Campylobacter jejuni Drives MyD88-Independent Interleukin-6 Secretion via Toll-Like Receptor 2[∇]

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Gastrointestinal disease caused by *Campylobacter jejuni* is characterized by localized inflammation and the destruction of the epithelial cell barrier that forms host innate protection against pathogens. This can lead to an imbalance in fluid transport across the gastrointestinal tract, resulting in severe diarrhea. The mechanisms of host cell receptor recognition of *C. jejuni* and downstream immune signaling pathways leading to this inflammatory disease, however, remain unclear. The aim of this study was to analyze the mechanisms involved in *C. jejuni* induction of the acute-phase inflammatory response regulator interleukin-6 (IL-6). Polarized intestinal epithelial Caco-2 monolayers responded to infections with *Salmonella enterica* serovar Typhimurium and eight isolates of *C. jejuni* by an increase in levels of expression and secretion of IL-6. No such IL-6 response, however, was produced upon infection with the human commensal organism *Lactobacillus rhamnosus* GG. The IL-6 signaling pathway was further characterized using short interfering RNA complexes to block gene expression. The inhibition of myeloid differentiation primary response protein 88 (MyD88) expression in this manner did not affect *C. jejuni*-induced IL-6 secretion, suggesting a MyD88-independent route to IL-6 signal transduction in *C. jejuni*-infected human epithelial cells. However, a significant reduction in levels of IL-6 was evident in the absence of Toll-like receptor 2 (TLR-2) expression, implying a requirement for TLR-2 in *C. jejuni* recognition. Caco-2 cells were also treated with heat-inactivated and purified membrane components of *C. jejuni* to isolate the factor responsible for triggering IL-6 signaling. The results demonstrate that *C. jejuni* surface polysaccharides induce IL-6 secretion from intestinal epithelial cells via TLR-2 in a MyD88-independent manner.

Campylobacter species are responsible for the majority of human bacterial gastrointestinal infections and have been associated with the more serious postinfectious sequelae Miller Fisher and Guillain-Barré syndromes (9, 45). Clinical features of campylobacteriosis range from mild, watery diarrhea to severe, bloody diarrhea and can include acute colonic mucosal inflammation (35). It is well established that *Campylobacter* initiates host innate immune responses; however, the mechanisms employed by *Campylobacter* to evoke such variable proinflammatory signals remain to be fully understood (13).

The intestinal epithelium forms a single layer of protection against bacterial pathogens that survive the journey into the human gastrointestinal tract. Currently, 11 members of the Toll-like receptor (TLR) family and two nucleotide-binding oligomerization domain (Nod) family members have been identified as being responsible for microbial recognition (2). These two families of pattern recognition receptors (PRRs) are able to mediate immune responses to infection by distinguishing between different bacterial products known as microbe-associated molecular patterns (10, 33). TLR and Nod receptors are found either on the surface or in the cytosol of both immune and nonclassical immune cells. Cell polarization

and the specific localization of these receptors are thought to be important for the prevention of continual activation by commensal microflora (17). Bacterial lipopolysaccharide (LPS) is one of the most common bacterial triggers of the innate immune system and stimulates cytokine and chemokine secretion via interactions with TLR-2 and TLR-4 (10). The activation of TLRs most commonly leads to signaling through the adaptor molecule myeloid differentiation primary response protein 88 (MyD88) to nuclear factor κ B (NF- κ B) transcriptional regulation of inflammatory cytokines (8). However, the existence of an LPS-inducible pathway independent of MyD88 has intensified research into alternative and complementary adaptor molecules (21, 62, 63). Immunogenic properties of *C. jejuni* capsular polysaccharides (CPS), lipooligosaccharide (LOS), flagella, and N- and O-linked protein glycosylation motifs have been proposed and investigated (18, 19, 30), yet their host cell recognition receptors remain to be identified. Paradoxically, only poor stimulation of the flagellum-associated TLR-5 was observed in *C. jejuni*-infected T84 intestinal epithelia (26, 59); thus, the molecules responsible for host recognition of this flagellated and encapsulated bacterium remain an intriguing unknown. CPS forms the heat-stable antigens used for the Penner serotyping method and has also been implicated as a modulator of host-pathogen interactions and virulence (4, 6). The structures of these carbohydrate modifications have been analyzed in greater detail using nuclear magnetic resonance spectroscopy (36, 40, 41, 54). Not only is there significant variability in CPS structures between strains, but the identification of phase-variable regions within the genetic loci encod-

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ing enzymes involved in CPS biosynthesis likely contributes to the avoidance of host recognition by *C. jejuni* (29, 31, 50).

The inflammatory cytokine interleukin-6 (IL-6) is important for the maintenance of the intestinal epithelium yet is also proinflammatory and plays a critical role in governing the transition from innate to acquired immunity (28). IL-6 is secreted from immune and nonimmune cells upon stimulation with bacterial pathogens. As IL-6 is secreted by and has effects on a wide range of cell types, it is now believed to be important for maintaining the homeostatic balance within tissues (39, 46). IL-6 is also thought to provide a protective effect on epithelial barrier maintenance within the gut (58) yet has been associated with the severity of inflammatory bowel diseases (44).

Although *C. jejuni*-stimulated secretion of the proinflammatory cytokine IL-6 has been reported for monocytic and dendritic cell lines, little is known about the signaling route to this response or whether the IL-6 proinflammatory response can be extended to epithelial cells (22, 27). The goal of this study was to identify the role of the acute-phase response cytokine IL-6 in the primary host response to *C. jejuni* infection.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *C. jejuni* strains used in this study were as follows: NCTC 11828 (81116) (48), 81-176 (5), clinical isolates from the University of Alberta Hospital (23-69, 25-19, 27-52, 25-55, and 26-56), and a chicken isolate from Alberta Agriculture (CJ 758). The Human Ethics Review Board (Biomedical Panel) at the University of Alberta approved the protocol to access the human isolates of *C. jejuni* to do this research. *C. jejuni* strains were grown in gas jars at 37°C under microaerobic conditions (created using Campy-Gen packs [Oxoid, United Kingdom]) overnight on *Brucella* agar or shaking (150 rpm) in *Brucella* broth (BD Biosciences, NJ). *Lactobacillus rhamnosus* GG (ATCC, VA) cells were grown with shaking in MRS broth (BD Biosciences) under microaerobic conditions as described above. *Salmonella enterica* serovar Typhimurium (LT2) cells were grown aerobically in Luria-Bertani broth (BD Biosciences).

siRNA transient transfection. Caco-2 or HEp-2 cells were reverse transfected with short interfering RNA (siRNA) using Lipofectamine 2000 (Invitrogen, ON, Canada) or HiPerFect (Qiagen, ON, Canada) transfection reagent. Lipofectamine 2000 (1.5 µl) was diluted in 25 µl Opti-MEM (Invitrogen) and incubated at room temperature for 10 min. Nontargeted AllStars negative control and MyD88-specific siRNA duplexes (Qiagen) were diluted to 30 nM in Opti-MEM to a 25-µl final volume, mixed with the diluted Lipofectamine 2000, and incubated at room temperature for 10 min. HiPerFect transfection reagent (3 µl) and 5 nM nontargeted AllStars negative control or TLR-2-specific siRNA duplexes (Hs_TLR2_2 [CAGGTAAAGTGGAACGTTAA]; Qiagen) were diluted in 50 µl Opti-MEM and incubated at room temperature for 10 min. The RNA-transfection agent complexes were dispensed into empty 24-well culture plates, and a 450-µl cell suspension at 1×10^5 cells ml⁻¹ (in growth media) was added and mixed gently. The transfection mixture was incubated at 37°C for 20 h before replacing growth medium. Cells were infected after 48 h and assayed for transfection efficiency by real-time PCR.

Epithelial cell infections. All reagents for tissue culture were obtained from Sigma-Aldrich (ON, Canada) unless otherwise stated. Caco-2 and HEp-2 epithelial cells were grown in modified Eagle's medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% fetal bovine serum in 75-cm² tissue culture flasks at 37°C in 5% CO₂. For infection experiments, Caco-2 cells were seeded into 24-well plates at 5×10^4 cells ml⁻¹ and maintained for 14 days until fully confluent and differentiated. HEp-2 cells were seeded into 24-well plates at a density of 2.5×10^5 cells ml⁻¹ and maintained for 48 h before infection. One hour prior to infection, medium was replaced with infection medium (modified Eagle's medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and 1% fetal bovine serum). Cells were infected with 1×10^9 exponentially growing *C. jejuni* cells resuspended in 500 µl infection medium to establish a multiplicity of infection (MOI) of 1,000. This MOI was determined to be optimal for maximum *Campylobacter* invasion and for making strain-to-strain comparisons (15). Phorbol 12-myristate 13-acetate (PMA) (500 ng ml⁻¹), *Escherichia coli* 0111:B4 and 055:B5 LPS (100 ng ml⁻¹), and IL-1β (10 ng ml⁻¹) were used as controls. Mock infections were treated with infection medium alone.

TABLE 1. Oligonucleotide primers used to amplify RNA transcripts

Primer	Sequence
GAPDH-F	GAGTCAACGGATTGGTTCGT
GAPDH-R	GACAAGCTTCCCCTTCTCAG
MyD88-F	TTGAGGAGGATTGCCAAAAG
MyD88-R	CATCTCCTGCACAAACTGGA
IL-6_F	ATGAGGAGACTTGCTGCTGGTG
IL-6_R	CAGGGGTGGTTATTGCATCT
β-Actin_F	CCAGAGCAAGAGAGGTATCC
β-Actin_R	CTGTGGTGGTGAAGCTGTAG

Infections were performed for up to 24 h under cell culture conditions. Cells were washed in phosphate-buffered saline (PBS) before extraction of RNA.

RNA extraction and reverse transcription-PCR analysis. Total RNA was extracted and purified using an RNeasy minicolumn kit (Qiagen) as described by the manufacturers. Total RNA was quantified by spectrophotometry, and up to 1 µg total RNA was reverse transcribed to cDNA at 50°C using 1 µl oligo(dT) (50 mM) primer (Invitrogen), 1 mM deoxynucleotide triphosphates (Fermentas, ON, Canada), and Superscript III reverse transcriptase (Invitrogen) in a 20-µl volume as described by the manufacturer. A total of 2 µl cDNA was routinely amplified using 1.25 µM specific oligonucleotide primer (Integrated DNA Technologies Inc., IL), 1 mM deoxynucleotide triphosphates, and 0.5 U HotStart *Taq* polymerase (Qiagen) in a 50-µl reaction mixture. Primer sequences are listed in Table 1. After 15 min of incubation at 94°C, a two-step amplification protocol was performed with denaturation at 94°C for 1 min and combined annealing and extension at 60°C for 15 s. The reaction was stopped after 27 cycles, and PCR products were analyzed on a 2% agarose gel.

Real-time PCR. Real-time cDNA amplification was performed using the Quantifast SYBR green PCR kit (Qiagen) in a Realplex multicycler (Eppendorf, ON, Canada). cDNA was diluted 1:4 to 1:16 in RNase-free, diethyl pyrocarbonate-treated water, and 2.5 µl was added to 5 µl SYBR green dye and 2.5 µl specific oligonucleotide primer (1.25 µM final concentration) (Integrated DNA Technologies Inc.). A 2-min incubation preceded a two-step protocol, as described above, for 40 cycles. Oligonucleotide primers (Table 1) were designed to cross the gene intron-exon boundaries, thus making them specific for the amplification of RNA transcripts.

IL-6 ELISA. Supernatants from mock-infected and infected Caco-2 cells were harvested, centrifuged at $12,000 \times g$ for 5 min to remove debris, and stored at -80°C until required for further analysis. The IL-6 concentration was assessed using an OptEIA human IL-6 enzyme-linked immunosorbent assay (ELISA) set (BD Biosciences) as described by the manufacturer, with the exception that 0.01 M citrate buffer, freshly supplemented with 0.03% hydrogen peroxide and 1 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS), was used as the substrate solution. Absorbance was read at 405 nm after shaking for 20 min in 100 µl substrate solution.

Crude CPS preparation. CPS extractions were prepared as described previously by Karlyshev et al. (32). Briefly, lawns of 81116 grown overnight were washed once in PBS and incubated at 50°C or 37°C for 1 h or 100°C for 10 min. After centrifugation to remove bacteria, the supernatant was collected, and the polysaccharide concentration was measured using the phenol sulfuric acid method described previously by Dubois et al. (14). Samples were treated with 1 mg ml⁻¹ proteinase K for 1 h at 50°C and then boiled for 20 min to denature any remaining enzyme.

Statistical analyses. Statistical analyses and graphical representation of results were performed using Prism 4 (GraphPad Software Inc., CA). Results are presented as the means $\pm$ standard errors of the means and are representative of three or more biological replicates. Statistical analyses were performed using analysis of variance and paired or unpaired two-tailed *t* tests as appropriate.

RESULTS

***C. jejuni* induces IL-6 release from intestinal epithelial cells.** Elevated levels of IL-6 secretion in response to *Campylobacter jejuni* infection in human promonocytic, monocytic, and dendritic but not epithelial cell lines have been shown previously (20, 22, 27). *C. jejuni*-stimulated IL-6 release from intestinal epithelial cells in this study was both dose and time dependent. Released IL-6 increased proportionally from an MOI of 10 to

FIG. 1. *C. jejuni* induces IL-6 secretion in intestinal epithelial cells. Cells were infected for 24 h before supernatants were harvested. IL-6 concentrations in supernatants harvested from infected cells were measured by ELISA. Data represent the means $\pm$ standard errors of the means of data from at least four experiments. (A) Caco-2 cell infection. (B) HEP-2 cell infection. Values were compared to those for the mock-infected control using a paired *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. PMA was used at 500 ng ml⁻¹; LPS was used at 100 ng ml⁻¹.

1,000 and reached a maximum at 16 h postinfection. Eight *C. jejuni* isolates were tested, and despite differences in dates and origins of isolation, all strains induced a significant increase in levels of IL-6 release from Caco-2 cells compared to mock-infected controls (Fig. 1A). Minimal cytotoxicity of *C. jejuni* to Caco-2 cells was confirmed by measurements of lactate dehydrogenase activity in cell supernatants and cellular uptake of neutral red dye. The gram-negative enteric pathogen *Salmonella* serovar Typhimurium also induced a strong IL-6 response in Caco-2 cells, although no response was elicited by the human commensal bacterium *L. rhamnosus* GG over a 24-h infection period (Fig. 1A). This *C. jejuni*-induced upregulation of IL-6 was reproduced in HEP-2 epithelial cells (Fig. 1B). To support these secretion data, the expression of IL-6 after a 3-h infection period showed upregulation in response to PMA and *C. jejuni* 81116 but not *L. rhamnosus* GG-induced Caco-2 cells (Fig. 2). Data were compared to β -actin expression controls. Subsequent experiments were performed using strain 81116 as it consistently stimulated the highest levels of IL-6 secretion in polarized epithelial cells.

***C. jejuni*-induced secretion and expression of IL-6 are independent of MyD88.** Microbe-associated molecular patterns such as peptidoglycan, LOS, and CPS are identified by PRRs present either intracellularly or imbedded in the cell membrane. The stimulation of PRRs typically leads to NF- κ B transcriptional regulation in either a MyD88-dependent or -independent manner (62). siRNA specific for the adaptor molecule MyD88 was used to investigate the signaling pathway responsible for the intestinal epithelial secretion of IL-6. Data were compared to data for nontargeted negative control siRNA-transfected cells and were presented as the mean percent expression of the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase). An 80% transient knockdown of MyD88 expression was demonstrated by real-time PCR in all experiments ($P < 0.01$), which had no effect on *C. jejuni* 81116-stimulated IL-6 expression or secretion (Fig. 3). As a control, levels of IL-6 expression stimulated by the MyD88-dependent

signal inducer IL-1 β (1) were significantly reduced in MyD88-specific siRNA (siMyD88)-transfected HEP-2 cells (Fig. 3A), confirming the activity of this siRNA construct in its inhibition of the MyD88 signaling pathway. Additionally, it was observed that infection with *C. jejuni* led to a significant ($P < 0.05$) decrease in MyD88 expression levels in both nontargeting negative control-specific siRNA (siNEG)- and siMyD88-transfected Caco-2 cells, implying a more complex role for *C. jejuni* in the modulation of immune responses (Fig. 3B).

FIG. 2. *C. jejuni* induces an increase in levels of IL-6 expression. RNA extracted from Caco-2 cells treated for 3 h was analyzed by reverse transcription-PCR using primers specific to β -actin and IL-6. Products were compared against the GeneRuler 100-bp DNA ladder (Fermentas) using 2% agarose gel electrophoresis. *Lactobacillus*, *L. rhamnosus* GG; RT control, cells treated with PMA but with no reverse transcriptase added. PMA was used at 500 ng ml⁻¹.

FIG. 3. Loss of MyD88 signaling does not affect *C. jejuni*-induced IL-6 production. Cells were reverse transfected with siNEG or siMyD88 for 48 h prior to infection. After a 3-h mock, IL-1 β , or *C. jejuni* 81116 treatment, MyD88 (□) and IL-6 (■) transcripts and IL-6 secretion levels were measured. Gene expression is presented as a percentage of GAPDH housekeeping gene expression levels. Data represent the means $\pm$ standard errors of the means of data from three independent transfection experiments. (A) HEP-2 cell infection. IL-1 β -stimulated expression of IL-6 is reduced in the absence of MyD88 (#, $P < 0.001$), whereas *C. jejuni*-stimulated IL-6 expression is unaffected. *, $P < 0.001$ compared to mock-infected controls. (B) Caco-2 cell infection. MyD88-independent *C. jejuni*-induced IL-6 expression was reproduced in Caco-2 cells. *, $P < 0.05$ compared to mock-infected controls (IL-6); +, $P < 0.05$ compared to mock-infected controls (MyD88). (C) IL-6 secretion by Caco-2 cells measured by ELISA. There was no statistical difference between IL-6 secretion in siNEG- or siMyD88-transfected *C. jejuni*-stimulated cells.

Nonviable bacteria are able to induce IL-6 secretion from Caco-2 cells. In order to identify which bacterial component was responsible for the MyD88-independent cytokine response, we sought to establish whether viable *C. jejuni* cells were required to initiate IL-6 secretion. Under normal conditions, viable bacteria could be recovered from Caco-2 cells infected with live *C. jejuni* 81116 cells for 3 and 24 h. Heat inactivation of *C. jejuni* 81116 (by boiling for 10 min) or sonication for 5 min (in 10-s bursts on ice) prior to infection failed to reduce IL-6 secretion levels (Fig. 4). Plate counts of sonicated or heat-inactivated preparations confirmed the loss of viability, and centrifugation at this stage to remove cell debris prior to the addition to Caco-2 cells also failed to reduce IL-6 secretion levels (data not shown). The high (or elevated) levels of IL-6 secretion maintained with heat-inactivated or sonicated *C. jejuni* cells suggest that viable bacteria were not required and that the stimulating factor was heat stable (Fig. 4). The treatment of *C. jejuni* with chloramphenicol (30 μ g ml⁻¹) for 1 h prior to (and during) infection also had no effect on IL-6

FIG. 4. Heat-inactivated 81116 stimulates elevated levels of IL-6 secretion. Caco-2 cells were treated with boiled preparations of *C. jejuni* 81116, and released IL-6 was measured by ELISA. Data represent the means $\pm$ standard errors of the means for at least three experiments. ***, $P < 0.0001$. HI, heat inactivated.

FIG. 5. *C. jejuni* polysaccharides induce IL-6 secretion in Caco-2 cells. Polysaccharides were extracted from *C. jejuni* cells at different temperatures as described in Materials and Methods. IL-6 concentrations in supernatants harvested from polysaccharide-treated cells were measured by ELISA. *, $P < 0.05$; ***, $P < 0.0001$. (Inset) Sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis of extracted polysaccharides compared against the PageRuler prestained protein ladder (Fermentas). Lane 1, 100°C treatment, Alcian blue stain; lane 2, 50°C treatment, Alcian blue stain; lane 3, 37°C treatment, Alcian blue stain. Lanes 4 to 6 are stained with silver. s/n, supernatant.

secretion, confirming that the synthesis of new proteins was also not required (data not shown).

***C. jejuni* surface polysaccharides induce IL-6.** To investigate the IL-6-stimulating factor present in heat-inactivated *C. jejuni* cells, strain 81116 was incubated at 100°C, 50°C, and 37°C in PBS for 1 h to extract polysaccharides, and proteins were subsequently removed by proteinase K treatment (see Materials and Methods). Supernatants devoid of bacterial contamination were run on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel stained specifically for polysaccharides using silver and Alcian blue (Fig. 5, inset). Samples incubated at 50°C showed a mid-low-range, 20-kDa band representative of CPS (Alcian blue staining) and a smaller (approximately 10-kDa) band possibly corresponding to LOS (silver- and Alcian blue-stained gels). These bands were either markedly reduced or absent in the samples treated at 37°C. Levels of IL-6 secretion from Caco-2 cells treated with the above-described supernatants were also compared and showed an association between polysaccharide concentration and stimulated IL-6 released from Caco-2 cells (Fig. 5). The stimulating factor present in supernatants was thus thermostable and resistant to proteinase K treatment, confirming that the stimulation of IL-6 secretion from Caco-2 cells was most likely polysaccharide driven. According to studies carried out by Karlyshev et al. (32), *C. jejuni* cells shed a lipid-free form of CPS preferentially at 50°C, suggesting that the IL-6-stimulating factor in our studies is most likely CPS. However, possible contamination with the higher- M_r LPS-associated polysaccharide B was described previously for this method of preparation (36).

FIG. 6. *C. jejuni* activates TLR-2 recognition in Caco-2 cells. Caco-2 cells were reverse transfected with siNEG or TLR-2-specific siRNA (siTLR-2) for 48 h prior to infection. RNA was extracted after a 3-h mock or *C. jejuni* 81116 treatment, and levels of IL-6 RNA transcripts were compared to those of GAPDH and are presented as the change in expression. Data represent the means $\pm$ standard errors of the means of three independent transfection experiments. ***, $P < 0.001$ compared to the respective siNEG controls.

***C. jejuni* recognition is dependent on TLR-2.** TLR-2 recognition of lipoproteins and polysaccharides and its location on the epithelial cell surface made this receptor a logical choice for investigations into *C. jejuni* recognition. The transfection of Caco-2 cells with siRNA specific to the PRR TLR-2 caused a reduction in TLR-2 expression by greater than 80%, confirming the efficient knockdown of the gene (data not shown). TLR-2-specific siRNA-transfected cells showed a 60% reduction in IL-6 expression levels at 3 h after 81116 infection compared to siNEG-transfected cells (Fig. 6). These data demonstrate an important role for the epithelial cell PRR TLR-2 in the recognition and signaling responses to *C. jejuni* infections.

DISCUSSION

The mechanisms and consequences of the host immune response to *C. jejuni* infection remain unclear, particularly with respect to its role in the development of inflammatory disease. It is likely that *C. jejuni*'s invasive nature and disruption of epithelial barrier integrity combined with the stimulation of host proinflammatory cytokines and defensins play a composite role in gastroenteritis (11, 15, 37, 64). However, our understanding of the sequence of events leading to postinfectious sequelae (for example, reactive arthritis and irritable bowel syndrome), now more frequently being associated with the bacterium (38, 51), is minimal. This study identifies and characterizes an IL-6-mediated proinflammatory pathway induced by *C. jejuni* infections, which has shown significant links to the development of autoimmune and inflammatory diseases.

Human intestinal epithelial cell transcriptional upregulation and secretion of antimicrobials (β -defensin) and chemokines (IL-8, monocyte chemoattractant protein 1, and macrophage inflammatory protein 1 β) have been proposed to play a role in *C. jejuni*-mediated intestinal inflammation (7, 23, 26, 64). Currently, it is believed that *C. jejuni* stimulates innate immune responses through the activation of NF- κ B signaling pathways via the mitogen-activated protein kinase family (11, 26, 42, 59). More recent evidence implicates the cytosolic Nod1 receptor

in the host cell recognition of *C. jejuni* cell components and IL-8 signaling (65). The marked upregulation of IL-6 secretion from intestinal epithelial cells in response to *C. jejuni* described here has not previously been reported. The IL-6 response was variable between strains, and data showed no correlation with the ability of *C. jejuni* to invade Caco-2 cells (M. Keelan, unpublished data). All of the strains tested showed statistically significant increases in levels of secreted IL-6 compared to mock-infected controls despite differences in isolation dates and origins (Fig. 1). Although *C. jejuni* 81116 stimulated the highest levels of IL-6 in Caco-2 cells, strain 23-69 was the highest-level stimulator in HEp-2 cells (Fig. 1B). These differences may be due to the unpolarized nature of HEp-2 cells and the comparative localization of TLRs available for bacterial recognition. *C. jejuni* variability within polysaccharide biosynthesis regions may also account for this variation (41, 50). Exponentially growing bacteria were routinely inoculated at an MOI of 1,000; thus, infections were all performed at equivalent and optimal bacterial densities (15). Consistent with previous work, our study showed significant IL-6 release induced by pathogenic *Salmonella* serovar Typhimurium but not by the commensal bacterium *L. rhamnosus* GG (57). This gives further credence to the *C. jejuni*-induced IL-6 response as a proinflammatory response to infection as opposed to a nonspecific homeostatic signaling mechanism (52). Performing both pro- and anti-inflammatory functions, IL-6 forms part of the acute-phase immune response by regulating the transition from innate to acquired immunity. Among its numerous biological activities, IL-6 is important for neutrophil clearance and apoptosis from sites of infection or inflammation while also promoting the recruitment and activation of lymphocytes, characteristics of acquired immunity (28). IL-6 pathway knock-in mutants and mouse models of induced colitis have shown how inappropriate IL-6 activation can lead to T-cell resistance to apoptosis and, similarly, that IL-6 deficiency disrupts the coordinated control of T-cell trafficking (3, 39, 55). It was previously suggested that the homeostatic balance of IL-6-activated signaling pathways is vital for immune regulation (55). In support of this, under physiological conditions, IL-6 has been described to be a mediator of epithelial barrier protection by its regulation of colonic keratin expression (58). In contrast, increased IL-6 serum levels showed a strong correlation with severity of chronic diseases including Crohn's disease and rheumatoid arthritis (44). The subsequent inhibition of IL-6 signaling (using antibodies to the IL-6 receptor) has displayed therapeutic benefits for chronic inflammatory bowel disease (25) and for preventing joint destruction in rheumatoid arthritis (47).

In our study, IL-6 secretion was induced after *C. jejuni* activation of the PRR TLR-2 yet independently of the common TLR adapter molecule MyD88. Interestingly, the recent development of a mouse model of *C. jejuni* colonization was based on the finding that MyD88 was essential for the eradication of *C. jejuni* infection in mice. The authors of that study also showed that the *C. jejuni*-induced secretion of IL-6 from macrophages was dependent on the presence of MyD88 (60). That study, together with our work, highlights the potential differences in signaling processes between cell types. The extent to which human epithelial cell responses to infection are governed by the adaptor protein MyD88 deserves further investi-

gation. The discovery of the additional adaptor proteins Toll-IL-1 receptor (TIR) domain-containing adaptor-inducing IFN- β (TRIF) and TIR domain-containing adaptor protein (TIRAP) has increased the number of regulatory networks connecting proteins of the innate immune signaling genetic map (8). The recognition of *C. jejuni* and epithelial cell signaling to IL-6 in a manner independent of MyD88 may indicate the involvement of TRIF or TIRAP (62, 63). Although the ability of epithelial cells to secrete cytokines upon the recognition of pathogens at the front line of the host's defenses is now widely accepted, these pathways deserve more-detailed investigations with nonimmune cells. Our findings that *C. jejuni* 81116 infection led to a slight but significant decrease in MyD88 expression levels (Fig. 3B) ($P < 0.05$) provide evidence to suggest that *C. jejuni* may employ a mechanism to avoid the host innate immune system. By reducing MyD88-driven signaling responses, *C. jejuni* may function to subvert the host response and potentially protect the bacterium from cell-mediated killing. Although all TLRs signal through MyD88, previous studies have shown that TLR-2, TLR-3, and TLR-4, in addition to Nod receptors, also activate an inflammatory response independent of MyD88 (12, 21, 24, 62). Intracellular Nod1 was also previously identified as an epithelial cell receptor for *C. jejuni* (65). By blocking TLR-2 expression using transfected siRNA, we have shown a significant reduction in levels of *C. jejuni* 81116-stimulated IL-6 expression. This requirement for the cell surface-exposed TLR-2 in IL-6 production supports evidence that nonviable *C. jejuni* and components thereof can function as immune stimulants independent of bacterial internalization.

The low level of IL-6 secreted by Caco-2 cells stimulated with *E. coli* LPS was unexpected, yet this was consistent up to nonphysiological concentrations of $1 \mu\text{g ml}^{-1}$ in both Caco-2 and HEp-2 cells and was reproduced using LPS from two different *E. coli* strains. Previous studies describing LPS induction of IL-6 either used different cell lines or showed low maximal stimulated levels (equivalent to mock-infected controls in our study) (49, 61). As IL-6 could be stimulated by PMA, it is likely that the LPS-induced signaling pathway is restricted or redundant in these cell lines. There is evidence to suggest that exposure of TLRs on the intestinal epithelium is restricted to prevent continual activation by the commensal flora (16, 34). Specifically, mature Caco-2 cells displayed low-level TLR expression and showed no stimulated upregulation of the LPS PRRs TLR-2 and TLR-4 (16). Further analysis of the antigenic component of 81116 required for IL-6 stimulation led us toward the isolation of surface polysaccharides from this strain. Our isolation method was based on that used previously by Karlyshev et al. (32) to preferentially purify the CPS membrane component, which was identified as a mid-range M_r band (20 to 30 kDa) by Alcian blue staining. However, contamination of this preparation with the higher- M_r LPS-associated polysaccharide B described previously by Kilcoyne et al. cannot be discounted and may also account for the higher-level IL-6 stimulation by the samples treated at 100°C (Fig. 5) (36). Heat inactivation of *C. jejuni* by boiling will release polysaccharide antigens into the supernatant; thus, a greater concentration of shed polysaccharides may have caused the observed increased IL-6 release from Caco-2 cells. Analysis of polysaccharide concentrations in these preparations suggests that tem-

peratures below 50°C are suboptimal for CPS shedding but remain quantifiable. The Penner serotype (based on the mid-range- M_r CPS) differs between the *C. jejuni* strains tested in this study (Keelan, unpublished), and multiple mechanisms of structural variability within the *C. jejuni* CPS were previously reported (29). These potentially variable levels of CPS and associated modifications may reflect the resulting variation in the IL-6 response from intestinal epithelial cells. Previous analyses of *C. jejuni* polysaccharides, and the lack of LPS-stimulated IL-6 secretion in these cell lines, support *C. jejuni* CPS as being responsible for IL-6 stimulation. However, further analysis of this CPS preparation is needed to confirm the absence of contaminating LOS or LPS.

It is well established that *Campylobacter* cells are able to invade and translocate intestinal epithelial layers both in vivo and in vitro (43, 56). Subsequently, it was proposed that the penetration of the host's primary innate response leads to a disruption of gastrointestinal equilibrium and fluid loss, presenting the main symptoms of campylobacteriosis. The work discussed here presents a novel, enterocyte-driven, MyD88-independent induction of IL-6 by *C. jejuni* through TLR-2. This is likely responsible for gastrointestinal inflammation through *C. jejuni* CPS-induced dysregulated IL-6 overexpression and a consequent breakdown in epithelial barrier homeostasis. In accordance with these data, the role of IL-6 and intestinal permeability in the association of *Campylobacter* with postinfectious irritable bowel syndrome warrants further investigation (38, 53).

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