

Signal transduction in *Campylobacter jejuni*-induced cytokine production

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Summary

***Campylobacter jejuni* is the leading cause of food-borne illness in the USA and one of the most common causes of diarrhoea worldwide. Central to its pathogenicity is its ability to induce the production of proinflammatory cytokines such as interleukin (IL)-8 in intestinal epithelial cells. Here, we demonstrated that *C. jejuni* infection of intestinal epithelial cells results in the activation of the ERK and p38 mitogen-activated protein kinases and that the ERK kinase pathway is essential for IL-8 production. We found that MAP kinase stimulation leading to IL-8 secretion requires *C. jejuni* gene products whose production is stimulated upon contact with epithelial cells. We also found that *C. jejuni* flagellin is a very poor stimulator of Toll-like receptor (TLR)-5 and therefore does not play a significant role in the stimulation of cytokine production.**

Introduction

Bacterial pathogens that have sustained a long-standing association with their hosts have evolved sophisticated mechanisms to subvert host cell processes to initiate disease. Many invasive bacterial pathogens are now known to interact with host cells via intimate biochemical crosstalk, triggering signalling cascades in both the bacterium and the host that ultimately lead to bacterial uptake as well as changes in host cell gene expression. One such bacterium, *Campylobacter jejuni*, is the leading cause of food-borne illness in the USA and one of the most common causes of diarrhoea worldwide (Mead *et al.*, 1999). Enteritis caused by specific serotypes of *C. jejuni* is also an important precondition for the development of Guillain-Barré paralysis (Allos, 1997).

Both *in vivo* and *in vitro* studies have demonstrated that *C. jejuni* both adheres to and invades the cells of the

intestinal epithelium (Newell and Pearson, 1984; De Melo *et al.*, 1989; Konkel and Joens, 1989; Wassenaar *et al.*, 1991; Babakhani *et al.*, 1993; Oelschlaeger *et al.*, 1993). Mutants defective in their ability to be internalized into cultured epithelial cells have reduced virulence in animal models (Yao *et al.*, 1997) suggesting that bacterial internalization is important in campylobacter pathogenesis. While little is known about the mechanism of *C. jejuni* invasion, one general theme that has emerged from studying this process is that motility and chemotaxis are closely linked to bacterial internalization (Wassenaar *et al.*, 1991; Yao *et al.*, 1994; 1997; Szymanski *et al.*, 1995). Strains carrying such mutations are severely impaired in their ability to invade intestinal epithelial cells.

In addition to invasion, infection of cultured intestinal epithelial cells with *C. jejuni* stimulates nuclear responses including the activation of the transcription factor, nuclear factor (NF)- κ B (Mellits *et al.*, 2002). NF- κ B has emerged as a central regulator of the epithelial cell innate immune response to infection by a variety of bacterial pathogens. Upon activation, NF- κ B mediates the production of many of the chemokines, cytokines and innate defence molecules involved in innate immunity to bacterial infections (Hobbie *et al.*, 1997; Savkovic *et al.*, 1997; Elewaut *et al.*, 1999; O'Neil *et al.*, 1999; Philpott *et al.*, 2000). Moreover, it has been demonstrated that *Campylobacter* interaction with cultured intestinal epithelial cells induces the production of the proinflammatory cytokine, interleukin (IL)-8 (Hickey *et al.*, 2000). It is currently unknown which bacterial factors are involved in promoting IL-8 secretion; however, IL-8 and other proinflammatory mediators are thought to be important in initiating the host mucosal inflammatory response, which is critical for both the induction of diarrhoea and the clearance of infection. IL-8 production has been attributed to two general mechanisms, one of which is mediated by attachment/invasion (Hickey *et al.*, 1999) and the other by cytolethal distending toxin (CDT) (Hickey *et al.*, 2000), a *Campylobacter* toxin that causes G2 cell cycle arrest in host cells by inflicting DNA damage (Whitehouse *et al.*, 1998; Lara-Tejero and Galan, 2000).

There are at least two general mechanisms by which bacterial pathogens can stimulate nuclear responses. One is by host cell recognition of microbial components. Higher eukaryotes have evolved a complex means of detecting and responding to microorganisms through the

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innate immune system. At the centre of this mechanism are Toll-like receptors (TLRs), which are differentially expressed on subsets of host cells including phagocytic and epithelial cells, and recognize conserved microbial structures called 'pathogen-associated molecular patterns' (PAMPs) (Means *et al.*, 2000; Akira *et al.*, 2001; Medzhitov, 2001; Schnare *et al.*, 2001). Stimulation of TLRs by their specific PAMP promotes activation of nuclear responses leading to changes in host cell gene expression. TLR-5, for example, which recognizes the bacterial flagellum (Hayashi *et al.*, 2001), is expressed in intestinal epithelial cells (Gewirtz *et al.*, 2001). Studies conducted with *Salmonella* have demonstrated that upon treatment with its flagellin, FliC, cells expressing TLR-5 show increased NF- κ B activation as well as IL-8 production (Eaves-Pyles *et al.*, 2001; Reed *et al.*, 2002). The TLR-mediated detection system is designed to engage a large variety of pathogens with relatively little specificity. However, it is becoming increasingly clear that pathogens have also evolved specific adaptations that can trigger innate immunity outputs such as proinflammatory cytokine production independent of TLRs. An example of these adaptations is the *Salmonella enterica* type III secretion system, which through the delivery of effector proteins into host intestinal epithelial cells can stimulate proinflammatory cytokine production (Hobbie *et al.*, 1997).

It is apparent that a nuclear response leading to IL-8 secretion is initiated upon infection with *C. jejuni* in cultured epithelial cells. However, the signalling mechanism by which *C. jejuni* induces the production of IL-8 is currently unknown. It is also unknown whether *C. jejuni* has evolved specific adaptations, other than conserved PAMPs, to stimulate proinflammatory cytokine production. Central to nuclear response regulation are two members of the mitogen-activated protein (MAP) kinase family, ERK and p38, which are ubiquitous components involved in a variety of signal transduction pathways. These signalling molecules have been implicated in stimulating nuclear responses in a variety of bacterial infection models, including *Salmonella typhimurium* (Tang *et al.*, 1994; Hobbie *et al.*, 1997; Keates *et al.*, 1999). In these studies, we have investigated the signalling mechanisms involved in the production of IL-8 during *C. jejuni* infection.

Results

Interaction of C. jejuni with intestinal epithelial cells leads to MAP kinase activation

Previous studies have shown that infection of cultured intestinal epithelial cells with *C. jejuni* leads to the induction of host cell nuclear responses, including activation of the transcription factor NF- κ B (Mellits *et al.*, 2002) and the production of the proinflammatory cytokine IL-8 (Hickey *et al.*, 1999). While these data suggest that

nuclear response and proinflammatory cytokines play a role during *C. jejuni* infection, the signalling mechanisms by which *C. jejuni* induces the production of IL-8 are unknown. Central to the mechanisms of induction of nuclear responses leading to proinflammatory cytokine production are two members of the MAP kinase family, ERK and p38, which are ubiquitous components of a variety of signal transduction pathways. These signalling molecules have been implicated in stimulating nuclear responses in a variety of bacterial infection models (Tang *et al.*, 1994; Hobbie *et al.*, 1997; Keates *et al.*, 1999), including *S. typhimurium*.

To gain insight into the signalling components leading to nuclear responses induced by *C. jejuni*, we examined ERK and p38 MAP kinase activation in infected cultured human intestinal epithelial T84 cells. Cells were infected with *C. jejuni* and the stimulation of ERK and p38 was assessed at different times during infection by Western immunoblot with antibodies specific to the phosphorylated (activated) form of these kinases. As shown in Fig. 1, infection with *C. jejuni* leads to the activation of both ERK and p38 MAP kinases. Robust but transient activation of ERK by *C. jejuni* occurred at 30 min after infection and then again, although weakly, at 120 min of infection. In contrast, stimulation of p38 was weak and only observed at 120 min of infection. As previously reported, infection with *S. typhimurium* activated both ERK and p38 MAP kinases 30 min after infection (Hobbie *et al.*, 1997). These results demonstrate that *C. jejuni* can activate MAP kinase pathways in cultured intestinal epithelial cells.

Differential contribution of ERK and p38 in C. jejuni-stimulated IL-8 production in intestinal epithelial cells

We next investigated whether the activation of MAP kinases during *C. jejuni* infection contributes to IL-8 production. To this end, we examined IL-8 synthesis during *C. jejuni* infection of cultured intestinal epithelial cells treated with U0126 and SB203580, which are specific inhibitors of MEK1 and p38 MAP kinases respectively. T84 cells were pretreated for 30 min with 10 μ M of each inhibitor, infected with *C. jejuni* for 8 h, and IL-8 secretion was monitored by ELISA. Addition of the MEK1 inhibitor U0126 significantly blocked (\approx 95% inhibition) IL-8 production induced by *C. jejuni* (Fig. 2A). In contrast, the addition of the p38 inhibitor did not affect IL-8 secretion, indicating that ERK (the downstream target of MEK1) but not p38 is required for *C. jejuni*-induced nuclear responses leading to proinflammatory cytokine production.

A previous report indicated that there is a correlation between *C. jejuni* invasion and IL-8 secretion in cultured epithelial cells (Hickey *et al.*, 1999), suggesting the existence of a common signalling pathway for both events. We therefore tested the involvement of ERK and p38 in bac-

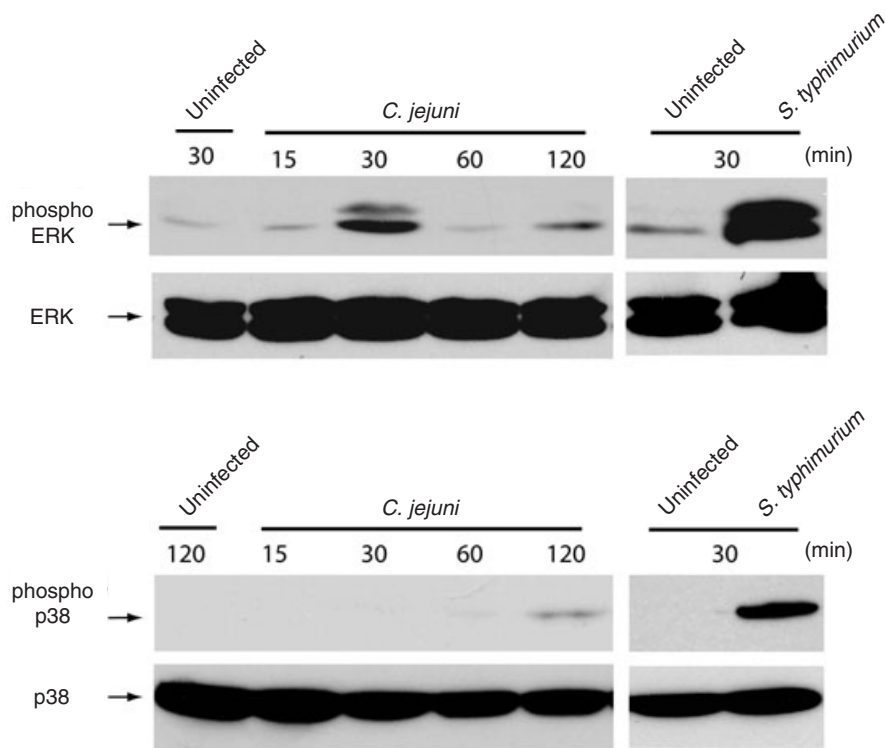


Fig. 1. Activation of MAP kinases during *Campylobacter* infection of intestinal epithelial cells. Human intestinal epithelial T84 cells were infected with *C. jejuni* or *S. typhimurium* for the indicated times. Activation of ERK and p38 were examined by Western immunoblot using monoclonal antibodies specific to the phosphorylated state of each kinase. The total ERK and p38 immunoblots confirm equal protein loading.

terial internalization by the gentamicin protection assay. Addition of the specific inhibitors U0126 or SB203580 did not affect the ability of *C. jejuni* to enter into cultured intestinal cells (Fig. 2B), indicating that neither ERK nor p38 are required for bacterial invasion. Taken together, these results demonstrate that the ERK MAP kinase pathway is required for *C. jejuni*-induced IL-8 production and that p38, although weakly activated by bacterial infection, does not contribute to IL-8 production.

Campylobacter jejuni flaA is defective in its ability to activate ERK in intestinal epithelial cells

The importance of motility in *C. jejuni* pathogenesis is well documented. For example, several reports have shown that *C. jejuni* strains defective in motility have a significant defect in their ability to attach and invade cultured intestinal epithelial cells (Wassenaar *et al.*, 1991; Grant *et al.*, 1993; Yao *et al.*, 1994; Szymanski *et al.*, 1995) as well as colonize the intestine of newborn chicks (Wassenaar *et al.*, 1993; Hendrixson and DiRita, 2004). In addition, non-motile strains of *C. jejuni* have been shown to be impaired in their ability to stimulate IL-8 secretion in cultured epithelial cells (Hickey *et al.*, 1999). We therefore investigated the role of *C. jejuni* motility in the activation of MAP kinases. Human intestinal epithelial T84 cells were

infected with either wild-type *C. jejuni* or its isogenic *flaA* mutant strain. To maximize bacteria–host cell contact, infected cells were centrifuged as indicated in *Experimental procedures*. The activation of the MAP kinases ERK and p38 MAP was monitored at different time points during infection by Western blot analysis using antibodies specific for the activated (phosphorylated) form of the kinases. In contrast to wild type-infected cells, no activation of ERK was observed in cells infected with the *flaA* mutant of *C. jejuni* shortly (15–30 min) after infection (Fig. 3A). However, a weak but reproducible activation of ERK was observed in cells infected with the *flaA* mutant 120 min after infection as observed with wild-type *C. jejuni*. In contrast, the ability of *C. jejuni* to induce weak activation of p38 was not affected by the introduction of the *flaA* mutation. (Fig. 3A). Infection of T84 cells with the *flaA* mutant also produced significantly lower levels of IL-8 compared with wild type (Fig. 3B). Taken together, these results indicate that the *C. jejuni flaA* mutant is defective in its ability to activate ERK MAP kinase leading to IL-8 production in cultured intestinal epithelial cells.

Campylobacter jejuni flagellin is severely impaired in its ability to stimulate nuclear responses

The significantly reduced ability of *C. jejuni flaA* mutant to

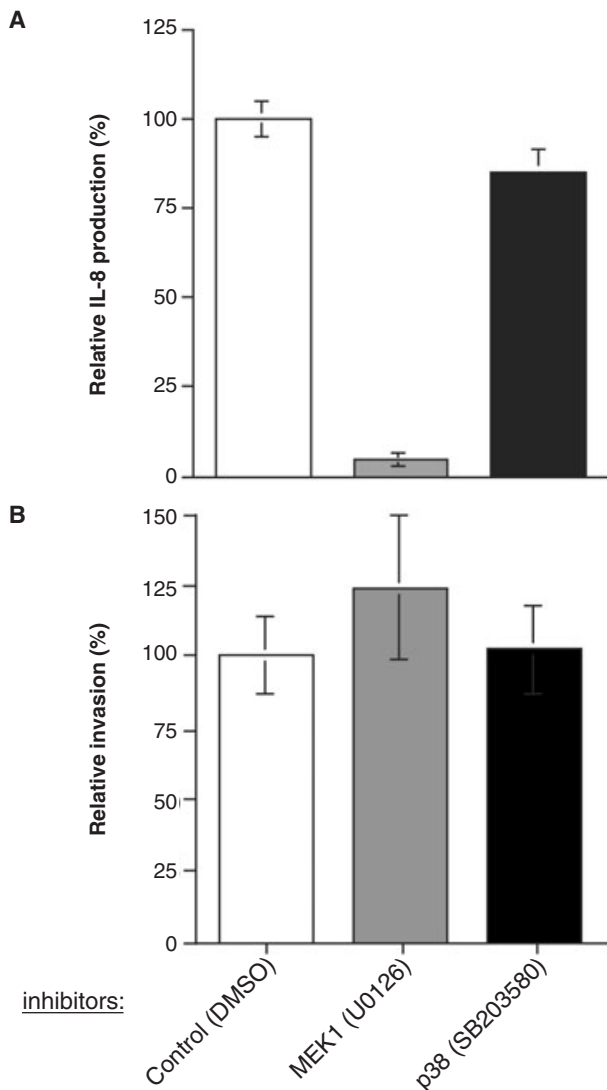


Fig. 2. *Campylobacter jejuni* induced IL-8 production requires ERK MAP kinase pathway.

A. T84 cells were treated with 10 μ M of the inhibitors of MEK1 (U0126) or p38 MAP kinase (SB203580), or the eluent alone (DMSO), 30 min before infection and kept throughout the experiment. IL-8 production was monitored by ELISA in supernatants collected 8 h after infection.

B. Effect of MAP kinase inhibitors on *Campylobacter* entry into host cells. Bacterial internalization was measured after 2 h of infection by the gentamicin protection assay as described in *Experimental procedures*.

All values are the mean and standard deviation of three independent determinations and are expressed as a percentage of the control (DMSO only), which was considered to be 100%. (The actual invasion value of the control was $2.7\% \pm 0.35\%$ of the initial inoculum.)

stimulate ERK activation and proinflammatory cytokine production suggested the possibility that, similar to many other virulence properties of *C. jejuni*, motility *per se* (or an intact flagellar system) may be essential for the stimulation of nuclear responses. Alternatively, as *C. jejuni flaA* does not express flagellin, the defect in the ability of this strain to stimulate ERK activation and proinflamma-

tory cytokine production suggested the possibility that wild-type *C. jejuni* may stimulate these responses through flagellin itself. Flagellins from many bacterial pathogens have been shown to stimulate proinflammatory cytokine production by serving as a ligand for TLR-5 (Hayashi *et al.*, 2001; Berin *et al.*, 2002; Reed *et al.*, 2002; Khan *et al.*, 2004), which is expressed and functional in intestinal epithelial cells (Gewirtz *et al.*, 2001; Bambou *et al.*, 2004). To gain insight into the mechanisms by which *C. jejuni* stimulates nuclear responses in cultured intestinal epithelial cells, we specifically compared the ability of *C. jejuni* flagellin to stimulate the activation of ERK and the generation of nuclear responses leading to cytokine production with that of *S. typhimurium* flagellin, a well-characterized agonist of TLR-5. Polyhistidine-tagged derivatives of both *C. jejuni* (FlaA) and *S. typhimurium* (FliC) flagellins were purified to homogeneity by affinity chromatography as indicated in *Experimental procedures*, and the purity of the preparations verified by SDS-PAGE and Coomassie brilliant blue staining (Fig. 4A). T84 cells were treated with equal concentrations of each purified flagellin preparation and the activation of ERK and p38 MAP kinases was assessed by Western immunoblot analysis. As shown in Fig 4B, *S. typhimurium* FliC stimulated potent p38 MAP kinase activation, although no detectable ERK activation. In contrast, *C. jejuni* FlaA induced very weak p38 activation and no ERK activation. We also tested the ability of *S. typhimurium* FliC and *C. jejuni* FlaA to stimulate the production of IL-8 in culture intestinal cells. Human T84 cells were treated with equal concentrations of FlaA or FliC, and IL-8 levels of the different samples were measured by ELISA. IL-8 production was significantly lower (≈ 10 -fold) in cultured epithelial cells treated with *C. jejuni* FlaA compared with *Salmonella* FliC (Fig. 4C). We then compared the ability of the *C. jejuni* and *S. typhimurium* flagellins (FlaA and FliC respectively) to stimulate cytokine production *in vivo*. To avoid potential background due to the presence of minor traces of lipopolysaccharide (LPS) in the samples of purified recombinant flagellins, we used TLR-4^{-/-} mice. TLR-4^{-/-} mice were injected intraperitoneally with equal amounts of purified flagellins, and 2 h after injection, the levels of tumour necrosis factor alpha (TNF α) and IL-6 were measured by ELISA in the serum of injected animals. Consistent with the *in vitro* experiments, *C. jejuni* FlaA was significantly less able to stimulate cytokine production *in vivo* when compared with the *S. typhimurium* flagellin FliC (Fig. 5). Taken together, these results indicate that *Campylobacter's* flagellin is a poor agonist of TLR-5-mediated nuclear responses leading to proinflammatory cytokine production. A comparison of the primary amino acid sequence of *C. jejuni* FlaA and *S. typhimurium* FliC reveals significant differences (Fig. 6). In particular this analysis reveals that residues known to be essential for

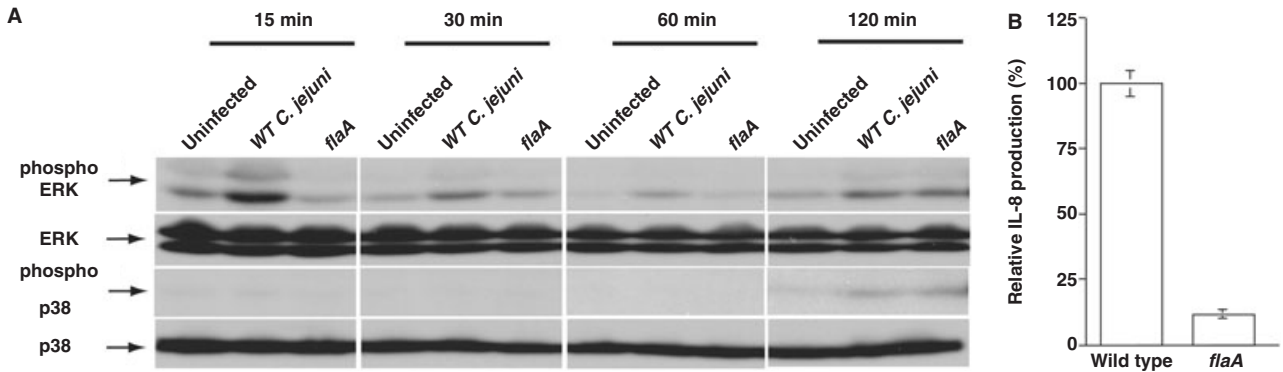


Fig. 3. Role of *C. jejuni* flagella in MAP kinase activation during infection. A. T84 cells were infected wild-type (WT) *C. jejuni* or the *flaA* mutant and activation of ERK and p38 were examined by Western immunoblot as described in the legend for Fig. 1. B. Role of *C. jejuni* flagella in IL-8 secretion during infection. IL-8 production was monitored by ELISA in supernatants collected 8 h after infection. The data represent the mean and standard deviation for three independent determinations and are expressed as a percentage of wild-type above background (the values of uninfected samples were subtracted from each experimental value), which was considered 100%.

the stimulation of TLR-5 by FliC (Mizel *et al.*, 2003; Smith *et al.*, 2003) are not conserved in *C. jejuni* flagellin. These results also indicate that the ability of *C. jejuni* to stimulate proinflammatory cytokine production is the result of a signalling pathway independent of TLR-5. This conclusion is further substantiated by the observation that stimulation of TLR-5 (by the *S. typhimurium* flagellin FliC) leads to the activation of p38 but not ERK. In contrast, *C. jejuni* infection leads to the activation of ERK but very limited activation of p38, which is not required for *C. jejuni*-induced cytokine production. Moreover, the p38 signalling observed during wild-type infection was also observed

during infection with the *flaA* mutant, suggesting that activation of p38 during *C. jejuni* infection is independent of flagellin. As TLR-5 is the only Toll-like receptor thought to be functional in T84 intestinal cells, these results suggest that *C. jejuni* has evolved specific adaptations to stimulate nuclear responses that operate independently of TLRs.

De novo protein synthesis is required for C. jejuni-mediated MAP kinase activation and IL-8 secretion in intestinal epithelial cells

It has been previously reported that *de novo* protein

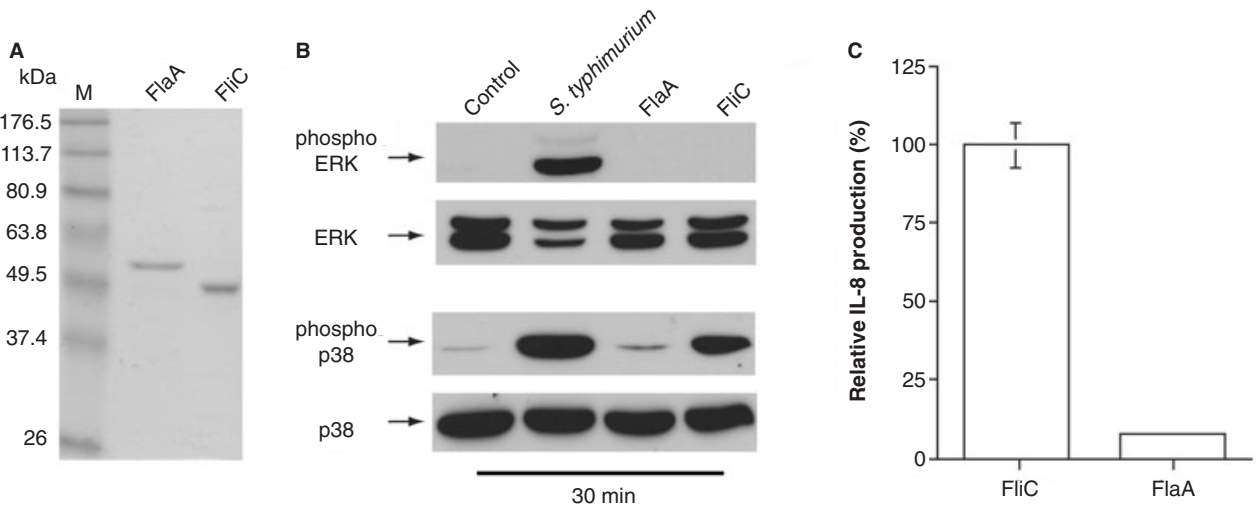


Fig. 4. *Campylobacter jejuni* flagellin has reduced ability to stimulate p38 MAP kinase and IL-8 secretion. A. Recombinant *C. jejuni* and *S. typhimurium* flagellin (FlaA and FliC respectively) were purified by affinity chromatography and quantified. Equivalent amounts were separated by SDS-PAGE, and stained using Coomassie brilliant blue to verify purity. M, molecular weight marker. B. MAP kinase activation in response to purified *C. jejuni* flagellin. Cells were treated with equivalent concentrations ($1 \mu\text{g ml}^{-1}$) of FlaA or FliC for 30 min and the activation of ERK and p38 was examined by Western immunoblot as described in the Fig. 1 legend. C. IL-8 secretion after stimulation of T84 cells with purified *C. jejuni* flagellin. Supernatants were obtained 8 h after addition of FlaA or FliC and IL-8 levels were monitored by ELISA as described for Fig. 3B.

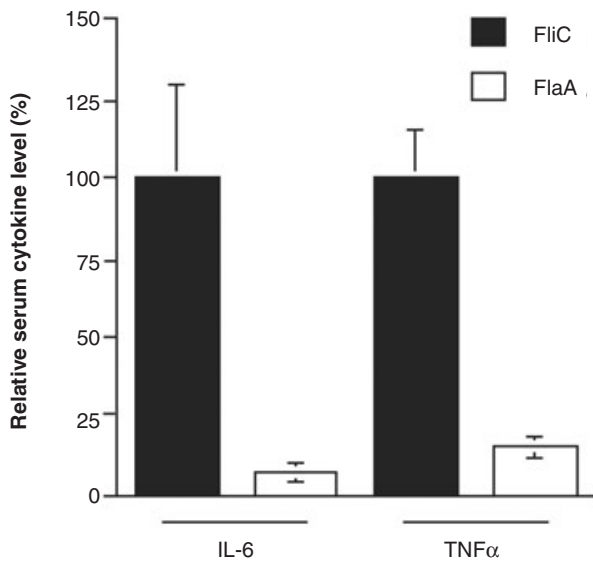


Fig. 5. *Campylobacter jejuni* flagellin has reduced ability to stimulate systemic IL-6 and TNF α production. TLR-4^{-/-} mice were injected with either 15 μ g of FlaA, FliC, or buffer alone and whole-blood cytokine levels were monitored at 2 h after inoculation. The data represent the mean and standard deviation for three mice and are expressed as a percentage of the control (FliC) above background (buffer alone values were subtracted out of each experimental value), which was considered 100%.

synthesis is necessary for internalization of *Campylobacter* into host cells (Oelschlaeger *et al.*, 1993) and that a subset of bacterial proteins necessary for these processes is synthesized upon contact with host cells (Konkel *et al.*, 1990). It is therefore possible that upon intimate contact with host cells *C. jejuni* induces the expression of

gene products responsible for both internalization into host cells and induction of a nuclear response. We therefore tested whether *de novo* protein synthesis is necessary for the stimulation of ERK MAP kinase and IL-8 production. Cultured intestinal T84 cells were infected with wild-type *C. jejuni* and ERK MAP kinase activation and IL-8 secretion were assessed by Western immunoblot and ELISA, respectively, either in the presence or in the absence of chloramphenicol, an antibiotic that specifically blocks bacterial protein synthesis. We found that in the presence of chloramphenicol, the ability of *C. jejuni* to stimulate ERK MAP kinase activation was effectively blocked (Fig. 7A). Consistent with the requirement for the *de novo* protein synthesis for the stimulation of nuclear responses, *C. jejuni*-mediated IL-8 secretion was also significantly impaired in the presence of chloramphenicol (Fig. 7B). These data indicate that bacterial *de novo* protein synthesis is required for ERK MAP kinase activation and IL-8 production during *C. jejuni* infection of intestinal epithelial cells. These results are also consistent with the hypothesis that *C. jejuni* has evolved specific adaptations to stimulate nuclear responses, presumably independently from the conserved pathogen-associated molecular patterns.

Discussion

A central feature of *C. jejuni* pathology is its ability to induce inflammatory diarrhoea. Although very little is known about the mechanisms by which *C. jejuni* induces diarrhoea, it is likely that its ability to stimulate the production of proinflammatory cytokines plays a central role

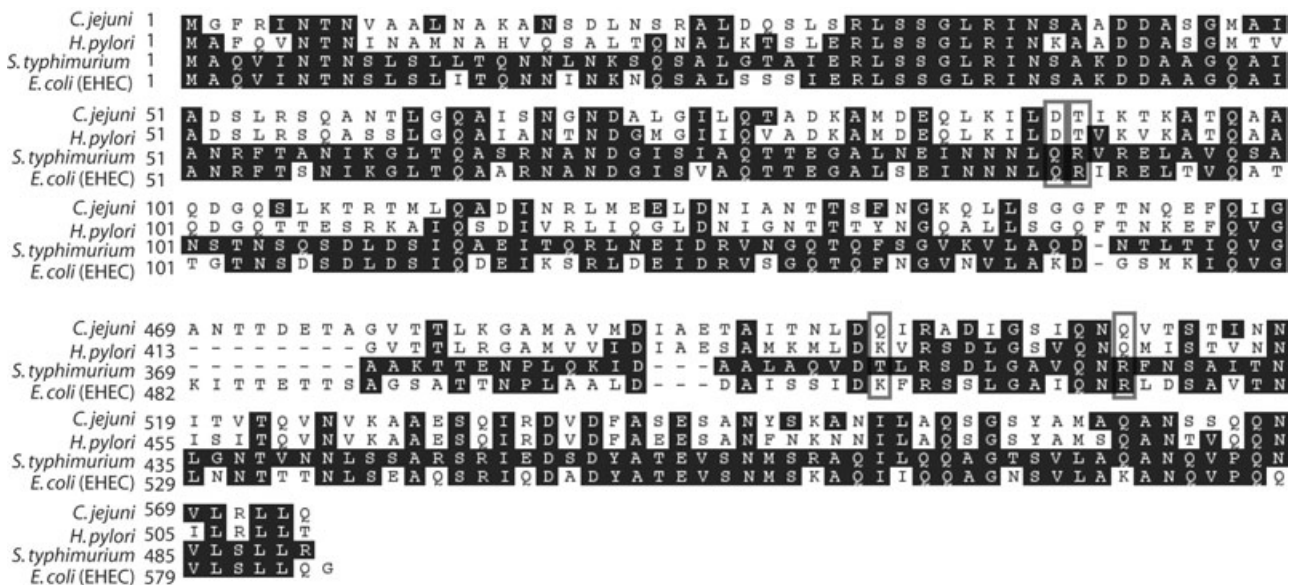


Fig. 6. Flagellin alignment comparing *C. jejuni* FlaA and *S. typhimurium* FliC. Sequences of flagellin proteins were aligned with CLUSTALW (residues identified to be important in TLR-5 recognition of flagellin; Smith *et al.*, 2003).

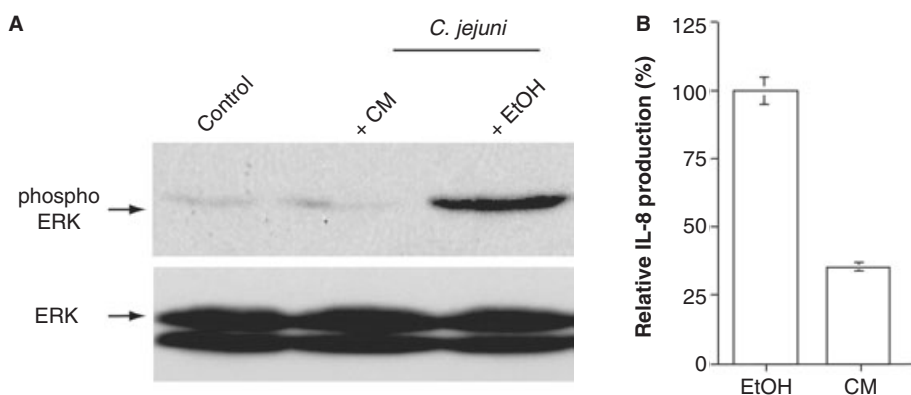


Fig. 7. *Campylobacter jejuni* *de novo* protein synthesis is involved in ERK MAP kinase stimulation and IL-8 secretion. T84 cells were treated with chloramphenicol (CM; 100 $\mu\text{g ml}^{-1}$) or solvent alone (ethanol) and infected with wild-type *C. jejuni*. Activation of ERK was examined by Western immunoblot at 30 min. IL-8 levels were monitored by ELISA in supernatants obtained at 8 h as described for Fig. 3B.

in this process. As one of the earlier barriers to contain bacterial infection, intestinal epithelial cells have the potential to produce a variety of cytokines that serve as 'early warning' signals for the presence of bacterial pathogens and may help to set the stage for the development of the inflammatory reaction that eventually leads to diarrhoea. Central to the signalling cascades leading to proinflammatory cytokine production are the MAP kinases ERK and p38. We found that *C. jejuni* induced a robust activation of ERK shortly (20 min) after infection and a more moderate activation after 2 h of infection. In contrast, *C. jejuni* induced a very modest activation of p38 MAP kinase. Consistent with these findings, inhibition of ERK completely abrogated the ability of *C. jejuni* to stimulate IL-8 production, while inhibition of p38 had no significant effect on this assay. These results indicate that the ERK kinase pathway is central to the *C. jejuni* signal transduction pathways leading to cytokine production.

Microbial pathogens possess specific as well as broad and conserved determinants that are capable of stimulating the production of proinflammatory cytokines. The relative importance of these adaptations in the generation of nuclear responses largely depends on the type of cell encountered by the pathogen. In general, when pathogens interact with cells of the immune system, the stimulation of proinflammatory cytokine production is the consequence of the agonistic activity of conserved determinants, such as LPS, peptidoglycan, and lipoproteins, which serve as stimulants of TLRs expressed in these cells (Takeda *et al.*, 2003). However, cells other than those of the immune system often do not express TLRs or when they do, they express a much narrower array of these receptors. In addition, polarized cells often restrict the expression of TLRs to surfaces that under physiological conditions are not available for interaction with bacterial agonists. We investigated whether the stimulation of proin-

flammatory cytokine production by *C. jejuni* was the consequence of the activity of specific adaptations or the result of stimulation by conserved bacterial determinants collectively known as PAMPs. Intestinal epithelial cells are not thought to significantly express any TLRs other than TLR-5. We therefore investigated whether *C. jejuni* flagellin, FlaA, was responsible for the stimulation of proinflammatory cytokine production as flagellin of many bacteria have been shown to be the agonist of TLR-5 (Hayashi *et al.*, 2001; Donnelly and Steiner, 2002; Reed *et al.*, 2002; Zhang *et al.*, 2003; Khan *et al.*, 2004). We first tested the ability of a *flaA* *C. jejuni* mutant to stimulate ERK kinase activation and IL-8 production. This mutant was defective in its ability to stimulate these responses, in particular shortly after infection. Although these results suggested the involvement of FlaA in the stimulation of signalling event leading to nuclear responses, several studies have shown that absence of motility or a functional flagellum significantly impaired many *C. jejuni* virulence properties (Wassenaar *et al.*, 1991; Grant *et al.*, 1993; Yao *et al.*, 1994; 1997; Szymanski *et al.*, 1995). Consequently, these results could not rule out that the absence of flagella or motility may indirectly affect the ability of *C. jejuni* to stimulate nuclear responses. We therefore investigated the ability of purified *C. jejuni* flagellin to stimulate ERK activation and the production of IL-8. Purified *C. jejuni* FlaA was not able to stimulate ERK or p38 MAP kinase activation nor did it stimulate cytokine production in cultured intestinal epithelial cells or in inoculated mice. In contrast, purified *S. typhimurium* FliC, a known agonist of TLR5, stimulated robust p38 MAP kinase activation and the production of proinflammatory cytokines, both in cultured intestinal cells and in inoculated mice. These results argue that *C. jejuni* flagellin is a poor agonist of TLR-5 and therefore does not play a significant role in the stimulation of proinflammatory cytokine production. This conclusion is supported by the finding that ERK activation plays an

essential role in the signal transduction pathways leading to IL-8 production stimulated by *C. jejuni*, while p38 MAP kinase plays no role in this process and is not significantly activated by *C. jejuni*. In contrast, TLR-5 activation by *S. typhimurium* FliC resulted in robust p38 activation and minimal ERK activation. Consistent with this finding, others have demonstrated that p38 is required for FliC stimulation of proinflammatory cytokine production (Khan *et al.*, 2004). The inability of *C. jejuni* flagellin to stimulate TLR-5-mediated responses is consistent with our observation that it lacks specific residues that have been shown to be essential for the agonistic activity of *S. typhimurium* FliC (Mizel *et al.*, 2003). Interestingly, the *C. jejuni* substitutions of key flagellin residues necessary for TLR-5 stimulation are also present in the flagellin of *Helicobacter pylori*, which some reports have also indicated to be a poor stimulator of TLR-5 (Lee *et al.*, 2003; Gewirtz *et al.*, 2004). It is therefore possible that flagellin from *C. jejuni*, and presumably from other pathogenic bacteria, have evolved to be poor stimulants of the innate immune system.

Our results suggest that, to stimulate signalling pathways leading to cytokine production in intestinal epithelial cells, *C. jejuni* has evolved specific adaptations, presumably different from those involving determinants capable of stimulating TLRs. This is similar to other intestinal pathogens such as *S. enterica*, which have also evolved specific determinants to stimulate proinflammatory cytokine production (Galán, 2001). However, the specific mechanisms must be different since to stimulate these responses *S. enterica* relies on a type III secretion system, which is absent from *C. jejuni*.

Addition of chloramphenicol immediately before infection effectively blocked the ability of *C. jejuni* to stimulate IL-8 production or ERK activation, indicating that *de novo* protein synthesis is required for the stimulation of cytokine production. It is therefore possible that genes that are induced upon contact with cells may be involved in this process. It has been previously shown that addition of chloramphenicol also blocked bacterial internalization into host cells. However, it is not clear whether there are commonalities in the signalling pathways leading to both these responses. The pathways are not entirely overlapping as inhibition of ERK activation did not affect bacterial entry but completely abrogated cytokine production. However, it is still possible that both these processes may have a common upstream triggering event (i.e. stimulation of a surface receptor). Furthermore, it is also possible that bacterial internalization may be a requirement to induce cytokine production through the stimulation of members of the Nod family of intracellular proteins, which are known to be able to respond to bacterial products (Kim *et al.*, 2004). More experiments will be necessary to distinguish among these possibilities.

In summary, we have identified ERK as a central component of the signalling pathways leading to *C. jejuni*-induced proinflammatory cytokine production in intestinal epithelial cells. In addition, we have shown that *C. jejuni* has evolved specific adaptations to stimulate these responses in a manner that is independent of flagellin, and presumably other TLR agonists.

Experimental procedures

Bacterial strains, cell lines and culture conditions

Wild-type *C. jejuni* 81-176 has been described previously (Black *et al.*, 1988). The *flaA* null mutant of 81-176 was constructed by gene replacement strategy. Briefly, *flaA* was polymerase chain reaction (PCR) amplified from *C. jejuni* 81-176 chromosomal DNA and cloned into a suicide vector. A 1.4 kb fragment containing a kanamycin resistance cassette (Km^R) derived from pILL600 (Labigne-Roussel *et al.*, 1987; Yao *et al.*, 1994) was digested and inserted into a unique site within the cloned sequence of *flaA*. The mutant gene was transformed into *C. jejuni* 81-176 as previously described (Wang and Taylor, 1990). The gene replacement insertion mutant was isolated as a non-motile Km^R colony and confirmed by PCR. *C. jejuni* strains were grown routinely on Brucella Broth (BB) agar and in brain heart infusion (BHI) broth under microaerophilic conditions at 37°C. Wild-type *S. typhimurium* strain SB300 has been previously described (Kaniga *et al.*, 1994). *Salmonella* was grown in Luria-Bertani (LB) broth containing 0.3 M NaCl at 37°C. T84 cells, a human intestinal epithelial cell line, were obtained from American Type Culture Collection (Rockville, MD) and were grown in DMEM supplemented with 10% fetal bovine serum containing penicillin (100 U ml⁻¹) and streptomycin (50 µg ml⁻¹).

IL-8 assay

T84 cells were serum starved overnight. Eight hours after infection, culture supernatants were collected and centrifuged for 10 min at 12 000 *g* to pellet residual bacteria and cells. The relative IL-8 levels were determined by human IL-8 ELISA according to the instructions of the manufacturer (BD Biosciences, Pharmingen).

ERK and p38 Western immunoblotting

To examine ERK and p38 activation during *Campylobacter* and *Salmonella* infection or treatment with flagellin, serum-starved T84 cells were infected at a multiplicity of infection (moi) of 100 and 40 for *Campylobacter* and *Salmonella*, respectively, or treated with flagellin (1 µg ml⁻¹) and directly lysed in 1× sample buffer at the indicated times. During infections, plates were centrifuged at 200 *g* for 5 min to maximize bacteria–host cell contact. Equal amounts of cell lysate were separated by SDS-PAGE and the activation of ERK and p38 MAP kinases were analysed by Western immunoblot using monoclonal antibodies specific to the phosphorylated state of each kinase as indicated by the manufacturer (New England Biolabs). To assess the total levels of these kinases in the different samples, blots were stripped and reprobed with antibodies directed against ERK and p38.

Inhibitors

Where indicated, cells were incubated with the specific MEK inhibitor U0126 (10 μ M) or p38 inhibitor SB203580 (10 μ M) (Cell Signaling Technology) dissolved in DMSO (Sigma), or chloramphenicol (100 μ g ml⁻¹) dissolved in ethanol 30 min before infection. Inhibitors were kept throughout the duration of the incubation period. All control cells were treated with the appropriate solvent for the same length of time.

Purification of recombinant *C. jejuni* FlaA and *S. typhimurium* FliC

Vectors expressing polyhistidine-tagged wild-type *C. jejuni* FlaA and *S. typhimurium* FliC were constructed by cloning PCR-amplified *flaA* and *fliC* into the pQE-60 vector (Quiagen). *Escherichia coli* BL21 strains carrying the different resulting plasmids were grown in LB at 37°C for 5 h, IPTG (isopropyl- β -D-thiogalactopyranoside) (10 μ M) was added to induce the expression of FlaA and FliC, and the cells were grown for an additional 3 h at 37°C. Bacterial cells were collected by centrifugation, suspended in lysis buffer [50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazol, 1 mM phenylmethylsulphonyl fluoride (PMSF)], and passed through a French press (1500 lb in⁻²). Lysates were cleared by centrifugation at 20 000 *g* for 20 min. Cleared lysates, which constituted the soluble fraction of recombinant flagellin, were loaded onto a Ni-nitrilotriacetic acid (Ni-NTA) column, and after several washes, FlaA and FliC were eluted with elution buffer (50 mM NaH₂PO₄, 10 mM Tris-HCl, 250 mM imidazol, pH 8.0). The bulk of FlaA protein remained in inclusion bodies in the pellet fraction of the bacterial lysates. For the isolation of FlaA from this fraction, the pellet was suspended in a denaturing lysis buffer (100 mM NaH₂PO₄, 10 mM Tris-HCl, 8 M urea, pH 8.0) and kept on ice for 1 h. After centrifugation to remove the debris, the supernatants were loaded onto a Ni-NTA column equilibrated with the same denaturing lysis buffer. FlaA was then eluted with denaturing elution buffer (100 mM NaH₂PO₄, 10 mM Tris-HCl, 8 M urea, pH 5.9), and refolded by dialysis in a refolding buffer [50 mM Tris-HCl (pH 7.5), 1 M NaCl, 5 mM MgCl₂, 2 mM CHAPS, 5% glycerol]. After centrifugation of the refolded protein sample to remove insoluble FlaA, the fresh flagellin preparations were used immediately in the appropriate assays.

Analysis of systemic responses to flagellin

Age-matched female TLR4^{-/-} mice (C57Bl/6) were injected intraperitoneally with 15 μ g of FlaA, FliC, or buffer alone in 200 μ l of saline (*n* = 3 for each treatment). Blood was sampled at 2 h after injection and IL-6 and TNF α levels were determined by ELISA (BD Biosciences, Pharmingen).

Invasion assay

T84 cells were split to 70% confluence in a 24-well dish. After washing three times with Hank's Balanced Salt Solution (HBSS), the epithelial cells were infected at an moi of 100 with *C. jejuni* grown to an OD₆₀₀ \approx 0.7 in BHI. The plates were centrifuged at 200 *g* for 5 min to maximize bacteria–host cell contact and incubated for 2 h at 37°C in 5% CO₂. Following the incubation, the monolayers were washed three times with HBSS and incubated

with complete media containing gentamicin (100 μ g ml⁻¹) for 2 h to kill extracellular bacteria. After three additional washes, the infected epithelial cells were lysed in PBS with 0.1% deoxycholic acid and the intracellular bacteria were enumerated by plating serial dilutions.

Acknowledgements

We would like to thank Dirk Hofreuter for helpful discussion, Carlos Briones for technical assistance, María Lara-Tejero for construction of the *flaA* mutant, and Olivia Rossanesse and other members of the Galán laboratory for critical review of this manuscript.

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