL-8
99 secretion, or (b) between either the quantity or function of flagella and MBLI properties (10). In
100 combination with our data, these results indicated that the lack of IL-8 induction by SK1144 does
101 not reflect an organic abnormality in the flagellin encoded by this strain. Since the flagellin of
102 SK1144 presumably still is able to serve as an inflammatory stimulus, we hypothesized that
103 SK1144 may suppress the inflammatory reaction of epithelial cells. To test this hypothesis, the
104 transfected HEK293 cells were inoculated with purified flagellin from SK1144 or *Salmonella* at
105 3 h after the cells were infected with a bacterial strain, and the amount of IL-8 then was
106 measured. Indeed, SK1144 cells significantly suppressed the flagellin-mediated induction of IL-8
107 to a level as low as that seen in uninfected cells not exposed to flagellin. In contrast, culture

supernatants of epithelial cells exposed to flagellin and infected with V64 (a patient strain) or with HB101 (a laboratory strain) contained high levels of IL-8 (Fig. 1B, C). The attenuation of IL-8 secretion was not due to selective degradation of the cytokine in the culture medium by the SK1144, given that exogenous IL-8 (added directly into the medium) was quantitatively (overridingly) recovered from the culture medium of HEK293 cells infected with SK1144. (Fig. 1D).

Signaling pathways that regulate cytokine induction. We examined whether SK1144 suppressed the transduction of the signal from TLR5. Although the pathways employed by tumor necrosis factor-alpha (TNF-α) and phorbol myristate acetate (PMA) to induce inflammatory reactions are distinct from the TLR5-mediated pathway that is used by flagellin, all of these pathways reportedly share NF-κB downstream signaling (11, 12). As seen for flagellin stimulation, the SK1144 strain suppressed IL-8 secretion in the transfected HEK293 cells stimulated by either TNF-α or PMA (Fig. 1E, F). Furthermore, expression of the secreted embryonic alkaline phosphatase (SEAP), an indicator of activation of NF-κB in the transfected HEK293 cells, was significantly suppressed by flagellin-SK1144 co-inoculation compared with expression in cells inoculated with flagellin alone (Fig. 1G). Similarly, SK1144 inoculation suppressed IL-8 production by HEp-2 cells exposed to each of the inducers (Fig. 1H). Effects on the secretion of interleukin 6 (IL-6), a representative inflammatory cytokine, also were assessed. Notably, SK1144 inhibited IL-6 induction in HEp-2 cells exposed to flagellin, TNF-α, or PMA (Fig. 1I); Intriguingly, even infection with *Salmonella* Enteritidis did not induce IL-8 or IL-6 secretion when the epithelial cells had been previously infected with SK1144 (Fig. 1H and I). These results implied that SK1144 possibly suppressed cytokine induction by blocking NF-κB.

Enhanced transcription of the IL-8 gene in HEK293 cells. The low-level IL-8 secretion

observed in epithelial cells infected with SK1144 might result from blockade of NF-κB activity. To test this possibility, the mRNA level of the IL-8 gene was compared between flagellin-stimulated epithelial cells cultured in the presence and absence of SK1144. Given that an inhibitory effect against IL-8 secretion was observed from the early hours (4–7 h) after exposure to flagellin (Fig. 2A); we inferred that SK1144 suppressed the transcription of *IL-8*. However, the level of *IL-8* mRNA in the HEK293 cells inoculated with both SK1144 and flagellin was similar to or higher than that seen in cells inoculated with flagellin alone (Fig. 2B, Table S1), that is, in cells that produced high levels of IL-8 protein. These observations suggested that bacterial suppression of IL-8 secretion occurs at a post-transcriptional step, rather than through the NF-κB signaling pathway.

Intracellular transport and secretion of IL-8. Since SK1144 inoculation did not decrease *IL-8* mRNA, we tested whether SK1144 instead interfered with the transport and/or secretion of IL-8 protein. When HEK293 cells were treated with brefeldin A (BFA), an inhibitor of eukaryotic intracellular transport of secreted proteins, little IL-8 was detected in the culture supernatant unless the cells were disrupted by sonication (Fig. 3A). In contrast, the level of IL-8 recovered from epithelia infected with SK1144 was low regardless of the ultrasonic disruption (Fig. 3A). Furthermore, fluorescent immunostaining revealed that HEK293 cells infected by SK1144 did not store IL-8 intracellularly (Fig. 3B).

Translational control of IL-8 in the endoplasmic reticulum (ER) stress response. Since the observed block in IL-8 production did not reflect decreases in either transcription or transport, experiments were performed to examine whether the translation of IL-8 was prevented by SK1144 infection. We examined whether SK1144's effects resulted from changes in protein synthesis at the ER. Thapsigargin (TG) and tunicamycin (TM) were used as ER stressors, and the

levels of C/EBP homologous protein (CHOP) and GRP78 (immunoglobulin heavy chain-binding protein, also known as BiP) were assayed as markers of ER stress (13). First, we tested whether ER stress was induced by SK1144. The results showed that transcription of the genes encoding BiP and CHOP was not increased by SK1144 infection, although the transcript levels of these markers were increased after the administration of the ER stressors (Fig. 4A, B). Next, ER stressors were assayed for suppression of IL-8 production. Notably, neither TG nor TM suppressed the level of IL-8 protein, in contrast to the suppression of IL-8 accumulation observed in SK1144-infected cells (Fig. 4C, D). These data indicated that IL-8 suppression by SK1144 is not due to the ER stress.

Effect of timing of flagellin inoculation on the inhibition of IL-8 production. We tested how many hours it took SK1144 to sensitize HEp-2 cells for the inhibition of IL-8 secretion. HEp-2 cells were infected with the bacteria and then inoculated with flagellin immediately or 3 h later. Suppression of IL-8 production by SK1144 was not observed when bacteria and flagellin were added at the same time (Fig. 5A); in contrast, 3 h of pre-exposure to SK1144 suppressed flagellin-induced IL-8 production to a level similar to that seen in the absence of flagellin exposure. We hypothesized that SK1144 synthesizes the inhibitory effector *de novo* after recognizing the presence of HEp-2 cells, a theory that we tested by adding chloramphenicol to HEp-2 cells immediately or 3 h after inoculation with SK1144. Notably, IL-8 secretion was high when SK1144 organisms were treated with chloramphenicol at the time of inoculation. In contrast, when chloramphenicol was added 3 h after inoculation with SK1144, IL-8 secretion was suppressed (Fig. 5B). These data suggested that SK1144 produces the anti-inflammatory effector(s) *de novo* after infecting epithelial cells.

IL-8 suppression requires direct bacteria-cell contact. To determine whether IL-8

suppression by SK1144 is mediated by humoral factors, Transwell insert cups were used to prevent direct contact between SK1144 and HEK293 cells. IL-8 induction was similar to that in the positive control (flagellin only) when the SK1144 strain was inoculated into the cup (Fig. 6A). These results implied that the suppressive effect requires contact between the bacteria and host cells. The patient strain V64 caused high IL-8 secretion even without inoculation with *Salmonella* flagellin (Fig. 6A); thus, the V64 bacteria may secrete an inflammatory substance that can pass through the membrane filter of the Transwell (14).

Separate experiments (without the Transwells) tested the effect of mutation of the SK1144 *afa* gene (Fig. S2A, B); the resulting mutant is unable to adhere to the host cells. As expected, the SK1144 Δ*afa* strain showed no diffuse adhesion to the epithelial cells (Fig. 6B); nonetheless, the mutant bacteria still inhibited IL-8 secretion in both transfected HEK293 and HEp-2 cells (Fig. 6C, D). Together, these data suggested that although direct bacteria-cell contact is pivotal for the IL-8 suppression by SK1144, intimate adherence is dispensable for this process.

Type-VI secretion system. Genome sequencing revealed that SK1144 possesses the type-VI secretion system (T6SS) that is reportedly used by bacteria to inject toxic proteins into eukaryotic cells (15, 16). Since SK1144 seemed to synthesize the anti-inflammatory effectors *de novo* after recognizing the presence of epithelial cells (Fig. 5), we examined whether the T6SS of SK1144 contributed to the inhibitory effect. A *clpV* deletion mutant (which lacks the T6SS ATPase) suppressed IL-8 secretion from the flagellin-stimulated epithelial cells (Fig. 6C, D; Fig. S2A, C). In contrast, a *hcp* deletion mutant (which lacks the T6SS needle shaft protein) yielded partial secretion of IL-8 from the flagellin-stimulated epithelial cells (Fig. 6C, D; Fig. S2D). The loss of the suppressive function of the *hcp* deletion mutant was completely recovered by complementing with *hcp* (Fig. 6E). These results suggested that Hcp itself is a candidate

anti-inflammatory protein (Fig. 7).

DISCUSSION

The present study showed that DAEC strain SK1144, which was isolated from a healthy individual, actively suppresses the inflammatory response, presumably via one or more effectors. Intriguingly, the flagellin of SK1144, like *Salmonella* flagellin, induced high-level IL-8 secretion by HEK293 cells. The flagellin of SK1144 presumably has a normal structure, permitting the protein to be recognized by TLR5 and thereby inducing proinflammatory responses via the signal induction pathway. However, the prominent suppression due to SK1144 was evident based on the pronounced attenuation of flagellin-induced IL-8 production in cells infected by this organism. Although it has been reported that the Group-A streptococcus protease SpyCEP cleaves IL-8 to evade attack by neutrophils (17), SK1144 did not decrease the level of IL-8 added to the culture medium. Thus, this DAEC does not exert its effect by producing a protease that degrades IL-8.

Neither flagellin, TNF-α, nor PMA caused IL-8 secretion from the epithelial cells upon infection by SK1144. Furthermore, the SK1144 strain inhibited IL-6 secretion in HEp-2 cells and SEAP production in transfected HEK293 cells, indicating that the observed suppression is not IL-8 specific. It was suggested that SK1144 may inhibit activation of NF-κB or the translocation of NF-κB, as many reports indicate that pathogenic bacteria can suppress the intracellular signaling cascade to disturb inflammatory reactions (18–20). Surprisingly, however, the level of *IL-8* mRNA was not reduced by SK1144. Transcription-independent mechanism(s) might be involved.

SK1144 did not prevent overexpression of the *IL-8* transcript in infected cells exposed to flagellin, although the bacterium did suppress an increase of IL-8 protein accumulation in the

tissue culture medium. These results strongly suggested that SK1144 impedes cytokine secretion by interfering with post-transcriptional events rather than with the signal induction systems. The *Shigella* type-III effector IpaJ seems to inhibit cytokine secretion via the disruption of Golgi morphology during infection (21). In the present study, strain SK1144 also seemed to minimize accumulation in the Golgi of GM130 in a manner similar to that seen with BFA, a known inhibitor of ER-Golgi transport (Fig. 3B). However, intracellular IL-8 did not accumulate in epithelial cells infected with SK1144; in contrast, BFA stopped the secretion of IL-8 and resulted in the intracellular accumulation of high levels of IL-8. *Chlamydia trachomatis* in host cells triggers Golgi fragmentation around the replicative vacuole, which is necessary for lipid acquisition and intracellular growth of that bacterium (22). SK1144 may inhibit cytokine secretion by inhibiting Golgi as chlamydia do; however, unlike chlamydia, SK1144 is not an obligate intracellular organism. Thus, it appears that the reduced Golgi body is secondary to the suppression of protein synthesis in SK1144-infected HEK293 cells, just as the reduced Golgi body is secondary to decreased protein transport to the Golgi in BFA-treated cells.

SK1144 may block cytokine synthesis at the translational level. Protein synthesis is reportedly suppressed under the condition of so-called ER stress. However, SK1144 disturbed IL-8 production in HEK293 cells without elevating ER stress markers. SK1144 may suppress cytokine synthesis using bacterial effectors that act independently of the endogenous ER stress response. Such effector(s) are presumably synthesized *de novo* in the bacterium after the DAEC recognizes the presence of epithelial cells.

Many DAEC have afimbrial adhesins (Afa) that recognize the CD55 (decay-accelerating factor, DAF) marker expressed on the eukaryotic cell surface, facilitating bacterial attachment to the host cells (23); bacterial binding to this marker affects the growth of the host cells (24).

Blocking bacteria-to-cell contact by use of the Transwell apparatus eliminated the ability of the bacterium to suppress IL-8 secretion. This result suggested that DAEC must make direct contact with the host cells to suppress the inflammatory response. Interestingly, a SK1144 Δ*afa* strain, which lacks the ability to adhere diffusely to host cells, still suppressed IL-8 secretion. These observations suggested that direct contact, but not strong adherence, is essential for SK1144's suppression of the inflammatory response.

Several species of bacteria are known to inject effector proteins into host cells by means of secretion systems that require contact between the bacterial and eukaryotic cells. Notably, enteropathogenic *E. coli* and enterohemorrhagic *E. coli* suppress the secretion of inflammatory cytokines by employing effectors injected into host cells via type-III secretion systems (25–27). Although the genome sequence of the SK1144 strain shows no genes for type-III secretion systems (data not shown), the organism does possess genes for a T6SS. Thus, SK1144 may employ a T6SS to deliver the effector(s) that decrease the IL-8 secretion. Notably, we found that a SK1144 Δ*clpV* mutant (lacking the T6SS ATPase-encoding gene) still suppressed IL-8 secretion, while a SK1144 Δ*hcp* mutant (lacking the T6SS needle shaft protein) was partially impaired for IL-8 suppression. The weakened suppression of IL-8 by the Δ*hcp* mutant was complemented by a recombinant plasmid harboring an intact *hcp*. Intriguingly, recombinant Hcp from *Aeromonas hydrophila* has been shown to reduce bacterial uptake by macrophages, and to inhibit the production of proinflammatory cytokines while inducing immunosuppressive cytokines (28). Our results suggest that Hcp similarly may be involved in the DAEC-mediated suppression of IL-8 production. However, the Hcp1 protein of the meningitis-causing *E. coli* K1 strain has been shown to induce IL-6 and IL-8 cytokine release in human brain microvascular endothelial cells (29). This last result is inconsistent with the data presented in the present work,

indicating that Hcp proteins may have both inhibitory and accelerant effects on the inflammatory responses in host cells. Further studies are in progress to determine whether the SK1144 Hcp protein is an effector of DAEC responsible for suppression of the inflammatory response in epithelial cells.

In conclusion, strain SK1144, a representative DAEC isolated from a healthy carrier, impedes cytokine secretion. Hcp, a T6SS component, may play a role in interfering with cytokine production by the host at a post-transcriptional step. DAEC strains lacking this inhibitory mechanism(s) would be enteropathogenic for the inflammatory properties associated with DEC. Further elucidation of these effector(s) is expected to facilitate the assignment of DAEC strains to distinct diarrheagenic and non-diarrheagenic groups. These inhibitory strains may in turn be candidates for use as anti-inflammatory probiotic strains of *E. coli* capable of regulating the exaggerated inflammatory responses induced in the intestine by pathogenic strains.

MATERIALS AND METHODS

Bacterial strains. The DAEC strains consisted of SK1144 (an isolate from a healthy person) and V64 (an isolate from a diarrheal patient) (5, 8). These two strains were used as representative low-IL-8- and high-IL-8-inducing strains (respectively) (8, 9). HB101 (Takara Bio, Shiga, Japan), a laboratory strain of *E. coli*, served as the negative control. *Salmonella enterica* subsp. *enterica* serovar Enteritidis strain PT1, originally isolated from an outbreak patient, was used as an IL-8-inducing strain. Bacterial inocula were prepared by culturing the strains in Luria-Bertani Broth (LB broth, Becton Dickinson and Co., Speaks, MD) at 37°C overnight. BL21 (DE3) (Novagen, Madison, WI) was used as the protein-expressing strain. Bacteria were cultured in LB broth or on an LB-agar plate at 37°C overnight. Transformants were selected on an LB-agar plate

292 containing ampicillin (Amp, 100 μg/ml, Wako, Tokyo, Japan), chloramphenicol (Cm, 25 μg/ml,
293 Wako), or kanamycin (Km, 50 μg/ml, Wako). The optical density of the medium at 600 nm
294 (OD600) was measured to estimate colony forming units (CFUs).
295 **Epithelial cell line.** HEp-2 cells were grown in Eagle's Minimal Essential Medium (EMEM,
296 Nissui, Tokyo, Japan) containing 2 mM L-glutamine, 0.15% $NaHCO_3$, 10% fetal bovine serum
297 (FBS, Biosera, Nuaille, France) and Non-essential amino acids for MEM Eagle (MP biomedical,
298 Irvine, CA). TLR5 NF-κB/SEAP-transfected HEK293 cells (IMGENEX, San Diego, CA), which
299 were transfected already and used to test flagellin activity, were grown in Dulbecco's Modified
300 Eagle Medium (DMEM, Nissui) containing 2 mM L-glutamine, 0.15% NaHCO3, 10% FBS, 500
301 μg/ml G418 (Sigma-Aldrich, St. Louis, MO), 10 μg/ml blasticidin (InvivoGen, San Diego, CA),
302 Non-essential amino acids for EMEM Eagle, and 1 mM sodium pyruvate (MP biomedical). Cells
303 were grown in 25-cm^2 polystyrene tissue culture flasks at 37°C in a 5% CO_2 incubator.
304 **Stimulants.** Flagellin purified from *Salmonella* Typhimurium (Novus Biologicals, Littleton,
305 CO), tumor necrosis factor alfa (TNF-α, Wako), or phorbol 12-myristate 13-acetate (PMA,
306 Wako) were inoculated with cells at 50, 100, or 2.5 ng/ml, respectively. The SK1144 flagellin
307 gene (*fliC*) was amplified by PCR with a primer pair consisting of fliC_pet_f and fliC_p/c_r. The
308 PCR product and plasmid vector (pET-30a, Novagen) were digested with restriction enzymes
309 (BamHI, XhoI) and ligated together. The resulting plasmid (designated pET-1144fliC) was
310 recovered by transformation into *E. coli* BL21 (DE3) with selection for kanamycin resistance.
311 After induction with isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM, Takara Bio) for 6 h at
312 25°C, His-FliC protein was purified using the His60 Ni Gravity Column Purification Kit (Takara
313 Bio); purity was confirmed by demonstrating that the protein ran as a single band on SDS-PAGE.
314 The SK1144 flagellin was inoculated to cells at 50 ng/ml or 400 ng/ml.

Cytokine induction test. HEp-2 and transfected HEK293 cells were seeded in 24- or 96-well tissue culture plates. When the cells achieved confluency (2 days), the tissue culture medium was replaced with fresh tissue culture medium containing D-mannose (1%, w/v) without FBS or antibiotics. Bacteria were grown in LB medium and the OD600 of the medium was measured. When the bacterial culture had achieved the desired density, bacteria were harvested by centrifugation. The resulting pellet was resuspended in cell culture medium containing D-mannose and adjusted by dilution to provide a bacteria-to-cell ratio of 100:1 and the mixtures were incubated at 37°C for 3 h in the CO_2 incubator. Subsequently, individual stimulants were added to each culture, and the cells were incubated for another 19 h. The supernatant of the culture medium then was assessed by enzyme-linked immunosorbent assays (ELISAs) for IL-8 and IL-6. IL-8 was measured using the Optimiser ELISA Kit (Siloam Biosciences, Cincinnati, OH). IL-6 was measured using the Human ELISA Kit (Invitrogen, Camarillo, CA). For the SEAP assay, culture supernatants of transfected HEK293 cells were harvested and analyzed using the Secreted Alkaline Phosphatase Reporter Gene Assay Kit (Luminescence) (Cayman Chemical Company, Ann Arbor, MI).

mRNA transcription measurement. For the early experiments, transfected HEK293 cells were infected with bacteria and flagellin and incubated for 1, 4, or 7 h. For other experiments, SK1144, thapsigargin (TG, 1 µM, Wako, Osaka, Japan) or tunicamycin (TM, 10 µg/ml, Wako, Osaka, Japan) was administered to HEK293 cells 1 h before inoculation of the epithelial cells with SK1144 flagellin; the epithelial cells were sampled 5 h later. Total mRNA was extracted from the epithelial cells using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). After removal of genomic DNA by treatment with DNase (Qiagen), cDNA strands were generated with reverse transcriptase (Qiagen). The transcription levels of genes involved in TLR signaling were

assessed by the RT² Profiler PCR Array Human Toll-Like Receptor Signaling Pathway (Qiagen). IL-8-, BiP-, and CHOP-encoding genes were detected using QuantiTect SYBR® Green PCR Kits (Qiagen). Real-time PCR was performed with the StepOnePlus Real-time PCR system (Life Technologies, Gaithersburg, MD). Expression of target mRNA was normalized to the expression of a reference mRNA (*actb*, encoding the housekeeping protein β-actin), and the fold-change was calculated based on the ΔΔCt method (30). The sets of primers were as described in previous studies (31, 32).

Intracellular cytokine measurement. To block Golgi transport, HEK293 cells were stimulated with flagellin for 7 h after a 1-h pre-incubation in the presence of brefeldin A (BFA, 50 μg/ml, Wako) or SK1144 organisms. Following collection, the epithelial cells were sonicated on ice, and IL-8 in the centrifuged supernatant was measured by ELISA.

Immunofluorescence microscopy. Transfected HEK293 cells were seeded on 4-well chamber slides (Thermo Fisher Scientific, Rochester, NY) and incubated for two days at 37°C in a 5% CO_2 incubator. The spent medium then was replaced with fresh medium, and the cells were inoculated with SK1144, flagellin, or BFA and incubated for 3 h. Cells were washed with Dulbecco's phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde/PBS for 15 min at room temperature. Fixed cells were washed with PBS, then blocked and permeabilized with PBS containing 1% bovine serum albumin (BSA, Wako) and 0.2% Triton X-100 at room temperature for 1 h. The cells then were incubated with primary antibodies diluted in PBS containing 1% BSA at room temperature for 1 h, washed with PBS, and incubated with secondary antibodies and DAPI (1 μg/ml, Biotium, Hayward, CA) for 1 h at room temperature. IL-8 was stained with anti-IL-8 antibody (1:63 dilution, R&D) and detected with a secondary antibody (FITC-conjugated anti-mouse antibody; 1:100 dilution, Sigma-Aldrich). Golgi body

was stained with anti-GM130 antibody (clone: 35/GM130, BD Transduction Labs, San Jose, CA) and detected with a secondary antibody (Alexa Flour 647-conjugated anti-goat antibody; 1:200 dilution, Abcam, Cambridge, MA). Stained cells were imaged using a FV1200 confocal scanning laser microscope (Olympus, Tokyo, Japan).

Transwell test. Transfected HEK293 cells were seeded on a 24-well plate, and 6.5-mm Transwell inserts with 0.4-μm-pore-size membranes (Corning Inc., Corning, NY) were placed in the wells. When the HEK293 cells achieved confluence, bacterial strains were inoculated into the Transwell inserts, such that the bacteria did not come into direct contact with the HEK293 cells. After 3 h of incubation, flagellin was applied to the HEK293 monolayer (within the wells, not in the cups). Following incubation for another 19 h, the culture medium was recovered from the lower well, and the centrifuged supernatant was assayed by ELISA.

Gene deletion. To construct the deletion mutants of *afa* (encoding afimbrial adhesin) or of *clpV* or *hcp* (encoding the ATPase and needle shaft (respectively) of the T6SS), we used the phage λ Red recombinase system (33). Using electroporation, the SK1144 strain was transformed with a temperature-sensitive plasmid, pKD46, that expresses the lambda recombinase, with Amp selection at 30°C, yielding a strain that was designated SK1144-pKD46. The *cat* (chloramphenicol acetyltransferase-encoding) gene was amplified by PCR using plasmid pKD3 as the template. The set of primers is shown in Table S1. Amplified recombinant fragments were purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). Electrocompetent SK1144-pKD46 cells were generated, electroporated with the purified fragments and selected on an LB-agar plate containing Cm. To delete *cat* from the chloramphenicol-resistant transformants of SK1144-pKD46, the SK1144-Cm^r mutants were electroporated with the temperature-sensitive plasmid pCP20 (34) expressing the Flp flippase,

and ampicillin-resistant transformants were selected at 30°C. For each strain, several colonies were picked and cultured at 43°C without antibiotics; the resulting bacteria were tested to confirm their sensitivity to both Cm and Amp. Single-gene recombinatorial deletion was confirmed using PCR.

Gene complementation. To construct the complementation plasmid, the *hcp* gene was amplified using KOD FX Neo polymerase (Toyobo, Osaka, Japan) with SK1144 as the template; the set of primers is shown in Table S1. The pCold VI plasmid (Takara Bio) was digested with enzymes NdeI and EcoRI (Takara Bio). The digested plasmid was purified with Wizard SV Gel and the PCR Clean-Up System (Promega) before ligation with In-Fusion HD Enzyme (Takara Bio) for 15 min at 50°C. Ligation products were transformed into DH5α competent cells (Takara Bio). Transformants were selected on LB plates containing Amp and the presence of the insert was confirmed by performing colony PCR using the pCold-F and pCold-R primers. After confirmation, the desired clone was cultured overnight, and the plasmid was extracted with the PureYield Plasmid Miniprep System (Promega). The sequence of the DNA insert was determined using the pCold-F primer. This plasmid, which was designated pCold VI-hcp, was used to transform the SK1144Δ*hcp* strain, yielding strain SK1144Δ*hcp*-pCold VI-hcp. Prior to inoculation to the cell line, strain SK1144Δ*hcp*-pCold VI-hcp was cultured for 3 h at 37°C in LB Amp, and then incubated for another 24 h at 16°C in fresh LB Amp containing 1 mM IPTG.

Adherence test. Bacterial adherence to the epithelial cells was assessed as described previously (8). Briefly, after HEp-2 cells achieved confluence, bacteria were added, and the cultures were incubated for 3 h. Monolayers were washed with PBS and fresh EMEM was added. After further incubation for 3 h, the monolayers were washed with PBS, fixed with methanol, and stained with Giemsa. The cells were evaluated by BX53 light microscopy

(Olympus).

Statistical analysis. Two-tailed t-test and analysis of variance (ANOVA) with post-hoc Tukey analysis were employed for the statistical analyses, which were performed with Microsoft Excel supplemented with the add-in software +Statcel 4 (OMS, Tokorozawa, Japan). $P < 0.05$ was considered significant.

Whole-genome sequence. Complete genome sequencing and hybrid assembly were performed with Illumina MiSeq and PacBio RS II platforms. The genome sequences were automatically annotated using the Microbial Genome Annotation Pipeline (MiGAP, http://www.migap.org). The reported nucleotide sequence data are available in the DDBJ databases under the accession numbers AP018784 and AP018785. Sequence analysis of the T6SS encoding genes was performed with SecReT6 (http://db-mml.sjtu.edu.cn/SecReT6/), a web-based resource for type-VI secretion systems (16).

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Figure legends

Fig 1. SK1144 inhibits IL-8 and IL-6 secretion in epithelial cells. (A) Flagellin from SK1144 and *Salmonella* were inoculated on TLR5/SEAP-transfected HEK293 cells, and the secreted IL-8 was assayed 16 h later. (B, C, E-G) *E. coli* organisms (V64 is a DAEC strain from a patient; HB101 is a laboratory strain used as the negative control) were inoculated to transfected HEK293 cells. Three hours later, the infected cells were inoculated with purified SK1144 flagellin (B), *Salmonella* Typhimurium flagellin (C, G), TNF-α (E), or PMA (F). IL-8 secretion was measured by ELISA 19 h later. (D) SK1144 were incubated with TLR5/SEAP-transfected HEK293 cells for 16 h with (+) or without (-) IL-8 (1 ng/ml). (G) SEAP activity in the culture supernatants was assessed based on a colorimetric reaction. Symbols indicate exposure of transfected HEK293 cells to stimulatory material (+) or vehicle (-). Values are the mean ± standard deviation of three independent experiments (*P <0.05 **P <0.01 vs. uninfected (-), †P <0.05 ††P <0.01 vs. uninfected (+), ANOVA with post-hoc Tukey analysis). (H, I) After HEp-2 cells were infected with SK1144 for 3 h, purified SK1144 flagellin, TNF-α, PMA, or *Salmonella* Enteritidis (SE, viable bacteria) were inoculated to the infected cells and culturing was continued for another 19 h. IL-8 (H) or IL-6 (I) secretion was measured by ELISA. HEp-2 cells were infected with the SK1144 strain (+) or with material only (-). Values are the mean ± standard deviation (n=3, *P <0.05 **P <0.01 ***P <0.001, Student's t-test).

Fig 2. Strain SK1144 does not suppress transcription of *IL-8* in transfected HEK293 cells. Transfected HEK293 cells were inoculated with flagellin and SK1144, and then culture medium and total mRNA from the inoculated cells were collected 1, 4, and 7 h later. (A) The culture supernatant was assessed by ELISA for IL-8. (B) IL-8 mRNA was assessed by quantitative

RT-PCR. The continuous line indicates the cells stimulated only by flagellin. The dotted line indicates co-stimulation by SK1144 and flagellin. Values are the mean ± standard deviation (n=3, *P <0.05 **P <0.01 ***P <0.001, NS: not significant, Student's t-test).

Fig 3. HEK293 cells infected by the SK1144 strain do not retain intracellular IL-8. (A) At 1 h after TLR5/SEAP-transfected HEK293 cells were infected with SK1144, purified recombinant SK1144 flagellin was added to the cells, and incubation was continued for another 7 h. Samples recovered without or with sonication (supernatant / whole cell + supernatant, respectively) were assessed by ELISA for IL-8. Values are the mean ± standard deviation (n=3, **P <0.01 vs. uninfected (-), ANOVA with post-hoc Tukey analysis). (B) After transfected HEK293 cells were cultured with SK1144, flagellin, or brefeldin A (BFA) for 3 h, the cells were fixed, stained by immunohistochemistry, and evaluated using confocal scanning laser microscopy. BFA was used to induce Golgi fragmentation. Fluorescence imaging was used to detect IL-8 (green), GM130 (cis-Golgi marker, red), and cell nuclei (DAPI, blue). Scale bars, 5 μm.

Fig 4. Inhibitory effect of SK1144 on IL-8 synthesis is independent of ER stress in HEK293 cells. TLR5/SEAP-transfected HEK293 cells were cultured with SK1144, thapsigargin (TG), or tunicamycin (TM) for 1 h, and then cells were inoculated with purified recombinant SK1144 flagellin and incubated for another 5 h. TG and TM were used to induce ER stress. Total RNA was purified from the cells and the expression of the genes encoding (A) BiP, (B) CHOP, and (C) IL-8 were assessed by quantitative RT-PCR, and the supernatants were assayed by IL-8 ELISA (D). Values are the mean ± standard deviation (n=3, *P <0.05 **P <0.01 vs. uninfected (-), ANOVA with post-hoc Tukey analysis).

Fig 5. Effect of timing of flagellin inoculation on SK1144-mediated inhibition of IL-8 production. (A) After HEp-2 cells were infected with bacteria, flagellin was inoculated immediately (0h+) or three hours later (3h+). Sixteen hours later, culture supernatants were assessed by ELISA for IL-8. Values are the mean ± standard deviation of three independent experiments (**P <0.01 ***P <0.001 vs. uninfected (-), †P <0.05 ††P <0.01 vs. uninfected (+), Student's t-test). Antecedent infection was required for SK1144 to suppress IL-8 production in HEp-2 cells. (B) After HEp-2 cells were inoculated with SK1144, flagellin (Fla), or chloramphenicol (Cm) was added immediately (0h treat) or three hours later (3h treat). Sixteen hours later, culture supernatants were assessed by ELISA for IL-8. Values are the mean ± standard deviation (n=3). Means with different letters are significantly different (**P <0.01 vs. nontreated group, ANOVA with post-hoc Tukey analysis).

Fig 6. IL-8 secretion inhibition by SK1144 requires direct contact and a type-VI secretion system component (Hcp). (A) HEK293 cells were grown in 24-well plates, then the Transwell insert cups were inserted and bacteria were inoculated into the inserted cups. *Salmonella* flagellin was inoculated beneath the insert cup, i.e., where the HEK293 cells were cultured (+); (-) indicates that no flagellin was added. Values are the mean ± standard deviation (n=3, *P <0.05 **P <0.01 vs. uninfected (-), †P <0.05 ††P <0.01 vs. uninfected (+), ANOVA with post-hoc Tukey analysis). (B) Adherence pattern of SK1144 on HEp-2 cells; in contrast to the wild-type (upper panel), the SK1144 Δ*afa* strain did not adhere to HEp-2 cells (lower panel). (C-D) HEK293 (C) or HEp-2 (D) cells were infected with SK1144 or deletion mutants lacking the

genes that are responsible for adhesion or T6SS. Three hours later, flagellin from SK1144 was added (+) and cells were incubated for another 19 h. Values are the mean ± standard deviation of five (HEK293) or three (HEp-2) independent experiments (*P <0.05 **P <0.01 ***P <0.001 vs. uninfected (-), Student's t-test). (E) The *hcp*-complemented strain was used to infect HEp-2 cells for 3 h before flagellin was added, and cells then were incubated for another 19 h. Values are the mean ± standard deviation of four independent experiments (**P <0.01 vs. uninfected (-) †P <0.05 ††P <0.01 †††P <0.001 vs. uninfected (+), Student's t-test).

Fig 7. Schematic of the hypothesized mechanisms of suppression of proinflammatory responses by DAEC strain SK1144. DAEC can dilate tight junctions, permitting flagellin to reaches TLR5 molecules located on the basolateral side of the epithelial cells. Subsequent signaling through the NF-κB pathway provides enhanced transcription of genes encoding proinflammatory cytokines. However, Hcp, a component of the T6SS needle shaft, employs an unknown mechanism to suppress the translation of the cytokine-encoding mRNAs.

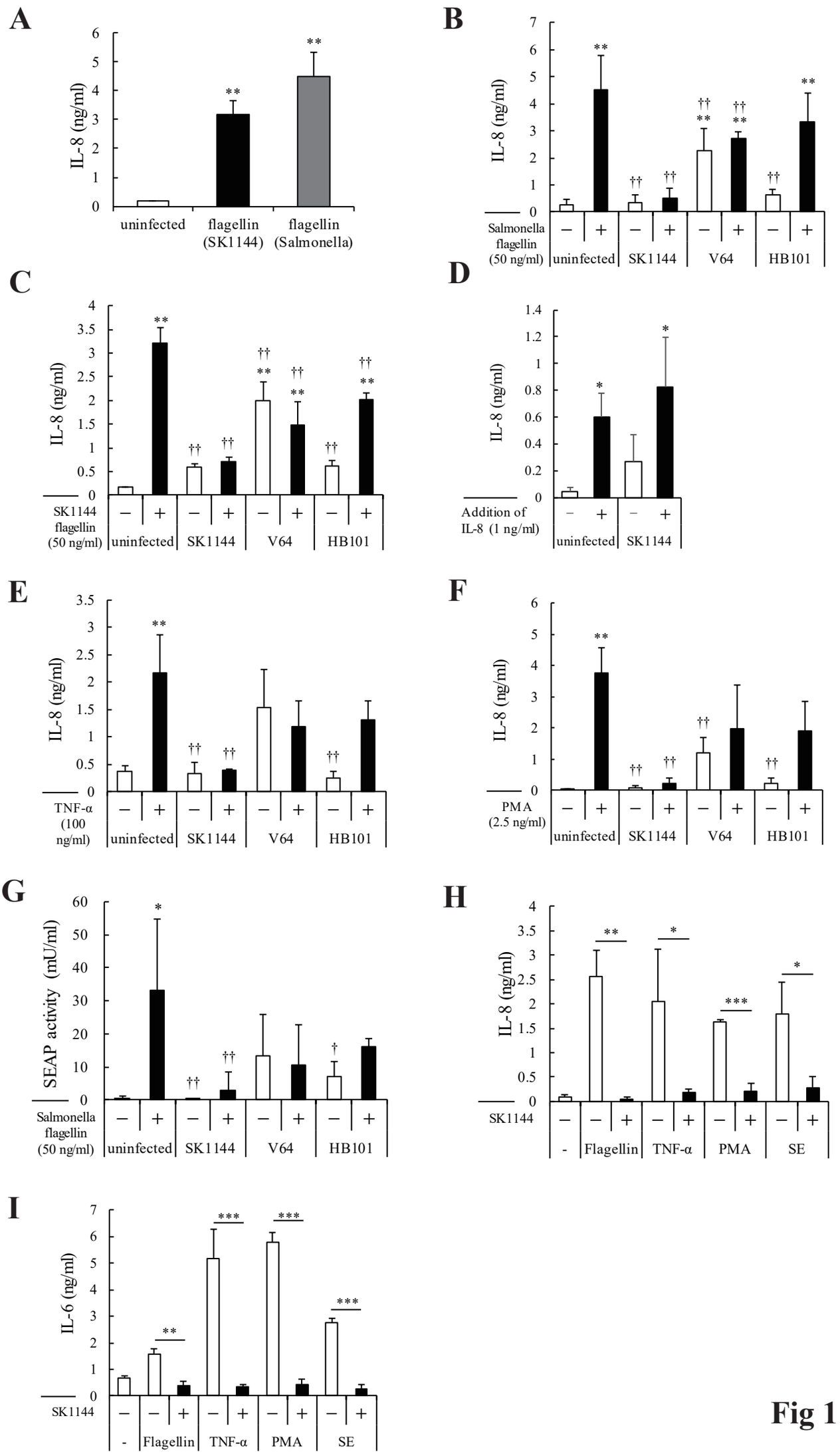
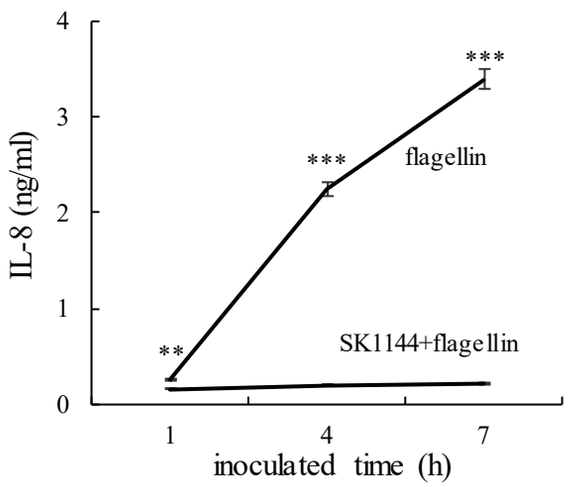

A
IL-8 (ng/ml)
uninfected
flagellin (SK1144)
flagellin (Salmonella)
B
IL-8 (ng/ml)
Salmonella flagellin (50 ng/ml)
uninfected
SK1144
V64
HB101
C
IL-8 (ng/ml)
SK1144 flagellin (50 ng/ml)
uninfected
SK1144
V64
HB101
D
IL-8 (ng/ml)
Addition of IL-8 (1 ng/ml)
uninfected
SK1144
E
IL-8 (ng/ml)
TNF-α (100 ng/ml)
uninfected
SK1144
V64
HB101
F
IL-8 (ng/ml)
PMA (2.5 ng/ml)
uninfected
SK1144
V64
HB101
G
SEAP activity (mU/ml)
Salmonella flagellin (50 ng/ml)
uninfected
SK1144
V64
HB101
H
IL-8 (ng/ml)
SK1144
-
Flagellin
TNF-α
PMA
SE
I
IL-6 (ng/ml)
SK1144
-
Flagellin
TNF-α
PMA
SE

Fig 1

A


IL-8 (ng/ml)
4
3
2
1
0
1
4
7
inoculated time (h)
**
***
***
flagellin
SK1144+flagellin

B

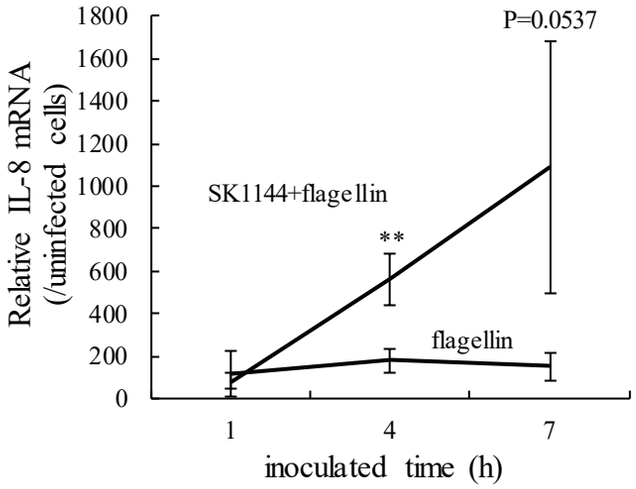

Relative IL-8 mRNA
(/uninfected cells)
1800
1600
1400
1200
1000
800
600
400
200
0
1
4
7
inoculated time (h)
P=0.0537
SK1144+flagellin
**
flagellin

Fig 2

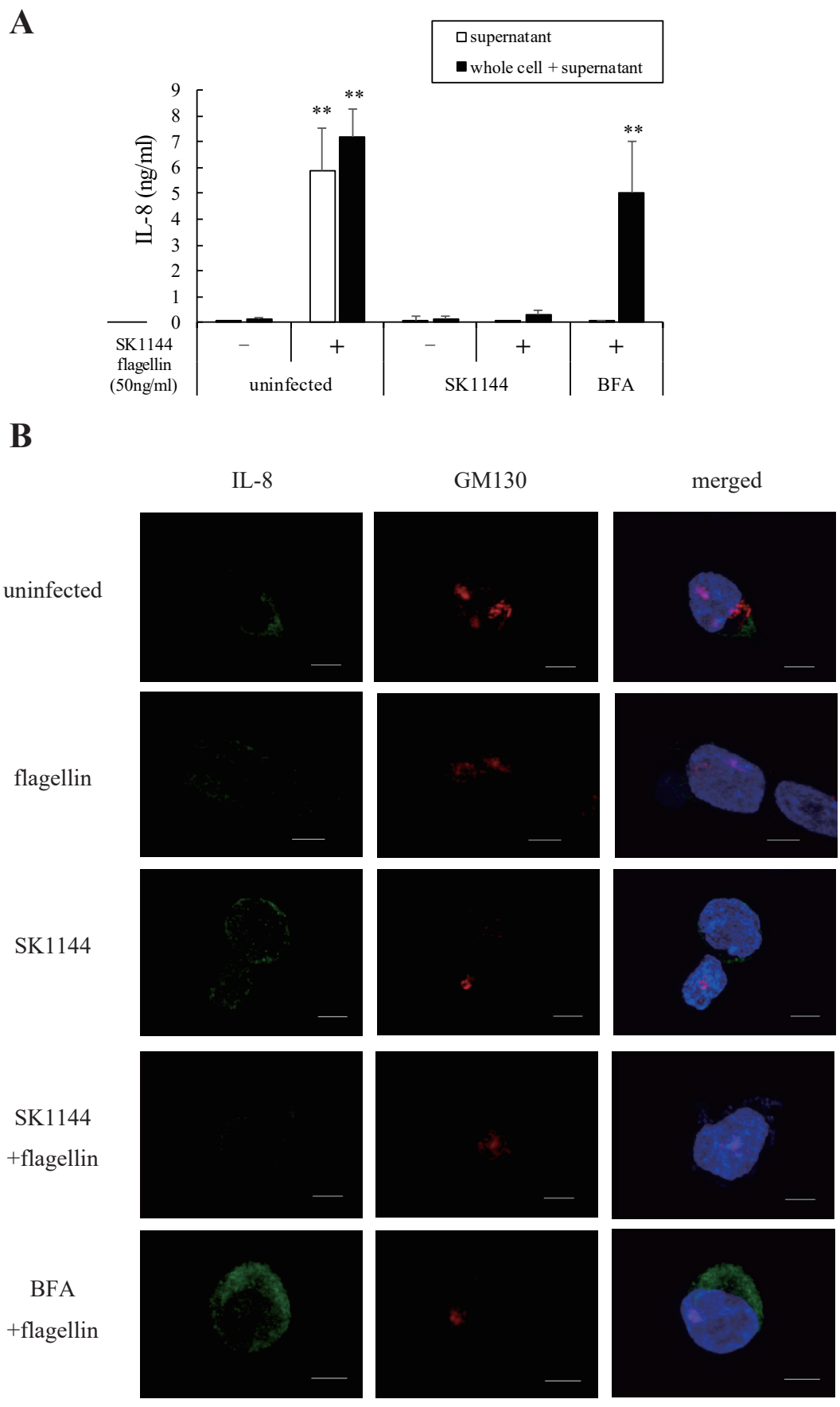

A
supernatant
whole cell + supernatant
IL-8 (ng/ml)
9
8
7
6
5
4
3
2
1
0
**
**
**
SK1144
flagellin
(50ng/ml)
–
+
–
+
+
uninfected
SK1144
BFA
B
IL-8
GM130
merged
uninfected
flagellin
SK1144
SK1144
+flagellin
BFA
+flagellin

Fig 3

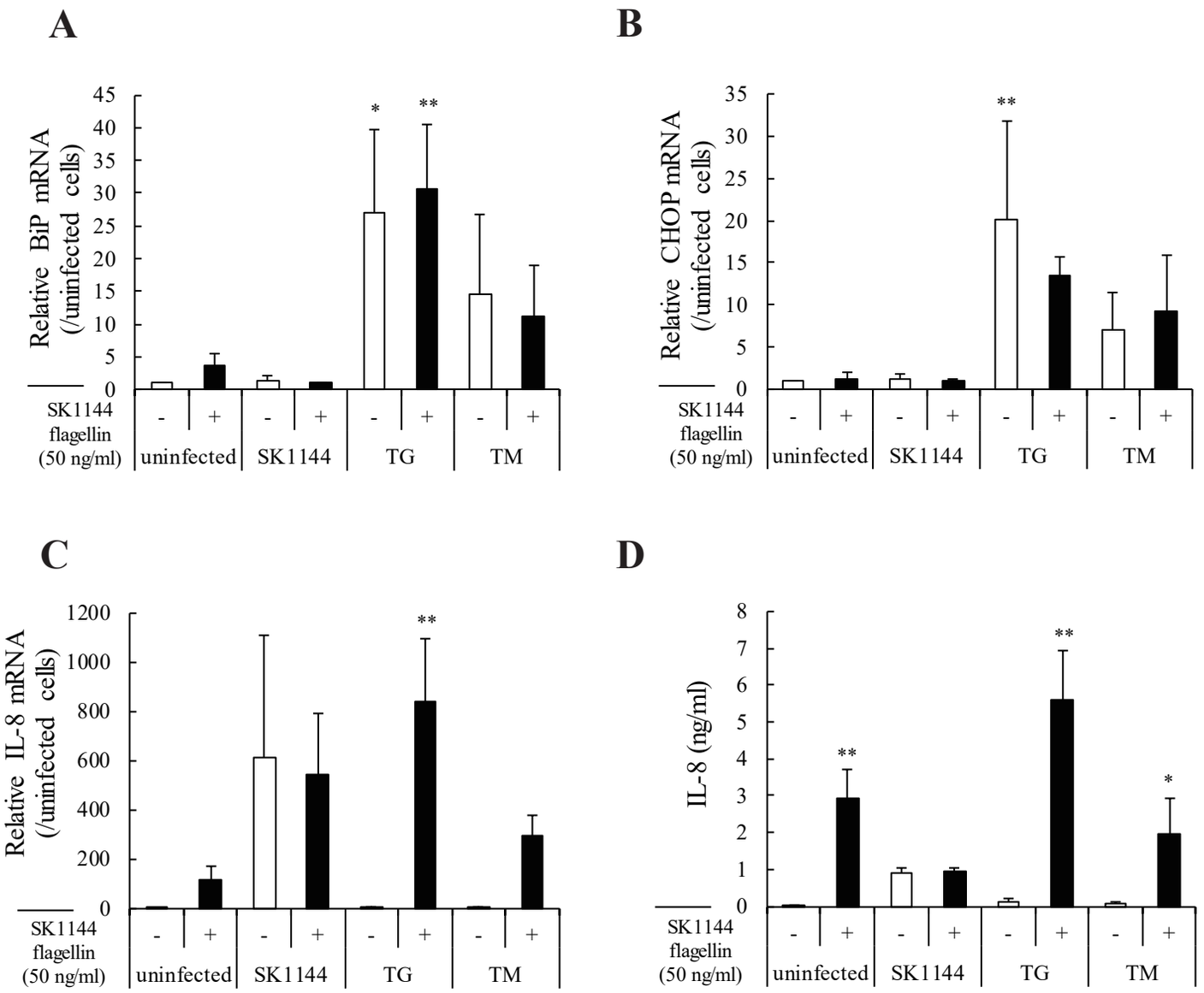

A
Relative BiP mRNA (/uninfected cells)
45
40
35
30
25
20
15
10
5
0
*
**
SK1144
flagellin
(50 ng/ml)
-
+
uninfected
SK1144
TG
TM
B
Relative CHOP mRNA (/uninfected cells)
35
30
25
20
15
10
5
0
**
C
Relative IL-8 mRNA (/uninfected cells)
1200
1000
800
600
400
200
0
**
D
IL-8 (ng/ml)
8
7
6
5
4
3
2
1
0
**
*

Fig 4

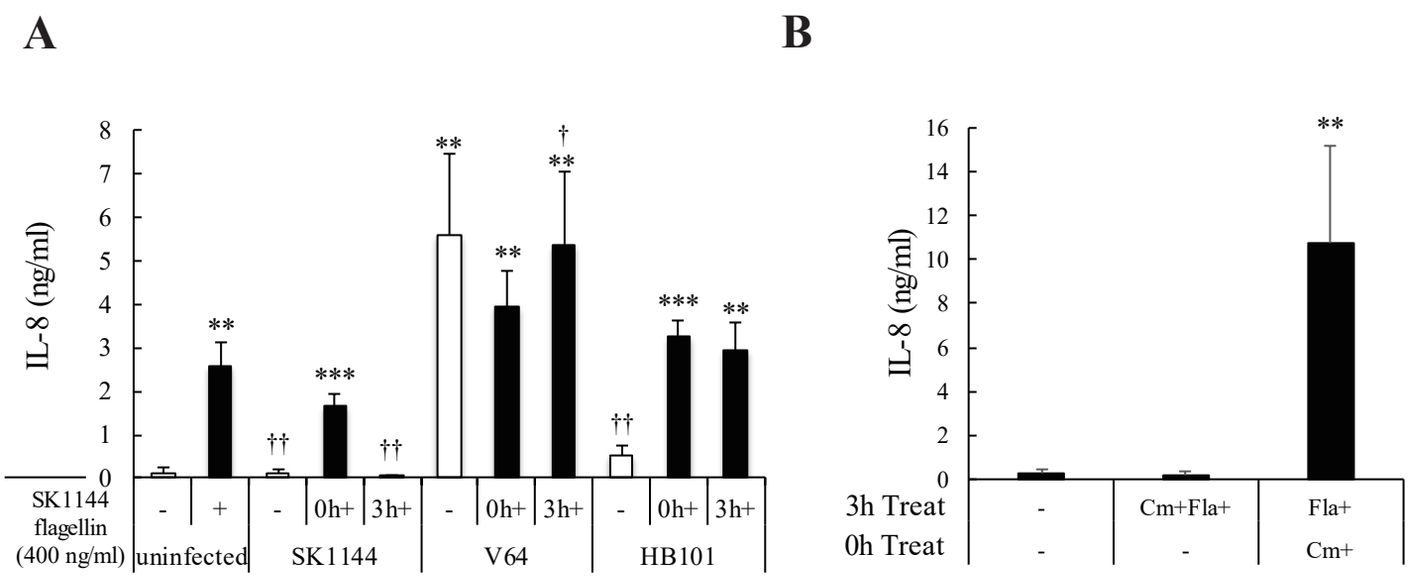

A
B
IL-8 (ng/ml)
SK1144 flagellin (400 ng/ml)
uninfected
SK1144
V64
HB101
0h+
3h+
3h Treat
0h Treat
Cm+Fla+
Fla+
Cm+

Fig 5

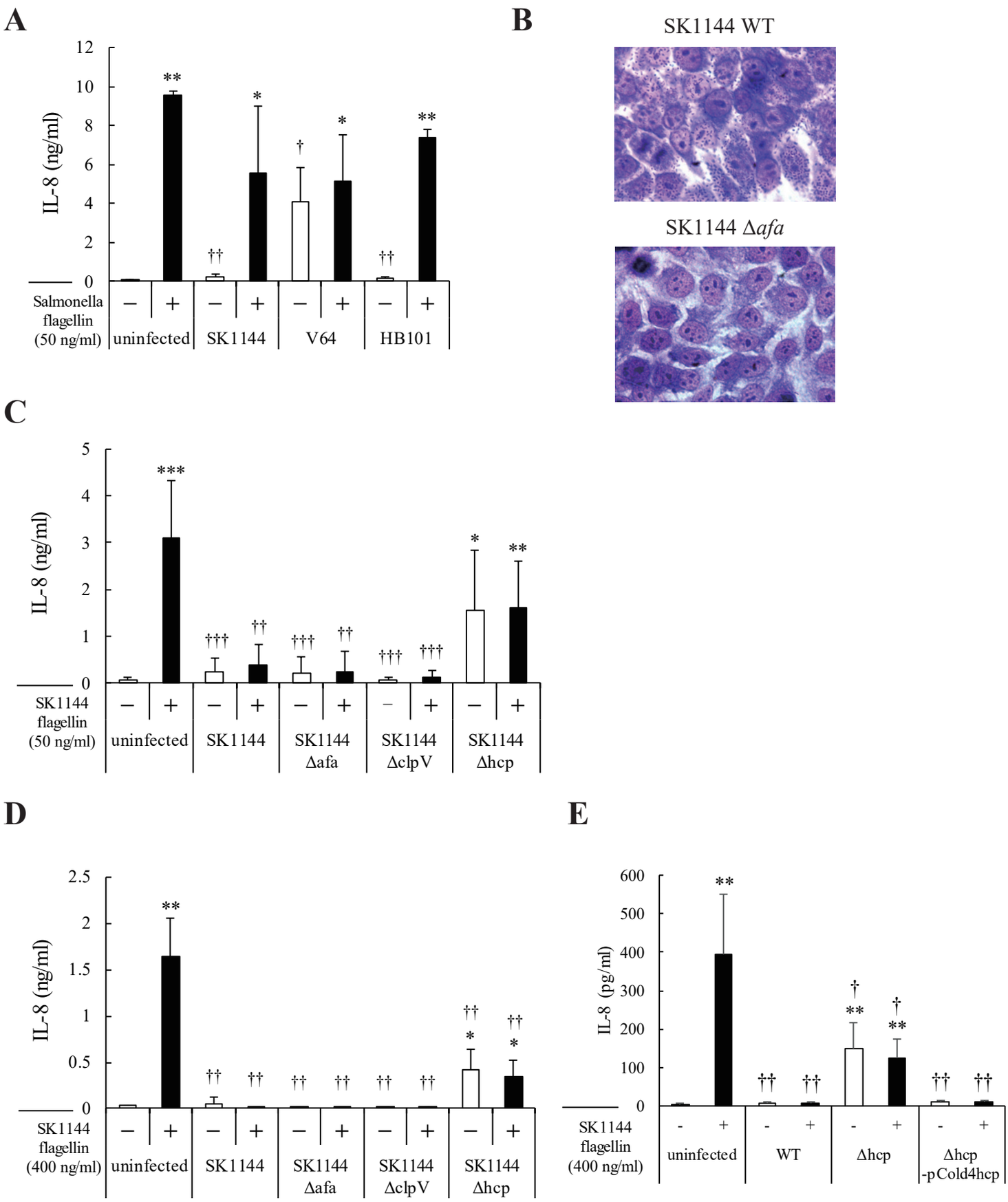

A
IL-8 (ng/ml)
Salmonella flagellin (50 ng/ml)
uninfected
SK1144
V64
HB101
B
SK1144 WT
SK1144 Δafa
C
IL-8 (ng/ml)
SK1144 flagellin (50 ng/ml)
uninfected
SK1144
SK1144 Δafa
SK1144 ΔclpV
SK1144 Δhcp
D
IL-8 (ng/ml)
SK1144 flagellin (400 ng/ml)
uninfected
SK1144
SK1144 Δafa
SK1144 ΔclpV
SK1144 Δhcp
E
IL-8 (pg/ml)
SK1144 flagellin (400 ng/ml)
uninfected
WT
Δhcp
Δhcp -pCold4hcp

Fig 6

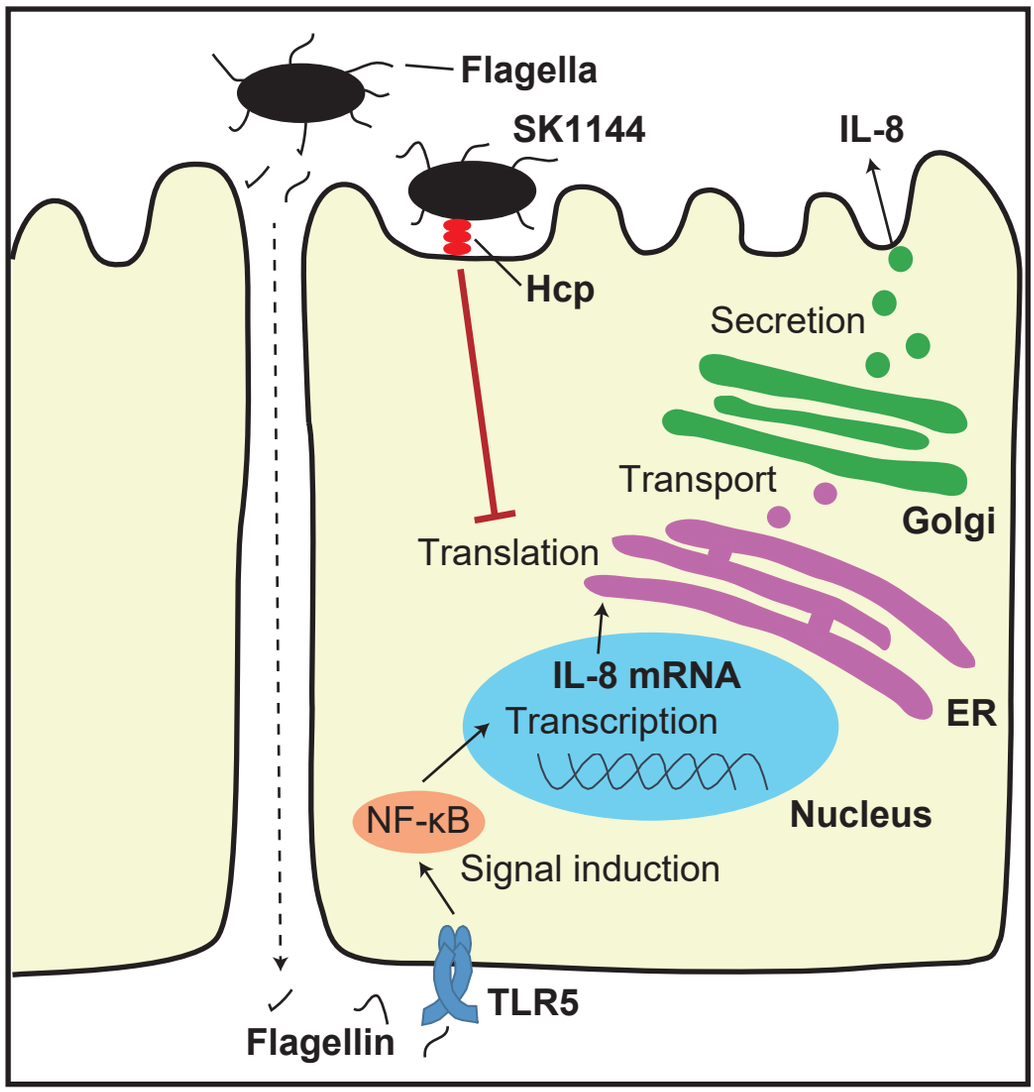

Flagella
SK1144
IL-8
Hcp
Secretion
Transport
Golgi
Translation
IL-8 mRNA
Transcription
ER
Nucleus
NF-κB
Signal induction
TLR5
Flagellin

Fig 7