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Recruitment of Dendritic Cells Is Responsible for Intestinal Epithelial Damage in the Pathogenesis of Necrotizing Enterocolitis by Cronobacter sakazakii

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Cronobacter sakazakii is a Gram-negative pathogen associated with the cases of necrotizing enterocolitis (NEC) that result from formula contamination. In a mouse model of NEC, we demonstrate that C. sakazakii infection results in epithelial damage by recruiting greater numbers of dendritic cells (DCs) than macrophages and neutrophils in the gut and suppresses DC maturation, which requires outer membrane protein A (OmpA) expression in C. sakazakii. Pretreatment of intestinal epithelial cell monolayers with supernatant from OmpA+ C. sakazakii/DC culture markedly enhanced membrane permeability and enterocyte apoptosis, whereas OmpA− C. sakazakii/DC culture supernatant had no effect. Analysis of OmpA+ C. sakazakii/DC coculture supernatant revealed significantly greater TGF-β production compared with the levels produced by OmpA− C. sakazakii infection. TGF-β levels were elevated in the intestinal tissue of mice infected with OmpA+ C. sakazakii. Cocultures of CaCo-2 cells and DCs in a “double-layer” model followed by infection with OmpA+ C. sakazakii significantly enhanced monolayer leakage by increasing TGF-β production. Elevated levels of inducible NO synthase (iNOS) were also observed in the double-layer infection model, and abrogation of iNOS expression prevented the C. sakazakii-induced CaCo-2 cell monolayer permeability despite the presence of DCs or OmpA+ C. sakazakii/DC supernatant. Blocking TGF-β activity using a neutralizing Ab suppressed iNOS production and monolayer leakage. Depletion of DCs in newborn mice protected against C. sakazakii-induced NEC, whereas adoptive transfer of DCs rendered the animals susceptible to infection. Therefore, C. sakazakii interaction with DCs in intestine enhances the destruction of the intestinal epithelium and the onset of NEC due to increased TGF-β production.

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Necrotizing enterocolitis (NEC) is the most common and lethal gastrointestinal disease of premature neonates (1–3). The incidence of this disease approaches 1 per 1000 live births and is associated with a high morbidity and mortality, ranging from 10 to 100%, depending on the extent of the intestinal injury (4–7). In its most severe form, NEC is characterized by full-thickness destruction of the intestine accompanied by intestinal perforation, peritonitis, bacterial invasion, sepsis, and death (8). The inciting event in the pathogenesis of NEC is believed to be the breakdown of the intestinal epithelial barrier due to prematurity or ischemic insults followed by translocation of pathogenic bacteria (9). Gram-negative bacteria have been culture from the blood of many septic patients with NEC (10). Cronobacter sakazakii, formerly known as Enterobacter sakazakii, is an emerging Gram-negative opportunistic pathogen, which is a common contaminant of powdered milk and infant formulas (11–14). The number of C. sakazakii-related infections has substantially increased during the past 10 years due to increased use of infant formula (15–17). The U.S. Food and Drug Administration has issued several warnings regarding C. sakazakii infection in newborn infants. Therefore, understanding the pathogenesis of NEC due to C. sakazakii is essential for developing strategies for prevention or treatment. Our group has shown that oral feeding of C. sakazakii to newborn rats under hypoxic conditions induces NEC-like inflammation (18). The interaction of C. sakazakii with enterocytes induced apoptosis in rat intestinal epithelial cells (IEC-6) both in vitro and in newborn rats. C. sakazakii that does not express outer membrane protein A (OmpA) could not bind to the intestine, indicating that OmpA may be playing a role in the interaction of C. sakazakii with enterocytes (18). In addition, we have shown that NO, an important second messenger and inflammatory mediator, plays a key role in intestinal barrier failure seen in NEC (19, 20). Subsequently, we have demonstrated that the enterocyte injury caused by C. sakazakii appears to be dependent on the production of NO, as inhibition of inducible NO synthase (iNOS) by small interfering RNA (siRNA) prevented apoptosis of IEC-6 cells (19). Although LPS has been shown to increase iNOS expression in the ileum of mice colonized with bacteria (21, 22), no studies to date have addressed how OmpA interaction with intestine induces NO.

Abbreviations used in this article: 7-AAD, 7-amino-actinomycin D; BM, bone marrow; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; iNOS, inducible NO synthase; LP, lamina propria; MDC, myeloid dendritic cell; MOI, multiplicity of infection; mr, mouse recombinant; NEC, necrotizing enterocolitis; OmpA, outer membrane protein A; PMN, polymorphonuclear cell; siRNA, small interfering RNA; TEBER, transepithelial electrical resistance; WT, wild-type.

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Dendritic cells (DCs) in the intestine are professional APCs required for the initiation of cell-mediated immune response (23–27). Nonetheless, several pathogens have developed strategies to suppress DC maturation so that they can thrive in hostile conditions (28). The stimulation of TLRs in DCs by pathogen-associated microbial patterns leads to the activation of various inflammatory signaling pathways and antimicrobial mechanisms (29). Our previous studies demonstrated that C. sakazakii interacts with DC-SIGN, present on DCs, to enter and subsequently suppress the maturation of DCs. However, the role of DCs at the mucosal surfaces in the pathogenesis of NEC is poorly understood. We hypothesize that the interaction of DCs with C. sakazakii expressing OmpA contributes to the epithelial barrier disruption through secretion of pro- or anti-inflammatory cytokines. Using an in vitro and an in vivo mouse model of NEC, we demonstrate that robust recruitment of DCs in the lamina propria (LP) of intestine enhances epithelial cell injury upon infection with OmpA+C. sakazakii. TGF-β production by DCs infected with OmpA+C. sakazakii promotes increased expression of iNOS from the epithelial cells and thereby enhances apoptosis and barrier dysfunction. In agreement with these studies, depletion of DCs in newborn mice protects the animals from OmpA+C. sakazakii-induced NEC, whereas adoptive transfer of DCs to DC-depleted mice restores the susceptibility to C. sakazakii infection. This, to our knowledge, is the first report to demonstrate the importance of DCs in the pathogenesis of C. sakazakii-induced NEC.

Materials and Methods

Bacterial strains, cells, and reagents

C. sakazakii (strain 51329) and CaCo-2 cells (human colon carcinoma cells; passage 18) were obtained from and maintained per the instructions of American Type Culture Collection. CaCo-2 cells were seeded in Transwells and maintained in enterocyte differentiation medium (BD Biosciences, San Jose, CA) to form monolayers. This medium promotes rapid growth of cells resulting in confluent monolayers of cells within 3–5 d. C. sakazakii were transformed with a GFP plasmid as previously described and grown in Luria broth containing ampicillin (31).

Isolation of bone marrow-derived and intestinal DCs and of enterocytes

Bone marrow (BM) cells were obtained by flushing the femurs and tibias with DMEM containing 5% FBS and subsequently washed and resuspended in appropriate medium. The BM cells were differentiated into DCs by resuspending the cells in RPMI 1640 supplemented with 10% FCS, 2.4 mM L-glutamine (Invitrogen, Carlsbad, CA), 2-mercaptoethanol (5×10⁻³ M), 100 IU/ml penicillin, 100 mg/ml streptomycin, 50 ng/ml mouse recombinant (mr) GM-CSF, and 20 ng/ml mrlIL-4 (PeproTech, Rocky Hill, NJ). On days 1 and 3 post-culture initiation, the contents of each well were divided in half, and the volume of each new well was brought to 2 ml with supplemented DC medium containing mrlGM-CSF and mLIL-4. On day 6–7, the same procedure was repeated for the final time (32). Subsequently, DCs were sorted by flow cytometry (BD Biosciences, San Jose, CA) after staining with anti-CD11c Ab. For isolation of intestinal DCs, intestines were harvested from mice, Peyer’s patches removed, and the specimen placed in RPMI 1640 medium. The samples were placed through a Percoll gradient for separation of enterocytes as described elsewhere (32). CD11c⁺ F4/80⁻ Gr-1⁻ cells were then sorted using flow cytometry.

Flow cytometry

Expression of DC maturation markers was detected by staining with appropriate fluorochrome-coupled mAbs or isotype-matched mAbs as described earlier (30). Cells were analyzed by four-color flow cytometry using FACSCalibur CellQuest Pro software (BD Biosciences) using CD11c as a gating marker, and at least 10,000 events within this gate were collected for analysis. Characterization of leukocytes derived from intestines of newborn mice was done using flow cytometry. Briefly, intestines were removed, transferred to ice-cold Hank’s buffer/3% FCS, homogenized using a glass potter, and passed through a stainless-steel sieve. The dissociated sample was collected by centrifugation and was digested for 60 min at 37°C with 1.4 ml of type II collagenase (0.95 U/ml; Sigma) and 104
U D Nase I (Sigma) in dissociation buffer (42 mmol/l MgCl₂/23 mmol/l CaCl₂/50 mmol/l KCl/153 mmol/l NaCl). The digested sample was pelleted and resuspended in PBS. Polymorphonuclear cells (PMNs) were identified using Gr-1 Ab, and macrophages were stained with F4/80 Ab. The presence of bacteria was measured using the GFP expression in the FITC gate. The expression of iNOS was detected in the intestines of infected mice by double staining for E-cadherin and iNOS using appropriate primary Abs (BD Biosciences) with two separate fluorochrome-coupled secondary Abs.

siRNA transfection

CaCo-2 cells were grown to 30% confluence in 6-well plates, then transfected with siRNA for iNOS or control siRNA using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. The siRNA primer sequence used for silencing iNOS expression is 5'-CCACCAGTATGCAATGAAT-3' (Invitrogen). The respective control-siRNA was also purchased from Invitrogen.

Animal experiments

The animal studies were approved by the Institutional Animal Care and Use Committee of the Saban Research Institute at Children’s Hospital Los Angeles and followed National Institutes of Health guidelines for the performance of animal experiments. Time pregnant C57BL/6 mice were obtained from Charles River at E18; after delivery, pups were kept with the mothers and housed at Children’s Hospital Los Angeles. Three-day-old pups were fed 10⁶ CFU OmpA⁺ C. sakazakii or OmpA⁻ C. sakazakii in 10 μl sterile PBS orally and then kept with the mother. The control animals received sterile PBS at day 3. The OmpA⁺ C. sakazakii-fed animals succumbed to infection on day 4 postinfection; other pups were euthanized at the same time. The animals were carefully monitored for any signs of disease, and intestinal tissues were collected from these animals at the moribund state but not from dead animals. The collected tissues were fixed in 10% formalin and stained with H&E. Intestinal sections were graded microscopically by a pathologist blinded to groups from grade 0 (normal) to grade 4 (severe) based on pathological manifestations including submucosal edema, villus core edema, epithelial sloughing/obliteration, neutrophil infiltration, intestinal perforation, and necrosis. In depletion studies, animals received six injections of CD11c Ab (Abcam, Cambridge, MA) in sterile PBS (50 μl) using i.p. injections starting on day 1. The last injection was given prior to infection with C. sakazakii. Depletion was confirmed prior to infection using flow cytometry for CD11c expression in liver, spleen, and the intestinal homogenates. Mouse DCs were derived from the BM aspirates from femurs and tibias of mice followed by culturing the cells in m rIL-4 and m rGM-CSF to differentiate the BM cells into DCs (bone marrow-derived dendritic cells; BMDCs) as described earlier and then sorted by flow cytometry based on CD11c staining and F4/80 and Gr-1 negative population. DCs (5 × 10⁶) were injected i.p. into the DC-depleted animals 6 h prior to oral infection with bacteria. The presence of DCs was confirmed by flow cytometry with anti-CD11c Ab in the homogenized intestinal tissue.

Cytokine measurements

Cytokine levels were measured in CaCo-2 cells as well as in the supernatant from fresh homogenized intestinal tissue after the animals were euthanized at the end of the experiment by ELISA using commercially available kits (Invitrogen). The tissue was weighed prior to homogenization, and the levels were calculated per gram of intestinal tissue.

Determination of apoptosis

Apoptosis was assessed by TUNEL staining using ApoTag kit (Chemicon, Temecula, CA) and annexin-V and propidium iodide staining (BD Biosciences) according to the manufacturers’ instructions.

Determination of CaCo-2 cell monolayer integrity

CaCo-2 cells seeded in Transwells in enteroocyte differentiation medium (BD Biosciences) were cultured for 48 h to reach a resistance of >300 Ω cm². C. sakazakii strains were added at a multiplicity of infection (MOI) of 10 in Transwells and incubated for varying periods. The monolayer integrity was determined by measuring transepithelial electrical resistance...
(TEER) and HRP leakage or monolayer permeability to FITC-conjugated dextran (4 kDa) as described previously (33, 34). Briefly, FITC-conjugated dextran was added to confluent CaCo-2 monolayers grown on Transwells and then infected with C. sakazakii. The leakage of FITC-conjugated dextran in the lower chamber was then determined using a fluorometer. The standard curve was calibrated using FITC-conjugated dextran, and the results are expressed in micrograms per milliliter. In some experiments, the Transwells containing CaCo-2 cells were flipped and seeded with human myeloid dendritic cells (MDCs), which were isolated as described (30) and cultured for 24 h to form double layers. In addition, C. sakazakii strains were incubated with MDCs for 24 or 48 h, the supernatants were collected, cleared of bacteria by centrifugation, and filtered through an 0.22-μm filter. The absence of bacteria was confirmed by culturing the supernatants on blood agar plates. The supernatants were stored at –70˚C until used in pretreatment experiments.

iNOS and NO measurement

CaCo-2 cells were infected with C. sakazakii at an MOI of 10 for 4 h; the cells were then washed, fixed in 2% paraformaldehyde, and subsequently permeabliized using Cytoperm solution (BD Biosciences). The cells were stained with fluorochrome conjugated anti-iNOS Ab for 1 h and analyzed using flow cytometry. The supernatants were collected and used to determine the production of NO by the Griess method (33).

Fluorescence microscopy

Sections of the intestine were cut with a cryostat (Leica, Wetzlar, Germany) and then stained with tight junction marker ZO-1 (31). Stained tissue sections were imaged with confocal microscope LSM710 and arranged using Adobe Photoshop 7.0.

Statistical analysis

Statistical significance was determined by Student t test, ANOVA, Wilcoxon signed-rank test, and Fisher exact test. The p values <0.05 were considered to be statistically significant.

Results

C. sakazakii induces NEC in newborn mice

Previous studies from this laboratory have demonstrated that oral feeding of C. sakazakii to newborn rats under hypoxic conditions induced NEC by day 4 (18, 19). To use knockout mice and to understand the role of various host and bacterial factors responsible for pathogenesis, it is necessary to establish a mouse

FIGURE 3. Pretreatment of CaCo-2 cells with OmpA+ C. sakazakii/DC supernatant compromises the tight junction integrity. Confluent monolayers of CaCo-2 cells grown in Transwell inserts were infected with OmpA+ C. sakazakii or OmpA– C. sakazakii, left alone, or pretreated with supernatants from C. sakazakii/DC cocultures. TEER (A), HRP (B), and FITC-conjugated dextran leakage (C) was measured as described in Materials and Methods. The results represent mean ± SD from four separate experiments performed in triplicate. The decrease in TEER or increase in the permeability of HRP or FITC-conjugated dextran was statistically significant: *p < 0.001 by ANOVA and Student t test. OmpA+ CS, OmpA+ C. sakazakii; OmpA– CS, OmpA– C. sakazakii.

FIGURE 4. OmpA+ C. sakazakii causes tight junction disruption. CaCo-2 cells grown on 4-well chamber slides were infected with OmpA+ C. sakazakii or OmpA– C. sakazakii for 4 h and stained for ZO-1. Infection with OmpA+ C. sakazakii resulted in disruption of tight junctions (yellow arrows), whereas OmpA– C. sakazakii infection did not affect the integrity of the tight junctions. The results are representative of five independent experiments. OmpA+ CS, OmpA+ C. sakazakii; OmpA– CS, OmpA– C. sakazakii (original magnification ×63).
model of NEC. Therefore, 3-d-old newborn mice were infected orally with 10^3 CFU C. sakazakii expressing GFP. In addition, C. sakazakii lacking OmpA expression was also used to determine the role of OmpA in the induction of NEC. The animals fed OmpA+ C. sakazakii succumbed to infection by 72–96 h post-inoculation, whereas those fed OmpA− C. sakazakii survived similar to the uninfected control mice (Fig. 1A). The mice fed OmpA+ C. sakazakii develop morphological signs of NEC including intestinal dilation and bowel wall discoloration as early as 48 h postinfection (Fig. 1B). The infected animals appeared sick with abdominal distention and decreased activity. OmpA− C. sakazakii-infected mice behaved like uninfected control mice until euthanized. Next, we examined the binding of OmpA+ and OmpA− C. sakazakii to the mouse intestine by plating the intestinal tissue homogenates on agar containing antibiotic. As shown in Fig. 1C, OmpA+ C. sakazakii bound to the intestine in significantly greater numbers compared with OmpA− C. sakazakii at 72 h postinfection. Of note, we observed that the binding of OmpA− C. sakazakii was similar to that of OmpA+ C. sakazakii up to 12 h postinfection (data not shown). Scoring of intestinal sections from the infected mice after staining with H&E by a pathologist blinded to specimens revealed severe epithelial injury as well as mucosal sloughing and villi destruction in mice fed OmpA+ C. sakazakii (Fig. 1D). The pathological changes were comparable with those seen in human infants with NEC (de-rangement of mucosa and villus sloughing) (Fig. 1E). In contrast, intestinal sections from OmpA− C. sakazakii-infected animals showed no such injury. The average pathology scores were significantly greater in mice infected with OmpA+ C. sakazakii compared with those in mice infected with OmpA− C. sakazakii (p < 0.001 by Wilcoxon signed-rank test).

C. sakazakii has been shown to manipulate the maturation of DCs by entering via DC-SIGN (30). DCs are important APCs in the gut, which sample pathogenic luminal bacteria. To assess whether DCs recruit to the LP as a result of OmpA+ C. sakazakii infection, intestinal DCs as well as PMNs and macrophages were isolated from digested intestinal tissue after separation by Percoll gradient. Flow cytometric analysis revealed that higher numbers of DCs were recruited to the LP in animals fed OmpA+ C. sakazakii compared with uninfected controls based on CD11c staining whereas the number of PMNs and macrophages increased only slightly (p < 0.001 by ANOVA and Fisher exact test, Fig. 2A). Notably, the number of DCs recruited to the LP was similar in OmpA− C. sakazakii-fed animals, indicating that the recruitment is not dependent on OmpA expression. Thirty-one percent of the immunocytes in the intestines of OmpA+ C. sakazakii-fed mice were

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**FIGURE 5.** Increased apoptosis in CaCo-2 cells pretreated with OmpA+ C. sakazakii/DC supernatant. Confluent monolayers of CaCo-2 cells were pre-conditioned with C. sakazakii/DC supernatants for 24 h and then infected with C. sakazakii or left uninfected. The monolayers were assessed for apoptosis by TUNEL staining using an ApoTag kit. Bacteria (green), apoptotic cells (red), and nuclei (blue) (A). Original magnification ×20; insets, original magnification ×63. The number of apoptotic nuclei per total nuclei was counted and graphed (B). In separate experiments, the cells were also stained with annexin-V/7-AAD and examined by flow cytometry (C). The error bars represent mean ± SD of five independent experiments performed in triplicate. *p < 0.01 by ANOVA and Student t test. CS, C. sakazakii; OmpA+ CS, OmpA+ C. sakazakii; OmpA− CS, OmpA− C. sakazakii.
CD11c+ compared with less than 1% in the gut of PBS-fed control animals. The intestinal DCs recruited to the LP were CD11c/CD103 positive and F4/80/Gr-1 negative as shown by flow cytometry (Fig. 2B). However, we observed that infection with OmpA+ C. sakazakii suppresses the expression of maturation markers (CD40, MHC class II, and CD86) in the intestinal DCs, whereas the OmpA− C. sakazakii-infected intestinal DCs express these markers (Fig. 2C). Confocal microscopy of the intestinal tissue from mice infected with OmpA+ C. sakazakii or OmpA− C. sakazakii upon staining with CD11c Ab also revealed significant recruitment of DCs to the LP in agreement with our flow cytometry data (Fig. 2D). Collectively, these results suggest that OmpA expression in C. sakazakii is critical for efficient binding to the intestine and that OmpA+ C. sakazakii can survive intrinsic DC defenses by suppressing DC maturation. In contrast, OmpA− C. sakazakii, despite showing some initial binding, could not survive in the intestine.

Preconditioning of CaCo-2 cells with OmpA+ C. sakazakii/DC supernatant compromises the monolayer permeability

Because the disruption of the intestinal barrier is an important feature of the pathogenesis of NEC, we hypothesize that OmpA+ C. sakazakii interaction with DCs might contribute to the pathogenesis of NEC by disrupting the epithelial barrier. To test this notion, we first examined the effect of C. sakazakii interaction on the permeability of CaCo-2 cell monolayers, which make very good tight junctions compared with IEC-6 cells, which were used in previous studies from this laboratory (18, 19). CaCo-2 cells were grown in Transwell inserts and at a baseline of 300 V × cm2 were infected with OmpA+ C. sakazakii or OmpA− C. sakazakii for varying periods. The TEER and the permeability of monolayers using the HRP or FITC–dextran leakage method were measured (33, 34). Because the baseline TEER for various monolayers in different sets of experiments vary, the results are expressed as percentage of TEER. At 1 h postinfection, OmpA+ C. sakazakii interaction with CaCo-2 cells induced a 20% decrease in TEER from a baseline of 316 V × cm2 to 253 V × cm2, whereas the TEER decreased by >60% after 4 h postinfection (Fig. 3A). OmpA+ C. sakazakii induced only a 15% decrease in TEER across the monolayers even after 4 h postinfection (329 V × cm2 at baseline versus 277 V × cm2 at 4 h). In agreement with the TEER data, HRP leakage across CaCo-2 monolayers was gradually increased with OmpA+ C. sakazakii infection up to 4 h postinfection, whereas a minor leakage of HRP was observed with OmpA− C. sakazakii (Fig. 3B). To examine further the monolayer permeability in response to C. sakazakii infection, we used FITC-conjugated dextran, which provides a finer measurement of paracellular flux and help in assessing tight junction integrity independent of cell viability. On par with our HRP data, we observed significant leakage of FITC-conjugated dextran across CaCo-2 monolayers infected with OmpA+ C. sakazakii (Fig. 3C). In contrast, very minimal leakage of FITC-conjugated dextran was observed in OmpA− C. sakazakii infected CaCo-2 monolayers.

FIGURE 6. OmpA+ C. sakazakii causes tight junction disruption prior to apoptosis. In separate experiments, CaCo-2 cells were pretreated with apoptosis inhibitor ZVAD (10 μM) and then infected with C. sakazakii. TEER (A), HRP (B), and FITC-conjugated dextran leakage (C) was then measured. Apoptosis was measured in these cells after treatment with ZVAD by staining with annexin-V/7-AAD and examination by flow cytometry (D). The results represent mean ± SD from five separate experiments performed in triplicate. The decrease in TEER or increase in the permeability of HRP or FITC-conjugated dextran between ZVAD-treated and untreated cells was not statistically significant. *p < 0.01, **p > 0.05 by ANOVA and Student t test. OmpA+ CS, OmpA− C. sakazakii; OmpA− CS infected; OmpA− C. sakazakii.
To examine further whether any soluble factors released into the culture supernatants are responsible for the permeability of CaCo-2 cell monolayers, supernatants obtained from the bacteria/DC infection experiments after clearing the bacteria were used to treat CaCo-2 cells. The monolayer permeability was assessed in the presence or absence of bacteria. The MDCs were isolated and cultured from human donor monocytes as a surrogate for our in vitro model to match CaCo-2 monolayers. These MDCs were cultured with OmpA⁺ *C. sakazakii* or OmpA⁻ *C. sakazakii* for 24 h, and the supernatants from the cocultures were collected after centrifugation and filtered through an 0.22-μm filter to remove bacteria. The CaCo-2 monolayers were pretreated with the supernatants for 24 h prior to infection with *C. sakazakii*, and TEER, HRP, as well as FITC-conjugated dextran leakage was measured. Pretreatment with supernatant from OmpA⁺ *C. sakazakii*/DC coculture resulted in significantly faster decline (>60%) of TEER by 2 h compared with that of untreated monolayers or pretreated monolayers in the absence of bacteria (Fig. 3A). In contrast, the supernatant from OmpA⁻ *C. sakazakii*/DC coculture induced no such decline. The HRP leakage across the monolayer was also significantly increased after pretreatment with OmpA⁺ *C. sakazakii*/DC supernatant (Fig. 3B). Similar results were also observed with FITC-conjugated dextran assay (Fig. 3C). In agreement with permeability data, CaCo-2 cells infected with OmpA⁺ *C. sakazakii* demonstrated the disruption of ZO-1 staining at the periphery of the cells compared with the staining in untreated or OmpA⁻ *C. sakazakii*-treated cells (Fig. 4). These results suggest that the interaction of OmpA⁺ *C. sakazakii* with DCs produces factors that synergistically promote the disruption of tight junctions in enterocytes.

**OmpA⁺ *C. sakazakii*/DC supernatant pretreatment increases apoptosis of CaCo-2 cells**

We have previously demonstrated that *C. sakazakii* induced apoptosis by 6 h postinfection in IEC-6 cells (18, 19). To examine whether the presence of soluble factors in DC/*C. sakazakii* supernatants enhance enterocyte apoptosis, CaCo-2 cells were pretreated with OmpA⁺ *C. sakazakii*/DC supernatant, and the number of apoptotic cells was determined by TUNEL staining as well as by annexin-V/7-amino-actinomycin D (7-AAD) method. Pretreatment with OmpA⁺ *C. sakazakii*/DC supernatant for 24 h prior to incubation with OmpA⁺ *C. sakazakii* significantly increased enterocyte apoptosis compared with pretreatment with OmpA⁻ *C. sakazakii*/DC supernatant or untreated cells (*p* < 0.001 by ANOVA test) (Fig. 5A). In addition, with the pretreatment alone did not induce apoptosis in CaCo-2 cells. Quantitative determination of apoptotic cells revealed that pretreatment of CaCo-2 cells with OmpA⁺ *C. sakazakii*/DC supernatant increased apoptosis by 4-fold compared with OmpA⁻ *C. sakazakii*/DC supernatant and by 2-fold compared with OmpA⁺ *C. sakazakii* alone (Fig. 5B). Similar results were obtained with annexin-V/7-AAD and propidium iodide staining (Fig. 5C and data not shown). Our hypothesis is that tight junction disruption occurs first followed by apoptosis of cells in response to *C. sakazakii* infection. To examine this hypothesis, we pretreated CaCo-2 cells with apoptosis inhibitor ZVAD and then infected with *C. sakazakii*. We observed decrease in TEER with increase in postinfection time similar to untreated infected cells (Fig. 6A). In agreement, the leakage of HRP and FITC-conjugated dextran remained increased with ZVAD pretreatment similar to that of OmpA⁺ *C. sakazakii*-infected cells (Fig. 6B, 6C). However, there was a significant decrease in apoptosis of CaCo-2 cells after ZVAD pretreatment despite infection with OmpA⁺ *C. sakazakii* (Fig. 6D). These results indicate that changes in tight junction occur prior to the onset of apoptosis.

*C. sakazakii* infection has been demonstrated to induce NO in IEC-6 cells by triggering the activation of iNOS, which is shown to be responsible for apoptosis of the cells (19). Therefore, to examine whether OmpA⁺ *C. sakazakii*/DC supernatant increases the production of NO, and thus causes an increased apoptosis of CaCo-2 cells, the cells were treated with the supernatants in the presence or absence of *C. sakazakii*. The expression of iNOS and the production of NO were then determined by flow cytometry and Griess reagent, respectively (33). As predicted, CaCo-2 cells treated with OmpA⁺ *C. sakazakii*/DC supernatant prior to infection with *C. sakazakii* showed higher iNOS expression, and enhanced production of NO by 2.5- and 6.0-fold, respectively, compared with those of infected cells alone (Supplemental Fig. 1A, 1B). In contrast, treatment with OmpA⁻ *C. sakazakii*/DC supernatant had no effect on the production of NO. Taken together, these results suggest that OmpA⁺ *C. sakazakii*/DC supernatants contain soluble factors that promote tight junction disruption of CaCo-2 cells.
followed by apoptosis of cells by increasing the production of inducible NO.

**TGF-β production significantly increases postinfection with OmpA+ C. sakazakii in vitro and in vivo**

We previously demonstrated that the interaction of OmpA+ C. sakazakii with myeloid DCs in vitro results in increased levels of TGF-β production (30). In agreement, a significant rise in TGF-β levels was observed in the intestines of infected mice upon infection with OmpA+ C. sakazakii compared with the levels induced by OmpA+ C. sakazakii infection (Fig. 7A). These results were also confirmed by RT-PCR using total RNA extracted from the mucosal scrapings of intestines (data not shown). Similarly, BMDCs and intestinal DCs infected with OmpA+ C. sakazakii in vitro showed higher levels of TGF-β that OmpA+ C. sakazakii induce significant increase in TGF-β production (30). In agreement, a significant rise in TGF-β levels was observed in the intestines of infected mice upon infection with OmpA+ C. sakazakii compared with the levels induced by OmpA+ C. sakazakii infection (Fig. 7A). These results were also confirmed by RT-PCR using total RNA extracted from the mucosal scrapings of intestines (data not shown). Similarly, BMDCs and intestinal DCs infected with OmpA+ C. sakazakii in vitro showed higher levels of TGF-β consistent with what was observed in MDCs (Fig. 7B, 7C). We believe that the significant rise in the levels of TGF-β is a result of the higher recruitment of DCs promoted by the OmpA+ C. sakazakii. These results suggest that OmpA+ C. sakazakii invades, survives within, and suppresses the maturation of intestinal DCs similar to that of myeloid-derived DCs for which OmpA expression is critical.

**Blocking of TGF-β with a neutralizing Ab prevents OmpA+ C. sakazakii-induced epithelial monolayer damage and apoptosis**

To delineate the role of TGF-β in OmpA+ C. sakazakii infection, we performed the experiments using CaCo-2 monolayers in the presence of TGF-β-neutralizing Ab. Our previous studies have shown that OmpA+ C. sakazakii also induces significant production of IL-10 in MDCs, therefore we also used neutralizing Abs to IL-10 along with TGF-β (30). CaCo-2 cells express both TGF-β and IL-10 receptors (35, 36). The TGF-β-neutralizing Ab binds all three isoforms of the protein. The cells were pretreated with the corresponding neutralizing Ab for 1 h prior to infection with bacteria for varying periods. Blocking of IL-10 did not affect the OmpA+ C. sakazakii-induced enterocyte apoptosis, whereas neutralizing TGF-β abolished OmpA+ C. sakazakii-induced apoptosis (Fig. 8A). Also, monolayer permeability and electrical resistance decreased significantly upon infection with OmpA+ C. sakazakii despite neutralizing IL-10. However, pretreatment with TGF-β-neutralizing Ab prevented the effects of OmpA+ C. sakazakii on TEER and monolayer permeability (Fig. 8B–D). Addition of TGF-β or IL-10 alone in the absence of bacteria did not induce any apoptosis or barrier permeability (data not shown). These results confirm that TGF-β expression exacerbates C. sakazakii-induced injury in enterocytes and is the putative culprit in the DC/C. sakazakii coculture supernatant that causes an increase in OmpA+ C. sakazakii-induced epithelial cell monolayer permeability and apoptosis. To determine whether the change in the levels of TGF-β has any effect on increased iNOS expression observed with OmpA+ C. sakazakii infection, flow cytometry was performed. The production of NO in the culture medium in the presence of neutralizing Abs was determined using the Griess method. Notably, blocking TGF-β prevented the upregulation of iNOS expression in CaCo-2 cells despite infection with OmpA+ C. sakazakii (Supplemental Fig. 2A, 2B). In agreement with the iNOS expression, NO production also was significantly decreased in

**FIGURE 8.** Blocking of TGF-β activity by a neutralizing Ab prevents OmpA+ C. sakazakii-induced CaCo-2 monolayer integrity and apoptosis, whereas blocking IL-10 had no effect. Confluent monolayers of CaCo-2 cells were pretreated with neutralizing Abs to TGF-β (against all three isoforms) or IL-10 for 1 h prior to infection with OmpA+ C. sakazakii or OmpA- C. sakazakii. Blocking of TGF-β prevented OmpA+ C. sakazakii-induced apoptosis as measured by annexin-V/7-AAD staining followed by flow cytometry (A). CaCo-2 monolayers grown to confluence on Transwells were pretreated with neutralizing Abs, then TEER (B), HRP (C), and FITC-conjugated dextran leakage (D) was determined at various time periods. Neutralizing TGF-β activity blocked OmpA+ C. sakazakii-induced monolayer dysfunction, whereas neutralizing IL-10 had no effect. *p < 0.001 by ANOVA. OmpA+ CS, OmpA+ C. sakazakii; OmpA− CS, OmpA− C. sakazakii.
cells pretreated with anti–TGF-β Ab. By contrast, anti–IL-10 Ab treatment did not prevent the increase in iNOS expression. These results suggest that TGF-β secreted by CaCo-2 cells upon OmpA + C. sakazakii infection could be responsible for enhancing the tight junction disruption and increasing apoptosis of these cells by inducing NO production.

Presence of DCs exacerbates the effect of C. sakazakii-induced epithelial monolayer permeability in a “double-layer” model

To mimic the presence of DCs in the LP of intestine, we used a “double-layer” model as described previously (32) and examined OmpA + C. sakazakii-induced epithelial barrier injury. CaCo-2 cells were cocultured to form monolayers on Transwells, and then DCs were seeded on the opposite side of the Transwells to form “double layers”. Bacteria were added in the top chamber at an MOI of 10 (cell to bacteria ratio 1:10) and incubated for various time points. The presence of DCs resulted in a rapid decline in TEER in the double layers after 1 h of incubation with bacteria (Fig. 9A). TEER dropped from 60% at 1 h postinfection to 30% by 4 h postinfection in the presence of OmpA + C. sakazakii. The steep decline in TEER is mirrored by an increase in permeability as demonstrated by rapid rise in HRP leakage through the monolayers (~400 pg/ml without DCs versus ~750 pg/ml with DC cocultures at 4 h postinfection; p < 0.01 by ANOVA) (Fig. 9B). Similar rise in values of FITC-conjugated dextran was also observed (Fig. 9C). OmpA− C. sakazakii could not induce such rapid changes in TEER and permeability despite the presence of DCs. The iNOS expression and amount of NO released into the medium in cocultures was robustly increased in the presence of DCs (Supplemental Fig. 2C, 2D). In a follow-up experiments, DCs were collected from the double layers at 4 h postinfection, and their maturation marker profile was measured by flow cytometry using Abs to MHC class II, CD40, and CD86. As previously demonstrated, the DCs infected with OmpA + C. sakazakii showed suppression of these markers in the presence of OmpA + C. sakazakii. However, OmpA− C. sakazakii-infected as well as the LPS-treated double layers showed maturation of DCs in the bottom layer with significant rise in the levels of these markers (Fig. 9D). No bacteria were observed in the bottom chamber in these experiments, indicating that the suppression of DC maturation is not due to overspill of the bacteria from the top chamber.

To confirm the role of iNOS in CaCo-2 cell monolayer integrity during C. sakazakii infection, the cells were transfected with siRNA to iNOS. These iNOS siRNA-transfected CaCo-2 cells form monolayers similar to untransfected cells. However, iNOS siRNA-transfected CaCo-2 cells infected with OmpA + C. sakazakii do not...
show increase in expression of iNOS and therefore NO production compared with untransfected cells (Fig. 10A, 10B). The monolayer permeability and HRP leakage was significantly reduced when the CaCo-2 cells were transfected with siRNA to iNOS compared with that of untransfected cells (Fig. 10C–E). In addition, iNOS expression was significantly elevated in enterocytes upon OmpA⁺ C. sakazakii infection. Therefore, increased TGF-β expression in DCs postinfection with OmpA⁺ C. sakazakii primes the enterocytes leading to an increase in iNOS expression and thus more apoptosis. CaCo-2 cells also produce TGF-β upon infection with OmpA⁺ C. sakazakii but the quantities are not as high as the levels produced by the DCs (Supplemental Fig. 3). Therefore, the presence of DCs exacerbates the response to bacteria, and increased production of TGF-β by DCs appears to be involved in the observed barrier injury.

**Depletion of DCs in newborn mice protects the animals from OmpA⁺ C. sakazakii-induced NEC**

To substantiate the requirement of DCs for disease severity in the pathogenesis of NEC, DCs were depleted in newborn mice by injecting anti-CD11c Ab at day 1 after birth. Control animals received isotype-matched Ab. Animals received six injections of anti-CD11c Ab prior to infection with the OmpA⁺ C. sakazakii on day 3. We found that CD11c and control Ab when used at concentrations of 5 µg were not toxic in mice. We observed no differences in the number of neutrophils, macrophages, monocytes, or CD4 and CD8 T cells between CD11c or isotype-matched treated animals and untreated animals at these concentrations in various tissues (data not shown). DC depletion was confirmed in the intestine prior to infection as well as postinfection using anti-CD11c Ab by flow cytometry (Supplemental Fig. 4A). As predicted, 100% of the DC-depleted mice survived up to day 7 postinfection, whereas control Ab-treated/infected and wild-type (WT)/infected mice succumbed to infection within 96 h postinfection (Fig. 11A). The intestinal bacterial load was significantly lower in DC-depleted/infected mice (log 1.23 CFU/g tissue) compared with that of WT/infected (log 5.67 CFU/g tissue) or control Ab-treated/infected mice (log 5.51 CFU/g tissue, p < 0.01 by ANOVA) (Fig. 11B). Histopathological examination revealed that the DC-depleted animals showed normal mucosa, whereas severe infiltration of neutrophils along with destruction of villus was observed in WT/infected and control Ab-treated/infected mice (Fig. 11C). The average pathology score was 0.5 in DC-depleted/infected mice, whereas it was 3.6, 3.85, and

![FIGURE 10. Silencing of iNOS prevents OmpA⁺ C. sakazakii-mediated monolayer permeability. CaCo-2 cells were transfected with siRNA to iNOS or control siRNA and then cocultured with DCs. There was an increase in expression of iNOS (A) and hence NO production (B) in iNOS siRNA-transfected CaCo-2 cells infected with C. sakazakii. TEER (C), HRP (D), and FITC-conjugated dextran leakage (E) were then measured. The error bars represent SD of four individual experiments performed in triplicate. *p < 0.001 by Student t test and ANOVA. OmpA⁺ CS, OmpA⁺ C. sakazakii; OmpA− CS, OmpA− C. sakazakii.](http://www.jimmunol.org/)

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3.5 in WT/infected, DC-repleted/infected, or control Ab-treated/infected animals, respectively (Fig. 11D, p < 0.001 by Wilcoxon signed-rank test). Control Ab injection did not affect the course of infection, and the animals histologically were similar to WT/infected controls (Fig. 11C, D). The bacterial load in the intestines of mice from experiments described in A were determined by plating the tissue homogenates and expressed as log CFU per gram of intestinal tissue (B). Histopathological examination of intestines from DC-depleted mice demonstrated intact mucosal architecture. Meanwhile, the animals that received DCs by adoptive transfer demonstrate villus destruction and inflammation similar to WT/infected controls (C). The pathology scores in these animals are comparable with their histological findings (D). The intestinal epithelial cells from the animals were isolated by Percoll as described in Materials and Methods. The cells were stained with E-cadherin and annexin-V/7-AAD. The cells obtained from DC-repleted/infected animals show similar percentage of apoptosis as WT/infected or control Ab-treated and infected mice, whereas DC/depleted epithelial cells showed only basal-level apoptosis (E). The data represent mean ± SD from three independent experiments and at least 15 animals in each experiment. *p < 0.01 by ANOVA, Student t test, and Wilcoxon signed-rank test. CS, C. sakazakii.

We next examined the expression of iNOS and production of NO by enterocytes in infected mice. Enterocytes from DC-depleted mice showed significant decrease in the expression of iNOS, whereas those from WT or control Ab-treated animals showed upregulation of iNOS (Supplemental Fig. 4B). In agreement with these results, intestinal tissues of DC-depleted mice showed very low levels of TGF-β (Supplemental Fig. 4C). These findings suggest that C. sakazakii exploits DCs to cause intestinal injury by upregulating the expression of iNOS protein and local NO production in enterocytes. To confirm the deleterious role of DCs in C. sakazakii-mediated intestinal insult, mouse DCs were adaptively transferred to DC-depleted mice by i.p. injection before infecting with OmpA* C. sakazakii. Flow cytometry confirmed replenishment of DCs in the gut of these adoptively transferred mice based on CD11c staining (Supplemental Fig. 4A). DC-depleted mice repleted with DCs succumbed to infection within 96 h postinfection similar to WT/infected mice (Fig. 11A). Notably, there was no statistical difference in intestinal bacterial load of DC-replenished and WT/infected mice (Fig. 11B). Histopathological examination of intestinal tissues revealed severe villus destruction and recruitment of inflammatory cells and hence comparable severity scores as WT/infected mice (Fig. 11C, D). In agreement with these findings, severe apoptosis of enterocytes was also seen in DC-replenished mice assessed by annexin-V/7-AAD staining (Fig. 11E, p < 0.001 by ANOVA). Higher expression of iNOS and TGF-β production was also demonstrated in the intestines of DC-reconstituted mice (Supplemental Fig. 4B, 4C). These findings clearly indicate that OmpA* C. sakazakii infection and intestinal injury in animals is a result of manipulation of DC function and recruitment by this bacterium.

Discussion
In this study, we have reported three novel observations regarding the pathogenesis of C. sakazakii-induced NEC: 1) DC recruitment
to LP upon infection with _C. sakazakii_ is responsible for the intestinal barrier dysfunction; 2) TGF-β production by DCs enhances tight junction disruption and apoptosis of enterocytes; and 3) OmpA expression in _C. sakazakii_ is important for these pathological changes observed in the onset of NEC. In addition, we have demonstrated for the first time, to our knowledge, that _C. sakazakii_ can induce NEC in mice under normal conditions. The recruited DCs send dendrites outside the epithelium to sample environmental microorganisms without compromising epithelial barrier function. It has been shown that DCs express tight junction molecules such as ZO-1, occludins, and claudins, thus the integrity of the epithelial barrier should be preserved due to their recruitment (37). In contrast, our studies demonstrate that DC recruitment disturbed the tight junctions both in the intestines of newborn mice in vivo and in a double-layer model in vitro. Therefore, the disruption of tight junction integrity could be due to the interaction of _C. sakazakii_ with DCs, which may modulate the expression of tight junction molecules. Alternatively, the cytokines or chemokines secreted by either epithelial cells and/or DCs modulate the function of the epithelial barrier. In agreement with the latter concept, _C. sakazakii_ interaction with DCs induced greater levels of cytokines such as IL-10 and TGF-β. Our data demonstrate that TGF-β induces tight junction disruption in the presence of _C. sakazakii_ as well as apoptosis of enterocytes. Although we did not observe bacterial traversing across the porous membrane used in Transwell experiments, _C. sakazakii_ could still modulate the function of DCs. Therefore, the interaction of _C. sakazakii_ with DC dendrites appears to be sufficient to suppress their activation. The presence of PMNs and macrophages does not appear to play a role in the _C. sakazakii_ infection in vivo as our studies demonstrated that the absence of PMNs or macrophages accelerates the course of infection (C.N. Emami, R. Mittal, L. Wang, H.R. Ford, and N.V. Prasadarao, unpublished observations). This is, to our knowledge, the first report to show that TGF-β causes damage to the epithelial barrier in the presence of a bacterial pathogen in the intestine.

A critical mediator of the inflammatory response associated with the pathogenesis of NEC is NO (38). High quantities of NO exert cytopathic effects on the intestine (39). Previous studies from our laboratory have shown that feeding _C. sakazakii_-contaminated formula to newborn rat pups causes severe pathological changes similar to NEC by inducing apoptosis of enterocytes under hypoxic conditions (18, 19). Attachment of _C. sakazakii_ to IEC-6 cells increased iNOS expression and NO production, which is shown to be responsible for causing enterocyte apoptosis. Although it was shown that release of bacterial LPS leads to prevention of enterocyte migration through activation of RhoA in a SHP-2-dependent manner, we demonstrate that despite having similar amounts of LPS as those of OmpA+ _C. sakazakii_, OmpA+ _C. sakazakii_ did not induce NEC (40, 41). It is noteworthy that anti-inflammatory cytokines IL-10 and TGF-β traditionally exhibit an inverse relationship with regard to iNOS activation (42–45). Nonetheless, our studies demonstrate that the supernatant from OmpA+ _C. sakazakii_/DCs, which contains a significantly high level of TGF-β, augments NO production, which in turn induces apoptosis and barrier injury. Of note, Maheswari et al. showed that TGF-β, particularly TGF-β2, is protective against NEC, which is in contrast to our data observed in this study (46). However, a hypoxia model of NEC has been used in this study without any bacterial component. Therefore, it is possible that OmpA+ _C. sakazakii_ induces greater quantities of TGF-β in the intestine, which along with other bacterial virulence factors contribute to the onset of NEC in mice.

The level of IL-10 produced by DCs upon _C. sakazakii_ infection did not affect epithelial apoptosis or barrier dysfunction. Therefore, the mechanism of _C. sakazakii_-induced epithelial injury might be distinctly tied to the expression and activity of TGF-β produced by a larger number of recruited DCs. The specific mechanism of this interaction is currently being elucidated. In addition, although TGF-β can be produced by macrophages recruited upon _C. sakazakii_ infection, depletion of these cells led to a higher number of mucosal DCs being recruited upon infection and an exaggerated response to _C. sakazakii_ infection (data not shown). Substantiating the role of DCs in _C. sakazakii_-induced NEC, depletion of DCs in the newborn pups protected against OmpA+ _C. sakazakii_-induced injury. Furthermore, adoptive transfer of DCs in DC-depleted animals resulted in reinfection, demonstrating that DCs are both necessary and sufficient for _C. sakazakii_-induced NEC in these animals.

Host–bacteria interactions play an important role in the pathogenesis of NEC. Bacteria-derived products activate TLR on intestinal epithelial cells resulting in activation of the inflammatory cascade that invariably leads to NEC (47). Of note, _C. sakazakii_ that does not express OmpA could not elicit the production of NO from intestinal epithelial cells despite the presence of LPS on its surface. Therefore, OmpA may bind a receptor on enterocytes to induce iNOS activation. It is possible that the OmpA receptor in enterocytes could be modulated by TGF-β produced by both DCs and enterocytes. Because OmpA is present in all _Cronobacter_ strains, it is possible that other virulence factors such as type VI secretion pathways, a newly described mechanism for protein transport across the membrane of Gram-negative bacteria that can enhance the apoptotic machinery in epithelial cells, may be expressed upon the interaction of OmpA with enterocytes (48–50).

In summary, we have demonstrated a novel observation that OmpA+–_C. sakazakii_ interaction with DCs exacerbates the production of NO in enterocytes and induces intestinal epithelial cell monolayer leakage and apoptosis. This interaction involves a significant increase in the levels of anti-inflammatory cytokines TGF-β and IL-10. However, the presence of TGF-β seems to be required for upregulation of iNOS and the ensuing epithelial injury. Identification of receptors on enterocytes to which OmpA of _C. sakazakii_ binds and/or inhibition of _C. sakazakii_ interaction with DCs can lead to the development of novel therapeutic strategies for preventing _C. sakazakii_-induced NEC.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**
