MAJOR ARTICLE

Host Attachment, Invasion, and Stimulation of Proinflammatory Cytokines by *Campylobacter concisus* and Other Non–*Campylobacter jejuni Campylobacter* Species

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Background. *Campylobacter concisus* and other non–*Campylobacter jejuni Campylobacter* species have been implicated in the initiation of gastrointestinal diseases. In the present study, we investigated the interaction between these bacteria and the human intestinal epithelium and immune cells.

Methods. The ability of *C. concisus, Campylobacter showae, Campylobacter hominis,* and *Bacteroides ureolyticus* to invade epithelial cells was examined using scanning electron microscopy and gentamicin protection assays. Proinflammatory cytokines generated by epithelial and immune cells in response to these bacteria were determined by enzyme-linked immunosorbent assay. Ussing Chamber, immunofluorescent stain, and Western blot were used to further elucidate the impact of *C. concisus* on intestinal barrier integrity and functions.

Results. Attachment of non–*C. jejuni Campylobacter* species to Caco-2 or HT-29 cells was mediated by flagellum-dependent and/or -independent processes. *C. concisus* was able to invade Caco-2 cells, generate a membrane-ruffling effect on the epithelial surface on entry, and damage epithelial barrier functions by preferential attachment to the cell-cell junctions. Proinflammatory cytokine profiles exhibited by epithelial cells, monocytes, and macrophages in response to *C. concisus* and other non–*C. jejuni Campylobacter* species were species and strain specific.

Conclusions. These findings demonstrate that *C. concisus* and other non–*C. jejuni Campylobacter* species may play a role in initiating gastrointestinal diseases.

Members of the *Campylobacter* genus are gram-negative spiral, curved, or rod-shaped organisms that usually inhabit the gastrointestinal tract of humans and animals. In recent years, an increasing number of *Campylobacter* species other than *Campylobacter jejuni* have been recognized as important human and veterinary pathogens; many of these have been implicated as causative agents of gastroenteritis of unknown etiology [1, 2]. *Campylobacter* species other than *C. jejuni* and *Campylobacter coli* are relatively fastidious and require specific hydrogen-enriched microaerobic environments for growth. Such conditions are not frequently used in routine clinical laboratories, which has led to their infrequent isolation and, thus, underestimation of their pathogenic potential [3, 4].

Very recently, we found an association between *Campylobacter concisus* and a number of other relatively unrecognized *Campylobacter* species and children with newly diagnosed Crohn's disease [5, 6], a chronic and debilitating disease of the gastrointestinal tract with an unknown etiology. After our initial findings, Lastovica [7] reported the isolation of *C. concisus* from children and adults with Crohn's disease. Early molecular studies

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have shown that *C. concisus* and other non–*C. jejuni Campylobacter* species may harbor a toxin similar to the cytolethal distending toxin [8, 9]. Furthermore, *C. concisus* isolates have been shown to secrete hemolysins, which affect human and animal red blood cells [10]. Specific strains of *C. concisus* may colonize the ileum, jejunum, and liver of wild-type BALB/cA mice and induce diarrhea, weight loss, and occasional liver microabscess formation [11]. The mechanism of interaction between *C. concisus* and human intestinal epithelial cells remains unknown.

Currently, none of the frequently detected *Campylobacter* species in children with newly diagnosed Crohn's disease is well-characterized, including *Campylobacter showae, Campylobacter hominis,* and *Bacteroides ureolyticus,* which is a misclassified *Campylobacter* species [12, 13]. Although the pathogenesis of *C. showae* is completely elusive, a case report documented isolation of *C. hominis* in a blood sample from a septicemic patient [14]. *B. ureolyticus* may cause a loss of ciliary activity and sloughing of epithelial cells lining human fallopian tubes and bovine oviduct organ cultures [15]. Clearly, our knowledge of the repertoire of pathogenic mechanisms used by these non–*C. jejuni Campylobacter* species to cause disease in humans is limited. In the present study, we investigated the interaction between *C. concisus* and other non–*C. jejuni Campylobacter* species and human intestinal epithelial cells in vitro, including their ability to attach, invade, compromise intestinal barrier integrity, and induce proinflammatory cytokine production.

MATERIALS AND METHODS

Bacterial species and strains. The following bacterial species and strains were used: *C. concisus* UNSWCD, *C. concisus* ATCC51561, *C. concisus* ATCC51562, *C. concisus* UNSWCS, *C. showae* UNSWCD, *C. hominis* UNSWCD, *B. ureolyticus* UNSWCD, *Salmonella* Typhimurium LT2, and *Escherichia coli* K-12 (UNSW Culture Collection).

Scanning electron microscopy. Human intestinal epithelial cell lines Caco-2 or HT-29 were grown on glass cover slips in 24-well plates at a concentration of 5×10^5 cells per well for 48 h. Cells were then infected with bacteria (multiplicity of infection [MOI], 200) for 6 h, washed with phosphate-buffered saline, and fixed overnight with 2% glutaraldehyde and 2.5% paraformaldehyde in 0.1 mol/L phosphate buffer (pH, 7.2). After dehydration in ethanol, monolayers were subjected to critical point drying with use of a critical point dryer (CPD 030; BAL-TEC), mounted on carbon tabs, and gold-coated in an Emitech K-550X Sputter Coater (Emitech). Samples were visualized on a Hitachi S3400 Scanning Electron Microscope (Hitachi).

Gentamicin protection assays. Caco-2 monolayers were infected with bacteria (MOI, 200) for 6 h, washed, and treated with 200 μg/mL of gentamicin (Gibco) for 1 h. Monolayers were lysed in 1% Triton X-100 and plated on suitable media. Colony-forming units were counted on plates after incubation for 2 days at 37°C. Time course experiments (30 min to 6 h) were also conducted for *C. concisus* UNSWCD. To determine the effect of pre-existing inflammation or cytoskeletal inhibitors on bacterial invasion, Caco-2 cells grown as described were treated with 40 ng/mL of tumor necrosis factor (TNF)–α or interferon (IFN)–γ, 2 μmol/L of cytochalasin D, or 10 μmol/L of colchicine (Sigma) for 1 h before the assay.

Transepithelial electrical resistance (TER) and the Ussing Chamber. Caco-2 cells were grown on 0.4-μm snap-well polycarbonate membrane supports (Corning). When TER reached a stable plateau, the monolayers were apically infected with *C. concisus* UNSWCD or *E. coli* K-12 (MOI, 200) and incubated for 6 h. Monolayers were washed with phosphate-buffered saline, and TER was measured using an EVOM and ENDOHM-24SNAP. Short-circuit current was measured using the Ussing Chamber (World Precision Instruments). To measure macromolecular permeability across the monolayer, horseradish peroxidase (HRP; Sigma) was added to the apical chamber to a final concentration of 1×10^{-5} mol/L at the baseline time. After 1 h, 500-μL aliquots were collected from the basal compartments, and HRP activity was estimated using an assay described elsewhere [16].

Immunofluorescent staining and confocal laser scanning microscopy. Caco-2 cells were grown on membrane supports and infected with *C. concisus* UNSWCD, as described above. Tight junctions were labeled with 10 μg/mL of anti–ZO-1 mouse monoclonal antibodies (Invitrogen), and *C. concisus* UNSWCD was labeled with rabbit anti–*C. concisus* serum (1:40) for 1.5 h. Goat anti-mouse secondary antibodies (Invitrogen) conjugated with Alexa Fluor 594 (5 μg/mL) and anti-rabbit secondary antibodies Alexa Fluor 488 (5 μg/mL) were then added and visualized using an inverted Olympus FluoView FV1000 Confocal Laser Scanning Microscope (Olympus).

One-dimensional polyacrylamide gel electrophoresis and Western blotting. Proteins were extracted using the method of Wroblewski et al [17], separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels, and transferred to methanol-treated polyvinylidine difluoride membranes with use of the Trans-blot cell transfer system (Bio-Rad). Membranes were probed in accordance with the Immun-Star WesternC Kit protocol (Bio-Rad). Membranes were immunolabeled with mouse monoclonal antibodies against ZO-1 (1:50), occludin (1:200), or β-actin (1:1000) (Santa Cruz). Goat anti-mouse IgG antibodies coupled to HRP (1:2000; Bio-Rad) were used as a secondary antibody.

Enzyme-linked immunosorbent assay. The level of interleukin (IL)–8 secreted in the supernatant by HT-29 cells was measured using the Quantikine Human CXCL8/IL-8 Kit ac-

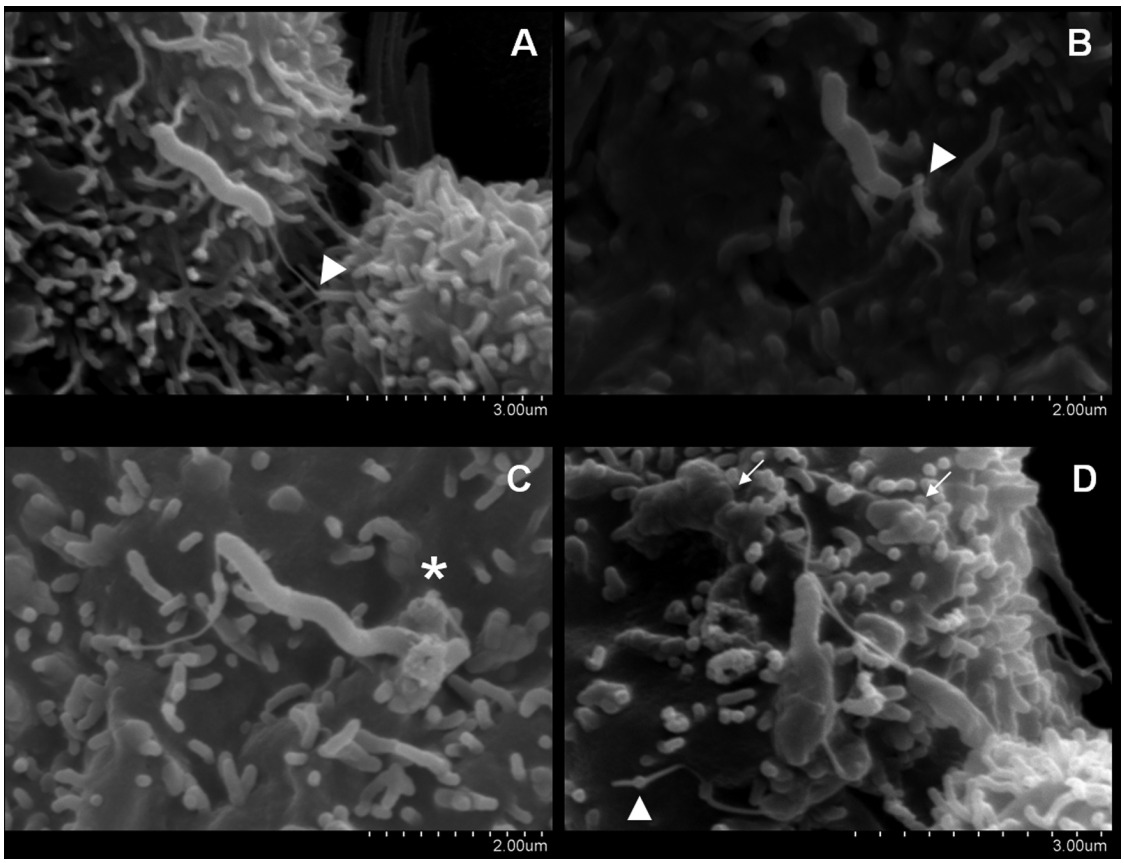

Figure 1. Host attachment and invasion by *Campylobacter concisus* UNSWCD. *A* and *D,* In Caco-2 cells, the polar flagellum of *C. concisus* UNSWCD mediated attachment to the microvillus tip (*triangles*). *B,* The flagellum appears to fold around the microvillus (*triangle*). *C, C. concisus* induced a membrane ruffling–like effect (*). *D, C. concisus* is observed half internalized in the host cell, resulting in a surface protrusion on the host cell membrane, and the flagellated half remains externally exposed. A host cell infected with multiple bacteria displays cell membrane irregularities and uneven texture because of bacteria-induced protrusions (*arrows*).

cording to the manufacturer's instructions (R&D Systems). The levels of IL-1β, IL-8, and TNF-α secreted in the supernatant by human monocytic leukemia cell line (THP-1) or primary macrophages were measured using enzyme-linked immunosorbent assay kits from BioSource.

Statistical analysis. The difference between 2 groups was analyzed using a paired Student's *t* test or 1-way analysis of variance for groups of $\geq$3, with all values corrected using the Tukey multiple comparisons test or the Dunnett multiple comparisons test (GraphPad Software). $P < .05$ was considered to indicate statistical significance.

RESULTS

Attachment and invasion by* C. concisus *and other non–*C. jejuni Campylobacter *species. Scanning electron microscopy revealed that the *C. concisus* UNSWCD polar flagellum mediated initial contact with host cells via a flagellum-microvilli interaction. The flagellum was observed to attach to $\geq$1 microvillus (Figure 1*A* and 1*C*) and to wrap around a microvillus (Figure 1*B*), allowing the bacterium to anchor to the intestinal epithelial cell. After initial attachment, *C. concisus* UNSWCD penetrated the host cell membrane from the nonflagellated end, inducing a membrane ruffling–like effect on the host cell membrane (Figure 1*C*). Invasion appeared to proceed from the nonflagellated end, inducing a protrusion on the surface of the host cell membrane (Figure 1*D*).

As observed in Caco-2 cells, *C. concisus* UNSWCD also appeared to use its polar flagellum to facilitate attachment to HT-29 cells (Figure 2*F*). In addition, the nonflagellated end of *C. concisus* UNSWCD mediated attachment by a mechanism resembling a sticky end to attract nearby microvilli (Figure 2*F*). *C. concisus* UNSWCD appeared to preferentially attach to areas resembling intercellular junctional spaces (Figure 2*A* and 2*B*). This spatial distribution was augmented in the presence of TNF-α (Figure 2*C*–2*E*).

Investigation of *C. concisus* ATCC 51562, *C. showae*, *C. hominis*, and *B. ureolyticus* attachment to Caco-2 or HT-29 intestinal epithelial cells showed that *C. concisus* ATCC 51562 and *C.*

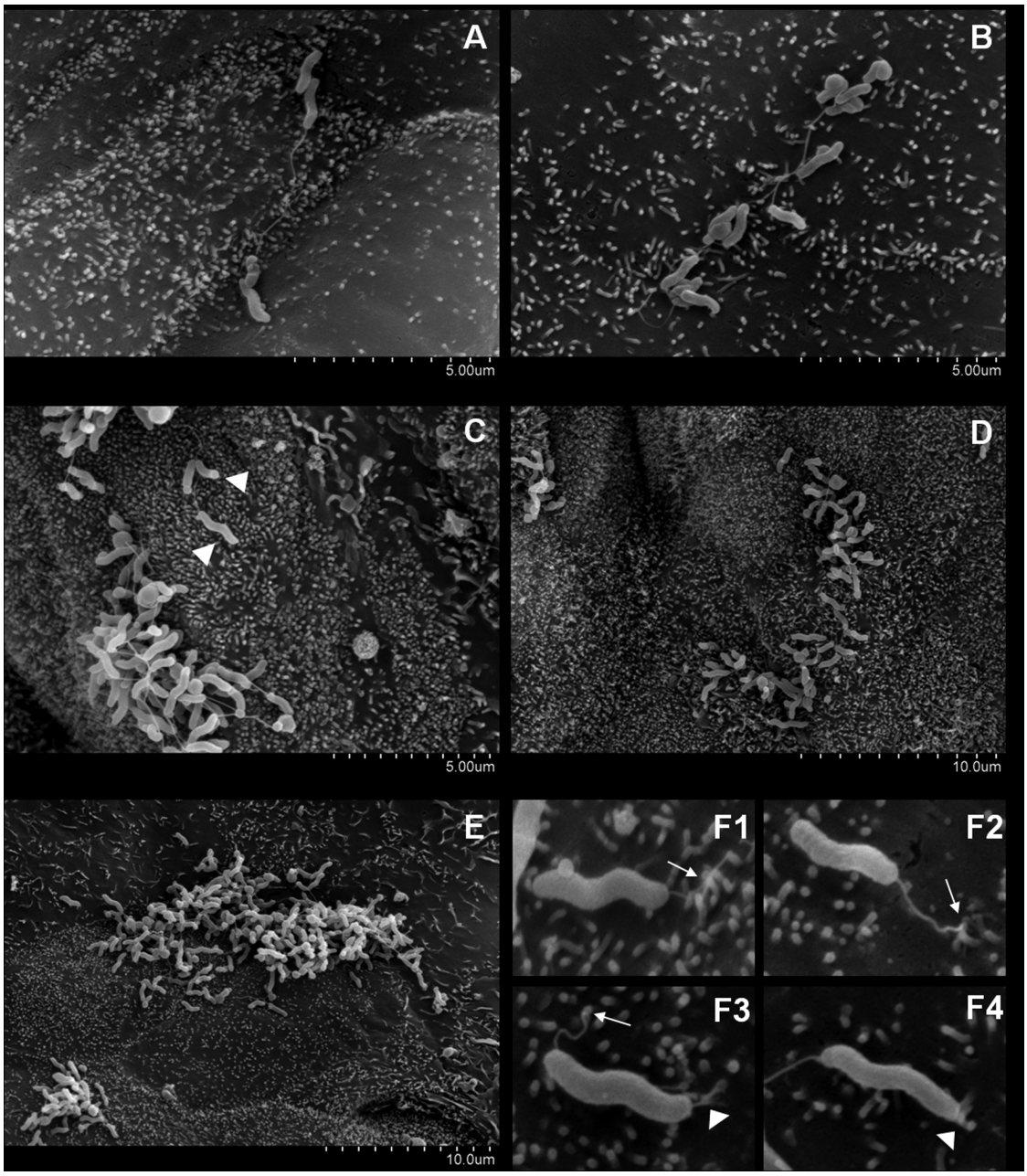

Figure 2. Preferential attachment of *Campylobacter concisus* UNSWCD to intercellular junctional spaces of HT-29 cells. *A* and *B, C. concisus* UNSWCD preferentially attached to areas resembling the intercellular junctional space. *F,* The process of attachment was mediated by the polar flagellum (*arrows*). Bacterial attachment was also mediated by the aflagellate end (*triangles*). *C–E,* When HT-29 cells were pretreated with tumor necrosis factor–α before infection, a higher density of bacterial attachment was observed, especially in areas resembling intercellular junctional spaces, but also at apical surfaces (*C, triangles*).

showae used flagellum-mediated attachment to microvilli. Similar to *C. concisus* UNSWCD, *C. concisus* ATCC 51562 (Figure 3*D*) and *C. showae* (Figure 3*B*) used their polar flagellum to adhere intimately to the microvilli. The nonflagellated end of *C. concisus* ATCC 51562 and *C. showae* also facilitated attachment by attracting neighboring microvilli on the bacterial surface with use of a sticky end mechanism (Figure 3*A*, 3*B*, and 3*D*). In Caco-2 and HT-29 cell lines, minimal attachment was observed for the aflagellate *B. ureolyticus* and *C. hominis*. Where attachment was observed, they also appeared to use a flagellum-independent mechanism of attachment (Figure 3*C*, 3*E*, and 3*F*).

Quantification of bacterial invasion levels. The ability of *C. concisus, C. showae, C. hominis,* and *B. ureolyticus* to invade Caco-2 cells at an MOI of 200 was further evaluated and quan-

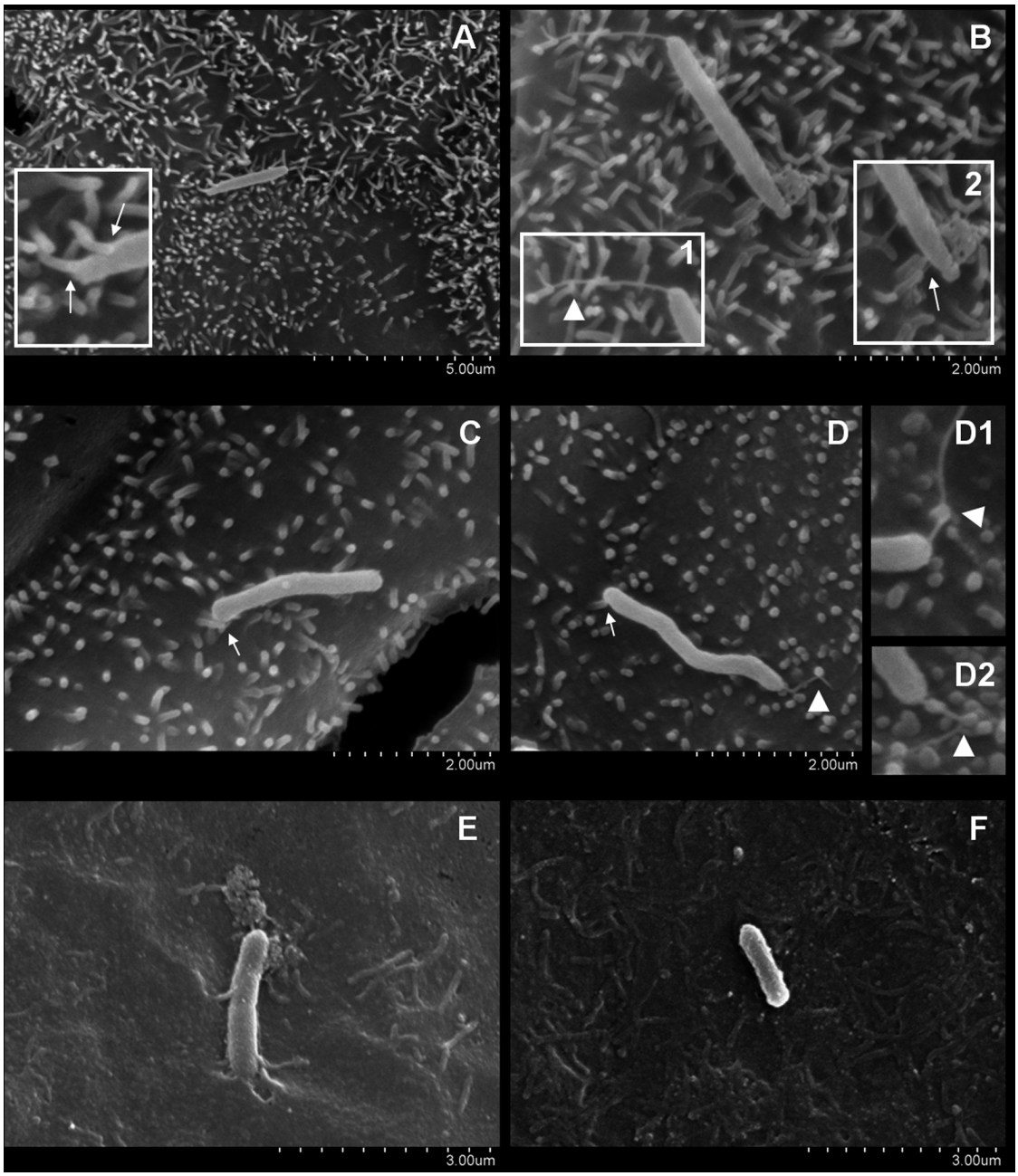

Figure 3. Attachment to HT-29 or Caco-2 cells by non–*Campylobacter jejuni Campylobacter* species, mediated by processes dependent or independent of a polar flagellum. Similar to *Campylobacter concisus* UNSWCD strains, *Campylobacter showae* (*B*) and *C. concisus* ATCC 51562 (*D*) used their polar flagellum to adhere intimately to the microvilli (*triangles*). *A, B,* and *D,* The nonflagellated end of *C. showae* (*A* and *B*) and *C. concisus* ATCC 51562 (*D*) also facilitated in the attachment process by attracting neighboring microvilli to the bacterial surface (sticky end mechanism; *arrows*). Aflagellate *Campylobacter* species also used a flagellum-independent mechanism of attachment to HT-29 cells (*C, Bacteroides ureolyticus; arrow* indicates sticky end) or Caco-2 cells (*E, B. ureolyticus*; *F, Campylobacter hominis*).

tified using gentamicin protection assays. Of the *Campylobacter* species examined, *C. concisus* UNSWCD was the most efficient in invading Caco-2 cells. The mean percentage (± standard error of the mean [SEM]) of invasion by *C. concisus* UNSWCD was 0.14% ± 0.04%. Less than 0.01% viable intracellular *C. concisus* ATCC 51562 and UNSWCS was recovered from Caco-2 cells. The level of invasion observed in *C. concisus* UNSWCD was 46 and 201 times higher than that of *C. concisus* UNSWCS and *C. concisus* ATCC 51562, respectively. *C. concisus* ATCC 51561, *C. showae, C. hominis,* and *B. ureolyticus* did not invade Caco-2 cells, consistent with our observations obtained using scanning electron microscopy. The positive control *S.* Typhi-

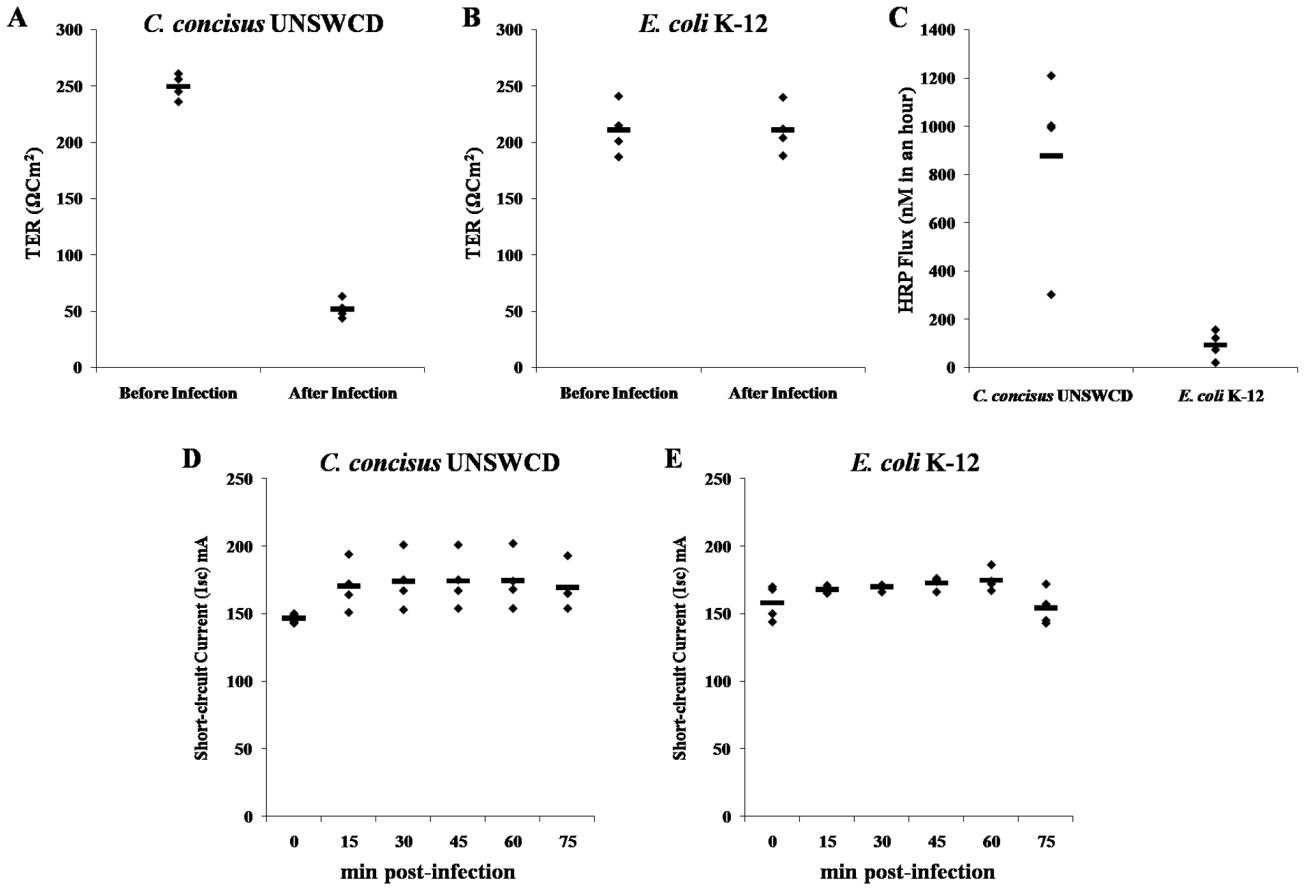

Figure 4. Increased barrier permeability but not ion transportation in Caco-2 cells after infection with *Campylobacter concisus* UNSWCD. After infection with *C. concisus* UNSWCD, the transepithelial electrical resistance (TER) of the Caco-2 monolayer decreased significantly (*A*), whereas no change in TER was recorded in Caco-2 monolayers infected with *Escherichia coli* K-12 (*B*). Movement of horseradish peroxidase (HRP) from the apical to basal compartment was measured (*C*), and a significantly higher flow-through was detected in Caco-2 monolayers infected with *C. concisus* UNSWCD, compared with *E. coli* K-12 ($P = .029$). No statistically significant difference was observed in the short-circuit current of Caco-2 monolayers infected with *C. concisus* UNSWCD (*D*), compared with *E. coli* K-12 (*E*) over 75 min after 6 h of infection ($P = .065$).

murium, as expected, exhibited the highest overall levels of invasion, with a mean level (±SEM) of 1.92% ± 0.31%. The number of viable extracellular bacteria after gentamicin treatment was nil or <1% of total recoverable bacteria.

C. concisus *invasion is dependent on time, pre-existing inflammation, and host cytoskeleton.* Because *C. concisus* UNSWCD showed the greatest capacity to invade human epithelial cells, a time course experiment was conducted to determine whether the process of invasion was dependent on time and host cytoskeleton. The maximum level of invasion was observed after 6 h of incubation and was therefore expressed as 100%. The mean percentage (±SEM) of *C. concisus* UNSWCD invasion after 30 min, 2 h, and 4 h of incubation was 6.5% ± 3.1%, 34.3% ± 9.5%, and 3.7% ± 2.3%, respectively.

Investigation of the potential impact of gut inflammation driven by cytokines, such as TNF-α [18] and IFN-γ [19], that was observed in patients with Crohn's disease on *C. concisus* UNSWCD invasion showed that the ability of *C. concisus* UNSWCD to invade Caco-2 cells treated with TNF-α was significantly increased by a mean percentage (±SEM) of 62% ± 7.6%, compared with untreated Caco-2 cells ($P = .015$). Similarly, a significantly elevated level of invasion was observed in Caco-2 cells treated with IFN-γ, compared with untreated Caco-2 cells (mean increase ± SEM, 36% ± 5.9%; $P = .027$). In addition, *C. concisus* UNSWCD invasion was significantly attenuated in the presence of colchicine (inhibits microtubule polymerization; $P = .006$), decreasing from 100% to a mean percentage (±SEM) of 15.7% ± 6.6%. Similarly, the level of invasion significantly decreased when Caco-2 cells were treated with cytochalasin D (inhibits microfilaments; $P = .001$), from 100% to a mean percentage (±SEM) of 28.2% ± 2.4%.

C. concisus–*induced movement of tight junction proteins significantly compromising TER and membrane permeability.* The effect of *C. concisus* UNSWCD on intestinal barrier function was further investigated by measuring TER, membrane

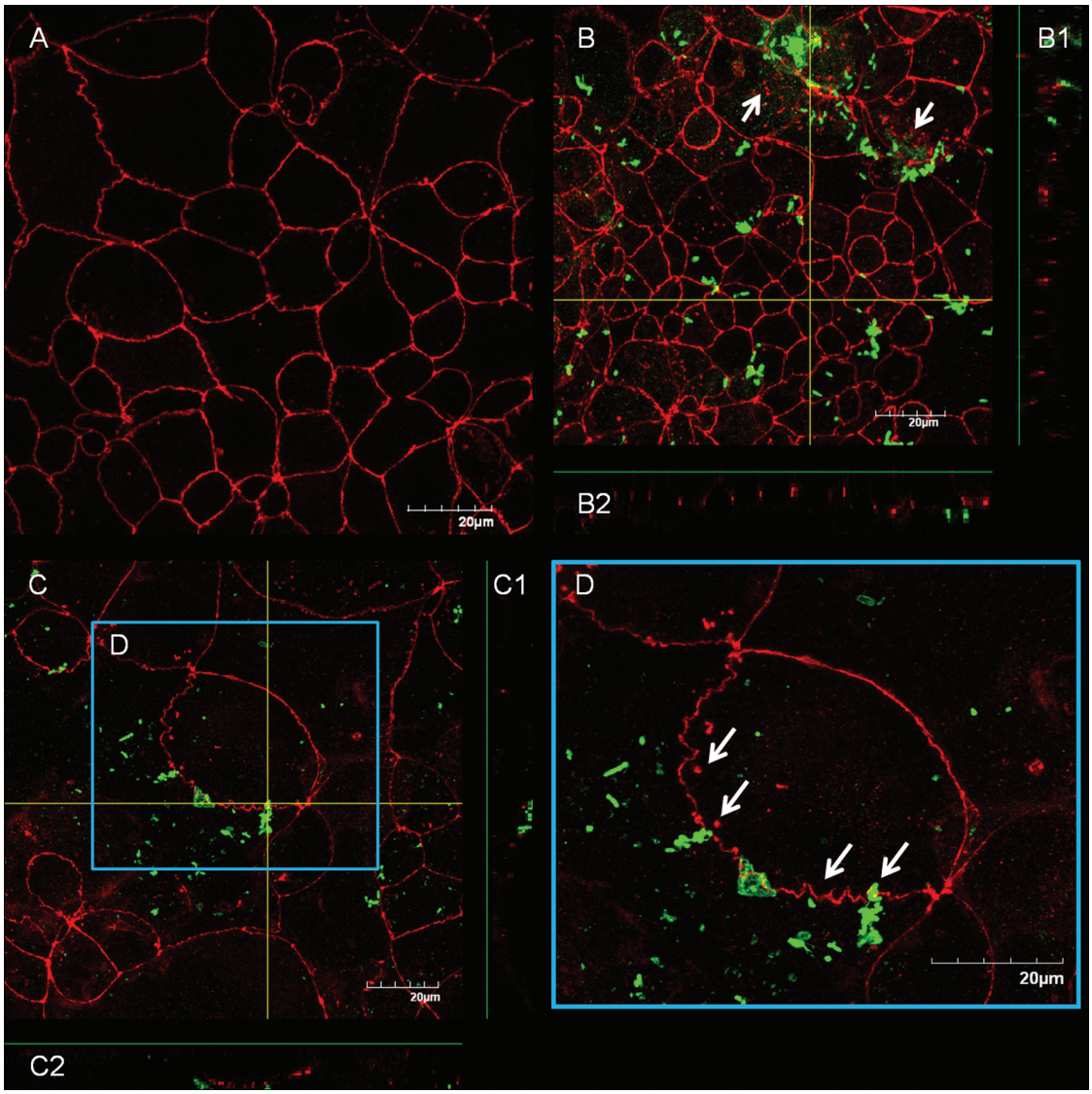

Figure 5. Redistribution of ZO-1 in Caco-2 cells infected with *Campylobacter concisus* UNSWCD. A Caco-2 monolayer in the absence of bacteria showing an intact ZO-1 boundary (*red*) confined to the intercellular junction (*A*). After infection with *C. concisus* UNSWCD (*green*), bacterial attachment and degradation of ZO-1 was observed after 6 h (*B*) or 48 h of infection (*C*). *B,* Disruption and movement of ZO-1 from the cell-cell boundary, as a result of infection with *C. concisus* UNSWCD for 6 h, is indicated by *arrows. C,* Loss of ZO-1 was more pronounced at 48 h. Views of cross-sectional images revealed the presence of *C. concisus* UNSWCD below ZO-1 proteins, indicating the occurrence of bacterial translocation across the cell-cell junction after 6 h (*B*) or 48 h of infection (*C*). *D,* Magnified view of a ZO-1 boundary, showing preferential attachment of *C. concisus* UNSWCD to the cell-cell junction and induction of ZO-1 internalization along the side with extensive bacterial attachment.

permeability, and ion transportation of infected Caco-2 monolayers. The TER value of Caco-2 cells stabilized and reached a plateau after 30 days of cultivation on cell culture membrane supports. After a 6-h infection of stabilized Caco-2 monolayers with *C. concisus* UNSWCD, the TER was significantly reduced (mean TER ± SEM, 52.0 ± 4.1 ohm/cm^2), compared with before infection (mean TER ± SEM, 249.5 ± 5.6 ohm/cm^2; $P < .001$) (Figure 4A). In contrast, the TER of Caco-2 monolayers was not affected after infection with *E. coli* K-12 (mean TER ± SEM before infection, 211.0 ± 11.5 ohm/cm^2; mean TER ± SEM after infection, 211.0 ± 10.9 ohm/cm^2; $P > .99$) (Figure 4*B*). In addition, increased membrane permeability as a result of infection with *C. concisus* UNSWCD was detected using HRP translocation in the Ussing Chamber (Figure 4*C*). Caco-2 monolayers infected with *C. concisus* UNSWCD showed significantly elevated levels of HRP in the basolateral compartment (mean level ± SEM, 877.3 ± 198 nmol/L), compared with monolayers infected with the *E. coli* K-12 negative control (mean level ± SEM, 93.4 ± 29.7 nmol/L; $P = .029$). After a 6-h infection with *C. concisus* UNSWCD, the short-circuit cur-

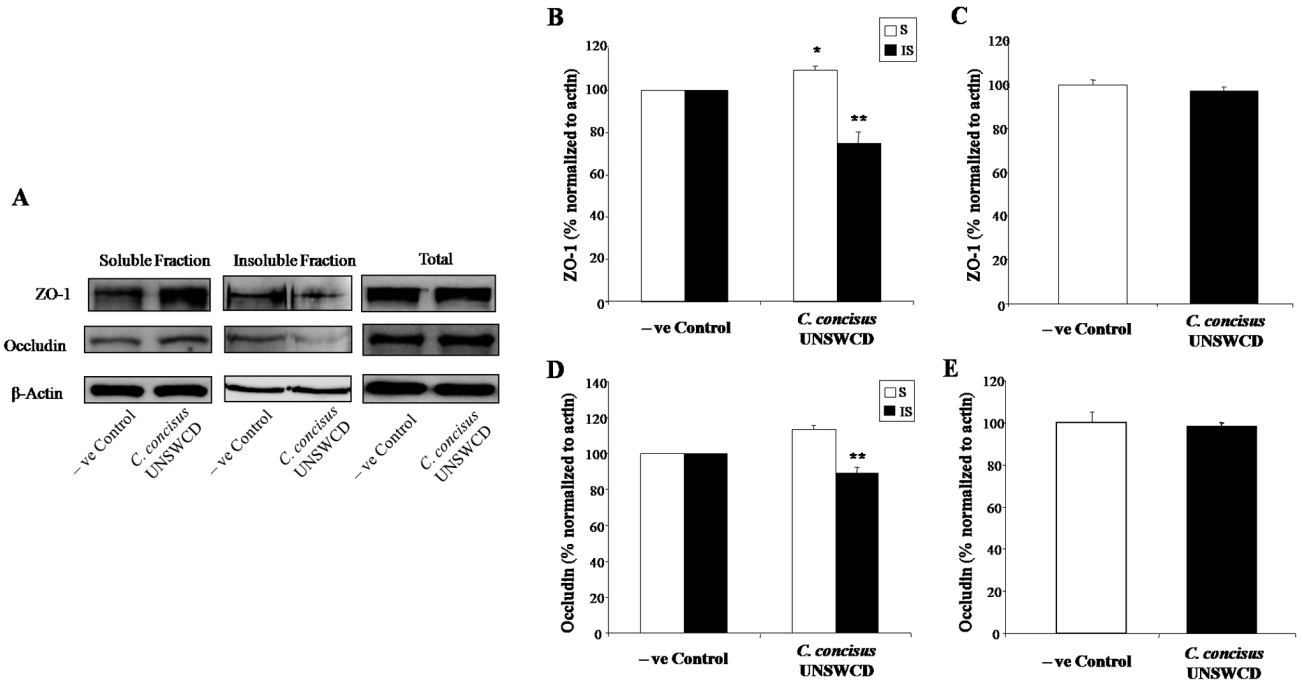

Figure 6. Movement of tight junction protein ZO-1 and occludin from host membrane to the cytosol of Caco-2 cells induced by *Campylobacter concisus* UNSWCD. *A, B,* and *D,* The level of ZO-1 or occludin significantly decreased in the membrane (insoluble; IS) fraction, and ZO-1 also increased in the cytosolic (soluble; S) fraction. *A, C,* and *E,* Total content of ZO-1 or occludin remained unchanged after infection with *C. concisus* UNSWCD for 48 h. β-actin was used as a loading control. Data from 3 independent experiments. $*P = .025$; $**P = .002$.

rent showed a slight increase during the subsequent 75-min period; however, this increase did not differ significantly from that of *E. coli* K-12 ($P = .065$) (Figure 4*D* and 4*E*).

Using immunofluorescent staining, intact tight junctions were observed in uninfected Caco-2 monolayers (Figure 5*A*). After infection with *C. concisus* UNSWCD, a substantial proportion of bacteria was observed to be preferentially attaching and congregating at or near the intercellular junction of the Caco-2 cells (Figure 5*B*–5*D*). This finding reinforced the scanning electron microscopy observations (Figure 2*A*–2*E*). After 6 or 48 h of infection with *C. concisus* UNSWCD, ZO-1 proteins migrated from the cell-cell junction to the cytoplasm (Figure 5*B*–5*D*). Translocation of bacteria to the basolateral surface was also observed (Figure 5*B* and 5*C*). Confirmation that viable *C. concisus* UNSWCD were recoverable from basolateral supernatants was assessed by culture (data not shown).

Western blotting was used to further analyze and confirm the effect of *C. concisus* UNSWCD on the expression of tight junction proteins in Caco-2 cells. Expression of ZO-1 and occludin significantly decreased in the membrane fraction of Caco-2 cells infected with *C. concisus* UNSWCD, compared with uninfected controls ($P < .05$), indicating a loss of membrane-associated tight junction proteins (Figure 6*A,* 6*B,* and 6*D*). Furthermore, the level of ZO-1 and, to a lesser extent, occludin also concomitantly increased in the cytosolic fraction of *C. concisus* UNSWCD–infected cells, suggesting internalization of these proteins from the cell membrane to the cytosolic compartment. Overall expression of ZO-1 and occludin in the total cellular fraction after infection with *C. concisus* UNSWCD was unchanged (Figure 6*A,* 6*C,* and 6*E*), further supporting the notion of migration of tight junction proteins from the membrane toward the cytosolic compartment, as opposed to loss of these proteins from the membrane to the supernatant. These results reinforced the observations of ZO-1 internalization made by confocal microscopy (Figure 5*B*–5*D*).

C. concisus *and other non*–C. jejuni Campylobacter *species stimulated production of IL-8 in intestinal epithelial cells.* Because the ability of *Campylobacter* species to invade the intestinal epithelium was species and strain specific, we explored pathogenic mechanisms other than invasion that may contribute to the ability of *C. concisus, C. showae, C. hominis,* and *B. ureolyticus* to cause disease. After 24 h of infection at MOI values of 100 or 200, all 3 strains of *C. concisus* (UNSWCD, ATCC 51561, and ATCC 51562) stimulated significantly higher levels of IL-8, compared with *E. coli* K-12 (Figure 7*A*). The highest level of IL-8 was induced by *C. concisus* ATCC 51562. In contrast, IL-8 levels induced by *C. showae, C. hominis,* or *B. ureolyticus* were not significantly elevated, compared with *E. coli* K-12. The lowest IL-8 level was induced by *B. ureolyticus.* Levels of IL-8 stimulated with heat-killed *C. concisus, C. showae,*

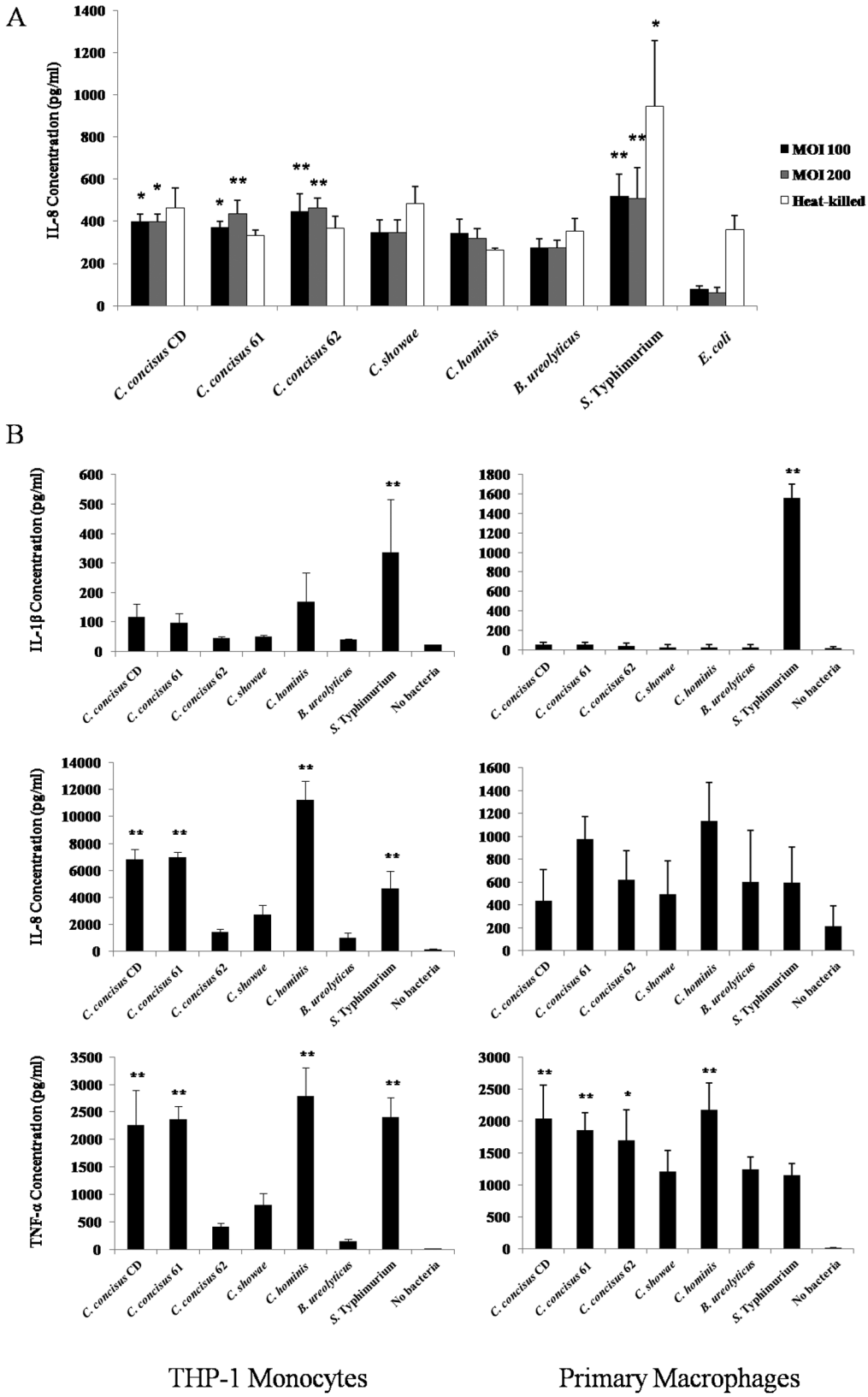

Figure 7. *A,* Levels of interleukin (IL)–8 produced by the human intestinal epithelial cell line HT-29 after infection with viable or heat-killed non–*Campylobacter jejuni Campylobacter* species. *B,* Levels of IL-1β, IL-8, and tumor necrosis factor (TNF)–α produced in the THP-1 human monocytic cell line and primary human macrophage. Data from 3 independent experiments ($\pm$ standard error of the mean), with each experiment performed in duplicate. MOI, multiplicity of infection. $^{*}P < .05$; $^{**}P < .01$.

and *B. ureolyticus* were comparable to those of their viable counterparts but were not significantly elevated, compared with that induced by heat-killed *E. coli* K-12.

C. concisus *and other non–*C. jejuni Campylobacter *species stimulated the production of proinflammatory cytokines in a monocytic cell line and primary macrophages.* After entry into the submucosa and bloodstream through a compromised epithelial barrier, pathogens encounter monocytes and macrophages. Examination of the THP-1 monocytic cell response to bacterial stimulation revealed that *C. concisus* UNSWCD, *C. concisus* ATCC 51561, and *C. hominis* induced significantly elevated levels of IL-8 and TNF-α, but not IL-1β, compared with unstimulated controls (Figure 7*B*). *C. hominis* induced the highest level of IL-8 and TNF-α in all bacteria tested. In contrast, *C. concisus* ATCC 51562, *C. showae,* and *B. ureolyticus* stimulated relatively low levels of IL-8 and TNF-α in THP-1 monocytes, suggesting that the ability of *Campylobacter* species to stimulate these proinflammatory cytokines is both strain and species dependent. All *Campylobacter* species tested induced relatively low IL-1β levels. Only *S.* Typhimurium stimulated a significantly higher level of IL-1β in THP-1 monocytes, compared with unstimulated monocytes ($P < .01$).

Primary human macrophages infected with *C. concisus* UNSWCD, *C. concisus* ATCC 51561, *C. concisus* ATCC 51562, and *C. hominis* produced a significantly higher level of TNF-α, compared with the unstimulated control, whereas *C. showae, B. ureolyticus,* and *S.* Typhimurium did not (Figure 7*B*). Only *S.* Typhimurium induced a significantly elevated level of IL-1β, compared with the unstimulated control. All other *Campylobacter* species induced relatively low levels of IL-1β. Although all *Campylobacter* species and *S.* Typhimurium induced increased levels of IL-8 in primary human macrophages, the levels observed were not significantly higher than those in the unstimulated control.

DISCUSSION

Although pathogenic mechanisms of *C. jejuni* are well documented, there have been limited studies investigating the pathogenesis of non–*C. jejuni Campylobacter* species. Non–*C. jejuni Campylobacter* species are important human and veterinary pathogens, and a number of these, including *C. concisus,* are currently considered to be emerging pathogens of the human intestinal tract. To investigate the potential of these organisms to initiate disease, we examined the pathogen-host relationships with use of human epithelial cell lines and immune cells. Our investigations using scanning electron microscopy showed that the polar flagella of both *C. concisus* and *C. showae* were involved in host cell attachment. The flagellum from these bacteria was observed to attach to the tips of microvilli, a process that may be required for colonization and, for *C. concisus* UNSWCD, penetration in the host cell. In *C. jejuni,* attachment is also necessary for facilitating the injection of *Campylobacter* invasion antigens into host cells [20]. Of interest, *C. jejuni* mutants defective in the major flagellin subunit K2–32 have reduced attachment and invasion capacity [21], suggesting the role of flagella in determining the invasiveness of *Campylobacter* species. After flagellum-mediated attachment, *C. concisus* UNSWCD initiated a membrane-ruffling effect on the host cell membrane in a manner not very dissimilar to that of *Salmonella* Typhi invasion [22].

In addition, we found visual evidence that a nonflagellate sticky end mechanism of attachment appears to exist for all *Campylobacter* species examined. The sticky end of these bacteria appears to attract nearby microvilli to the bacterial cell surface, possibly mediated by the presence of bacterial surface adhesins. This mechanism of attachment represents another strategy by which members of the *Campylobacter* genus adhere to intestinal epithelial cells, which is particularly advantageous for the aflagellate *B. ureolyticus* and *C. hominis.* Although *B. ureolyticus, C. hominis,* and *C. showae* had the ability to attach to intestinal epithelial cells, they did not appear to be able to invade. A strain of *C. concisus* isolated from a child with Crohn's disease had the greatest ability to invade Caco-2 intestinal epithelial cells, compared with the 3 other non–Crohn's disease strains. Comparison of the level of invasion by *C. concisus* UNSWCD with those of previously documented *Campylobacter* species revealed striking similarities [23, 24] and suggests that only a small population of *Campylobacter* may invade or survive in intestinal epithelial cells.

In addition to a transcellular route of infection through host invasion, we showed that *C. concisus* UNSWCD preferentially attached to intercellular junctional spaces and possibly translocated across the epithelium through a paracellular route. Such spatial distribution was concomitantly associated with a striking decrease in TER, an increase in HRP flux, and a loss of membrane-associated ZO-1 and occludin in Caco-2 monolayers after *C. concisus* UNSWCD infection. The observed preferential attachment may allow rapid disruption of tight junctions, either by direct *C. concisus*–host contact or by release of secretory proteins directly to the target sites, resulting in damage to the structural integrity and function of intestinal epithelial cells. Although the precise signal required is currently unknown, it is possible that internalization of ZO-1 and occludin from the cell membrane to the cytosol induced by *C. concisus* UNSWCD is attributable to a zonula occludens toxin, which has been identified in the complete genome of *C. concisus* strain 13826 [25]. Although the mechanism of zonula occludens toxin in *C. concisus* remains to be deciphered, studies in *Vibrio cholerae* have shown that zonula occludens toxin significantly increases tissue permeability, initiates actin polymerization, and increases intestinal secretion [26, 27].

In addition to attachment and invasion, our results showed

that *C. concisus* strains, irrespective of their invasive abilities, stimulated significantly elevated levels of IL-8, compared with *E. coli* K-12, in HT-29 cells. Furthermore, direct contact with viable *C. concisus* and other non–*C. jejuni Campylobacter* species was not required for IL-8 production. *C. concisus* also exhibited a strain dependency in the ability to stimulate IL-8 and TNF-α in monocytic THP-1 cells. *C. hominis* stimulated the highest levels of IL-8 and TNF-α in both monocytes and macrophages of all bacteria tested, including *S.* Typhimurium. Whether the potent stimulatory ability of *C. hominis* contributed to a reported fatal septicemia [14], in which *C. hominis* was isolated from the blood of a patient, is intriguing.

In conclusion, we revealed with use of an in vitro model that non–*C. jejuni Campylobacter* species, in particular *C. concisus,* have the potential to invade and modulate barrier permeability and cytokine production, suggesting that they are unlikely to be commensals of the intestinal tract and have the potential to cause disease. Such findings provide a novel insight into the role of non–*C. jejuni Campylobacter* species in inflammatory bowel diseases and other infectious gastrointestinal diseases with unknown etiology. Examination of additional clinical isolates is required to ascertain whether the virulence factors displayed by *C. concisus* and the other non–*C. jejuni Campylobacter* species are reproducible.

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