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Nucleotide-Binding Oligomerization Domain 1 Mediates Recognition of Clostridium difficile and Induces Neutrophil Recruitment and Protection against the Pathogen

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Clostridium difficile is a Gram-positive obligate anaerobic pathogen that causes pseudomembranous colitis in antibiotics-treated individuals. However, host immune protective mechanisms against C. difficile are largely unknown. In this study, we show that C. difficile possesses potent stimulatory activity for nucleotide-binding oligomerization domain 1 (Nod1), an intracellular pattern recognition molecule that senses bacterial peptidoglycan-related molecules. Nod1−/−, but not Nod2−/−, mice exhibited increased lethality in response to C. difficile intestinal infection despite comparable levels of intestinal damage and epithelial permeability in Nod1−/− and control mice. The enhanced lethality was accompanied by impaired C. difficile clearance, increased bacterial translocation, and elevated levels of endotoxin and IL-1p in the serum of Nod1−/− mice. Histological and flow cytometric analyses revealed that Nod1−/− mice had defective recruitment of neutrophils, but not macrophages, to the intestine after C. difficile infection. The reduced recruitment of neutrophils correlated with impaired production of CXCL1, but not CCL2, XCL1, and other cytokines/chemokines, in infected Nod1−/− mice. The influx of neutrophils also was reduced when C. difficile was administered i.p., suggesting that Nod1 directly recognizes C. difficile to induce the recruitment of neutrophils to the infected site. These results indicate that Nod1 regulates host susceptibility to C. difficile and suggest that Nod1-mediated neutrophil recruitment is an important immune response against the enteric pathogen. The Journal of Immunology, 2011, 186: 4872–4880.

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Abbreviations used in this article: BHI, brain–heart infusion; BMIM, bone marrow-derived macrophage; DGGE, denaturing gradient gel electrophoresis; HEK293T, human embryonic kidney 293T; MDP, muramyl dipeptide; MLN, mesenteric lymph node; Nod1, nucleotide-binding oligomerization domain 1; Nod1−/−, Nod1-deficient; PRR, pattern recognition receptor; TcdA and TcdB that mediate damage to the intestinal epithelium and subsequent induction of local and systemic inflammation. Although infected patients suffer from inflammation, host responses to C. difficile are protective because immunocompromised and elderly individuals are more susceptible to the morbidity and mortality from C. difficile infection. However, the host mechanisms that mediate recognition and immune protection against C. difficile remain largely unknown.

Mounting evidence indicates that innate immune responses mediated by bacteria-recognizing receptors provide the first line of defense against bacterial pathogens. These host receptors include pattern recognition receptors (PRRs), which are involved in the detection of specific microbial molecules that are commonly found in pathogens. Upon activation, they induce resistance against a wide array of pathogens at the early stage of infection (2). PRRs include membrane-bound TLRs and cytosolic nucleotide-binding oligomerization domain (Nod)-like receptors, RIG-I-like receptors, and AIM2-like receptors that are involved in host recognition of bacteria and viruses (2, 3). Stimulation of these PRRs by pathogens mediates the induction of several immune molecules, including antimicrobial peptides, cytokines, chemokines, and adhesion molecules through the activation of the transcription factors NF-kB, AP-1, and Elk-1 or the induction of type I IFNs through IFN regulatory factors (2). The PRRs stimulated by C. difficile have not been identified, although MyD88, an essential mediator for TLR/IL-1/IL-18 receptor signaling, has been suggested to play a role in the pathology associated with C. difficile-induced colitis (4). Moreover, the role of PRRs in the clearance and resistance against C. difficile remains unknown. In this study, we show that C. difficile is recognized by Nod1 to stimulate production of CXCL1. Importantly, mice lacking Nod1 were more susceptible to C. difficile. Although the epithelial damage caused by pathogenic intestinal C. difficile infection was similar in Nod1-deficient (Nod1−/−) and wild-type (WT) mice, Nod1−/− mice showed impaired neutrophil recruitment and bacterial clearance. To our knowledge, this is the first demonstration of an innate PRR that is critical for host defense against C. difficile.
Materials and Methods

Bacteria strains, culture cells, plasmids, and synthetic immunostimulatory molecules

C. difficile strains 630, VPI 10463, and VPI 11186 and Clostridium tetani ATCC700157 were obtained from the American Type Culture Collection (Manassas, VA). Bacillus cereus N1464, B. pumilus N1408, Escherichia coli K-12, Listeria monocytogenes EGD, Salmonella typhimurium SL134, and Staphylococcus aureus ATCC25923 were described previously (5). C. difficile and C. tetani were cultured and maintained at 37°C in brain–heart infusion (BHI) medium (BD Biosciences, Franklin Lakes, NJ) in an anaerobic environment (BBL GasPak Plus system; BD Biosciences), whereas other bacteria were grown at 37°C in BHI medium under aerobic conditions. Human embryonic kidney 293T (HEK293T) cells were cultured and transfected as described (5). The vector pcDNA3, the expression plasmids producing NOD1 and NOD2, and reporter plasmids as well as the synthetic compounds y-glutamyl-meso-diaminopimelic acid, muramyl dipeptide (MDP), and KF1B have been described (5). E. coli O55:B5 LPS was purchased from Sigma-Aldrich (St. Louis, MO). LPS was free of contamination with Nod1- and Nod2-stimulatory activity as detected by the HEK293T bioassay as described (5).

Preparation of culture supematant and heat-inactivated bacterial cell extracts

All of the bacteria were cultured at 37°C for 48 h as described above. Bacterial growth was monitored by measuring OD at 600 nm. The culture supernatant was prepared by filtration though a sterilizing filter (pore size 0.22 μm; Corning, Corning, NY), followed by removal of bacterial cells by centrifugation at 15,000 rpm for 10 min at 4°C. The bacterial pellets were washed with water once, resuspended in the original volume of water, and heat-inactivated at 95°C for 30 min.

Mice

C57BL/6 were obtained from The Jackson Laboratory (Bar Harbor, ME). Nod1−/− and Nod2−/− mice on a C57BL/6 background were described previously (6, 7). All of the mice were housed, bred, and fed Laboratory Rodent Diet 5001 (LabDiet; Purina Mills) under specific conditions. All of the mice were infected by oral challenge. All of the mice were infected by oral challenge. All of the mice were infected by oral challenge. All of the mice were infected by oral challenge.

Induction of neutrophil recruitment by i.p. administration of C. difficile

A total of 107 CFU C. difficile in 100 μl PBS were injected into the peritoneal cavity of 8-wk-old WT and Nod1−/− mice. To assess recruitment of immune cells, peritoneal cells were collected from C2E- euthanized mice 2 h postinjection by PBS lavage. After centrifugation (Cytospin 2; Shandon), cells were fixed and stained with a commercial kit (Diff; Dade Behring) according to the manufacturer’s recommendations. Cell types were determined by morphological features of stained cells as described (10).

Intestinal permeability

Mice were fasted for 4 h prior to the administration of 0.6 mg/g FITC-dextran (4 kDa; Sigma-Aldrich). Serum was collected 4 h later and diluted 1:3 in PBS, and the amount of fluorescence was measured using a fluorescence spectrophotometer with emission at 488 nm and absorption at 525 nm.

Preparation of intestinal immune cells

Cecum and colon were removed and washed with PBS. The tissues were cut into small pieces and washed three times in calcium and magnesium-free HBSS (Sigma-Aldrich) containing 0.5% heat-inactivated FBS (BioSource, Camarillo, CA). After being washed, tissues were incubated in HBSS containing 1 mM DTT (Sigma-Aldrich) for 15 min at room temperature to remove mucous and then incubated twice in HBSS containing 1 mM EDTA (Sigma-Aldrich) for 45 min at 37°C to remove epithelial cells. After being washed three times with HBSS, tissues were collected and incubated in HBSS containing 40 U/ml collagenase type 3 and 0.1 mg/ml DNase I ( Worthington Biochemical, Freehold, NJ) for 90 min at 37°C. The digested samples were resuspended in a 40% Percoll solution (Amersham Biosciences, Piscataway, NJ) and then layered on 75% Percoll before centrifugation at 2000 rpm for 20 min at room temperature. Viable cells were recovered from the 40–75% layer interface.

Flow cytometric analysis

Cell surface fluorescence was assessed using a FACSCalibur analyzer and analyzed using FlowJo software (Tree Star). Dead cells were excluded with 7-aminoactinomycin D staining. FITC-labeled mAb against CD11b (M1/ 70) and allopurinol-cyanin-labeled mAb F4/80 (BMS) were from eBioscience. PE-labeled Ly6G mAb (1A8) was purchased from BD Biosciences. Isotype-matched Abs (BD Biosciences) were used for control staining.

Immunostimulation assay

For the stimulation of mouse cells, bone marrow-derived macrophages (BMMs) and mesothelial cells were prepared from 6-wk-old mice as described (10). The homogenized tissue samples were heated at 98°C for 30 min. Samples diluted 1/10 were incubated with 1 × 106 BMMs or 2 × 104 mesothelial cells in 0.2 ml medium. After 12 h of stimulation, levels of CXCL1 and IL-6 in the culture supernatant were determined by ELISA (BD Pharmingen, San Diego, CA). Nod1−, Nod2−, or TLR4-specific stimulatory activity was determined by the HEK293T bioassay as described (5).

Limulus amebocyte lysate assay

LPS levels in homogenized tissue samples were determined using the Limulus amebocyte lysate assay kit (Lonza, Walkersville, MD) in accordance with the manufacturer’s instructions.

Statistical analysis

Statistical significance between groups was determined by two-tailed t test with unequal variance (Aspin–Welch t test). The survival rate of infected mice was analyzed using the log-rank test. Differences were considered significant when p values were <0.05.

Results

C. difficile possesses potent Nod1-stimulatory activity

To begin to assess how C. difficile is recognized by host cells, we determined the ability of C. difficile to induce CXCL1 and IL-6 in primary mesothelial cells and BMMs that are representative of
nonphagocytic and phagocytic cells, respectively. C. difficile induced more CXCL1 and IL-6 secretion in mesothelial cells than in macrophages (Fig. 1A, 1B). In contrast, both cell types secreted IL-6 upon KF1B (Nod1 agonist) stimulation in the presence of the limited amounts of LPS. These results suggest that the C. difficile-stimulating host receptors are expressed primarily in nonimmune cells (Fig. 1A, 1B). Furthermore, both toxigenic (630) and non-toxin-producing (VP11188) strains of C. difficile induced CXCL1 secretion, suggesting that the secretion of CXCL1 was independent of TcdA and TcDb. Because mesothelial cells are known to be highly sensitive to stimulation with Nod1 agonists and C. difficile is a Gram-positive bacterium that produces mesodiamino-pimelic acid-type peptidoglycan (5, 11), we hypothesized that C. difficile possesses Nod1-stimulatory activity. Indeed, we found that mesothelial cells prepared from Nod1−/− mice produced significantly less IL-6 and CXCL1 than cells from WT mice (Fig. 1A, 1B). Moreover, C. difficile produced high levels of Nod1-stimulatory activity (26.3 ± 7.3 kU/ml culture) (Fig. 1C), which are as high as those found in Bacillus species, which have been reported to express very high levels of Nod1-stimulatory activity (5). Unlike Bacillus species, however, C. difficile had trace levels of Nod2-stimulatory activity (Fig. 1C), indicating that not all of the Clostridium species produce high levels of Nod1-stimulatory molecules. Previous studies demonstrated that soluble Nod1-stimulatory molecules are released from bacterial cells to stimulate nonphagocytic cells (5, 10). Consistently, the major Nod1-stimulatory activity for mesothelial cells, as detected by CXCL1 production, was found in the culture supernatant of C. difficile rather than in the bacterial pellet (Fig. 1A, 1D). Collectively, these findings indicate that C. difficile produces high levels of Nod1 ligand, which is highly stimulatory for nonimmune cells.

**Nod1-dependent recognition of C. difficile and neutrophil recruitment**

The above in vitro studies suggested that C. difficile-induced cytokine/chemokine secretion is mainly dependent on Nod1. We tested next whether C. difficile-induced CXCL1 secretion was dependent on Nod1 in vivo by injecting bacteria into the i.p. cavities of Nod1−/− and WT mice. Both toxigenic (630 and VPI 10463) and toxinless (VP11188) C. difficile strains induced transient secretion of CXCL1 in WT mice, and this response was impaired in Nod1−/− mice (Fig. 2A–C). Thus, production of CXCL1 induced by C. difficile infection is not toxin-mediated, which is consistent with the fact that Nod1-stimulatory molecules are small peptidoglycan-related molecules (6, 10). In addition, i.p. infection of WT mice with C. difficile induced an influx of neutrophils that was greatly diminished in Nod1−/− mice (Fig. 2D). These results indicate that C. difficile induces CXCL1 secretion and neutrophil recruitment via Nod1. Although IL-17 has been reported to mediate neutrophil recruitment in a model of chemically induced colitis (12) and after i.p. infection with E. coli (13), we failed to detect IL-17 in the peritoneal fluid or serum of mice infected with C. difficile or injected with KF1B (data not shown). These results suggest that Nod1 mediates direct recognition of C. difficile and induces neutrophil recruitment via CXC chemokine(s).

**Nod1−/− mice are susceptible to C. difficile**

To determine the role of Nod1 in C. difficile-associated disease, we infected WT and Nod1−/− mice with C. difficile by gastric gavage (14). Mice received antibiotics for 5 d prior to infection and were infected with 10⁶ CFU C. difficile VPI10463, a strain that produces TcdA and TcDb. Notably, 91% of the WT mice survived, whereas 38% of the Nod1−/− mice succumbed to infection (Fig. 3A). In contrast, Nod2−/− mice showed no significant increase in susceptibility to C. difficile infection (Supplemental Fig. 1). Consistent with previous reports (4, 14), antibiotic treatment was required for lethality because all of the mice that were infected with 10⁶ or 10⁷ CFU C. difficile without antibiotic treatment survived (data not shown). Lethality of the Nod1−/− mice was observed within 3 d after C. difficile infection, suggesting that protection against infection is mediated through innate mechanisms. Histological analysis revealed that C. difficile infection in mice pretreated with antibiotics was associated with pathology largely confined to the cecum and colon and characterized by epithelial damage, marked submucosa edema, and infiltration of acute inflammatory cells into the submucosa and lamina propria (Fig. 4). There was a slight increase in the overall pathology score in WT mice when compared with that in Nod1−/− mice, but the difference did not reach statistical significance (Fig. 3B). We did not find significant differences in the levels of epithelial damage between WT and Nod1−/− mice (Fig. 3C).

**Reduced infiltration of neutrophils in the intestine of Nod1−/− mice infected with C. difficile**

Although the overall colitis score after C. difficile infection that includes such parameters as epithelial cell damage and infiltration by inflammatory cells including macrophages, neutrophils, and lymphocytes was similar in WT and Nod1−/− mice, close inspection of the histology of the cecum/colon in infected Nod1−/− and WT mice revealed reduced numbers of neutrophils in the cecum/colon of Nod1−/− mice (Fig. 4). Because no neutrophils were present in the intestines of both Nod1−/− and WT control mice before and after antibiotic treatment (data not shown) in the absence of C. difficile infection, these results suggested that Nod1 might affect neutrophil recruitment after C. difficile infection. To confirm this, we analyzed the neutrophil population in the colon and cecum of infected Nod1−/− and WT mice by flow cytometry (Fig. 5). The number of CD11b+Ly6G− cells (typical monocyte/macrophage) was similar in WT and Nod1−/− mice (Fig. 5A, left panels). Similarly, there were no significant differences in CD11b+ F4/80+ markers (CD11b+ macrophage). In contrast, the number of CD11b+Ly6G− and CD11b+F4/80− cells (CD11b− neutrophils) was greatly increased in WT mice infected with C. difficile compared with WT control mice (Fig. 5B, right panels). Together, these observations suggest that Nod1 regulates the recruitment of neutrophils in response to C. difficile infection in the intestine.

**Nod1 regulates the clearance of C. difficile and prevents translocation of commensal bacteria**

Because neutrophils are known to be important for the elimination of bacteria, we next determined if Nod1 plays a role in the clearance of C. difficile. Notably, there was a modest but significant increase (~5-fold) in the number of C. difficile in the feces of infected Nod1−/− mice compared with that in WT mice (Fig. 6A). Because C. difficile induced severe epithelial damage in both WT and Nod1−/− mice (Fig. 4), we examined the extent of bacterial translocation from the intestine of infected mice to other organ sites. Bacteria were detectable in the spleen, kidney, MLNs, and lung of both Nod1−/− and WT mice after C. difficile infection (Fig. 6B). Notably, more bacterial translocation was observed in Nod1−/− mice than in WT mice (Fig. 6B). Total bacterial numbers were similar in the feces of Nod1−/− and WT mice (Fig. 6B), eliminating the possibility that increased translocation is due to different numbers of intestinal bacteria in Nod1−/− mice. Consistent with
enhanced translocation, the levels of endotoxin and IL-1β in the serum were increased to a greater extent in Nod1/−/− and control WT mice after C. difficile infection (Fig. 6C, 6D). To determine if neutrophils can regulate bacterial translocation, we treated mice with a mAb that depletes neutrophils or a control Ab and challenged antibiotic-treated mice orally with C. difficile. We found that depletion of neutrophils enhanced bacterial translocation after C. difficile infection (Fig. 6E). These results suggest that reduced neutrophil recruitment in Nod1/−/− mice increases bacterial translocation after C. difficile infection.

FIGURE 1. C. difficile possesses Nod1-stimulatory activity. CXCL1 (A) and IL-6 (B) production from mesothelial cells and BMMs (B) from Nod1/−/− and control WT mice were determined by ELISA. Mesothelial cells and BMMs were infected with C. difficile or S. aureus at multiplicity of infection of 1:100 (living bacteria) or incubated with 100-fold diluted bacterial cell extract or medium supernatant from overnight cultures of the bacteria or left untreated. As controls, mesothelial cells were incubated with 5 μg/ml synthetic Nod1 ligand KF1B, 5 μg/ml synthetic Nod2 ligand MDP, or 50 ng/ml E. coli LPS. BMMs were incubated with 10 ng/ml E. coli LPS with or without 5 μg/ml KF1B. Twenty-four hours after stimulation, the levels of CXCL1 and IL-6 were determined by ELISA. C and D, The Nod1- and Nod2-stimulatory activity of heat-inactivated indicated bacteria (C), bacterial cell pellet, and culture supernatant (D) were determined by the HEK293T bioassay as described in Materials and Methods and given as kU/ml. The stimulatory activity of bacteria is given as kU per milliliter of the original culture volume. One unit of the Nod1- and Nod2-stimulatory activity is equivalent to that of 1 ng of synthetic γ-δ-glutamyl-meso-diaminopimelic acid and MDP, respectively. *p < 0.01.

FIGURE 2. Nod1-dependent recognition of C. difficile and neutrophil recruitment. A–C, WT and Nod1/−/− mice were infected with C. difficile VPI 10463 (A), 630 (B), or VPI 11186 (C) i.p. Serum levels of CXCL1 at indicated times after i.p. injection were determined by ELISA. D, Twenty-four hours postinfection, total peritoneal cells were obtained (in this experiment 1.2 × 10^7 ± 7 × 10^6 and 1.0 × 10^7 ± 6 × 10^6 cells from WT mice and Nod1/−/− mice, respectively), stained, and analyzed histologically. Randomly chosen representative fields of peritoneal cells from WT mice and Nod1/−/− infected with C. difficile were counted. The results are representative of at least three experiments. *p < 0.001.
Nod1 does not regulate intestinal permeability in C. difficile-infected mice

We next determined whether oral infection with C. difficile affected levels of Nod1-, Nod2-, and TLR4-stimulatory activity within the feces. The analysis revealed that fecal Nod1-stimulatory activity increased after C. difficile infection, whereas there was no significant increase in fecal Nod2- or TLR4-stimulatory activity (Supplemental Fig. 2). Because the composition of the gut microbiota may affect susceptibility to C. difficile infection, we examined the microbiota of Nod1<sup>−/−</sup> and control mice before and after C. difficile infection by denaturing gradient gel electrophoresis (DGGE) (15). We found that the composition of the intestinal microbiota was comparable in Nod1<sup>−/−</sup> and heterozygous Nod1<sup>+/-</sup> littermates in the absence of infection (Supplemental Fig. 3A). Furthermore, Nod1<sup>−/−</sup> and WT mice exhibited similar microbiota after C. difficile infection (Supplemental Fig. 3B). Together with the above findings, these results suggest that Nod1 protects mice from C. difficile-induced lethality, which cannot be explained by obvious differences in the composition of the intestinal microbiota in Nod1<sup>−/−</sup> and WT mice.

To dissect whether the increased bacterial translocation in Nod1<sup>−/−</sup> mice is due to greater intestinal permeability, we assessed the intestinal permeability to orally administered FITC-dextran in infected mice. No significant difference in FITC-dextran permeability was found between WT and Nod1<sup>−/−</sup> mice (15 ± 29 and 15 ± 12 fluorescence units per microliter of serum, respectively, after C. difficile infection; 1.2 ± 0.4 and 1.1 ± 0.2 fluorescence units per microliter of serum, respectively, before infection; n = 7 per group). These results suggest that the increased bacterial translocation observed in Nod1<sup>−/−</sup> mice is not due to differences in intestinal permeability.

Impaired CXCL1 production in cecum and colon of Nod1<sup>−/−</sup> mice infected with C. difficile

Our previous studies showed that neutrophil-recruiting chemokines (CXCL1, CXCL2, and human IL-8) are secreted from non-phagocytotic cells such as fibroblasts and intestinal epithelial and mesothelial cells upon Nod1 stimulation (5, 10). Therefore, we hypothesized that neutrophil recruitment induced by C. difficile infection is mediated by CXC chemokines. Indeed, we detected the production of CXCL1 in the serum of WT mice after C. difficile infection, which was reduced significantly in Nod1<sup>−/−</sup> mice (Fig. 7A). The reduced production of CXCL1 was specific to Nod1<sup>−/−</sup> mice because Nod2<sup>−/−</sup> mice showed similar levels of

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Nod1<sup>−/−</sup> mice are more susceptible to C. difficile. A, Survival of WT (n = 28) and Nod1<sup>−/−</sup> (n = 23) mice infected with C. difficile was monitored for 14 d. All of the mice were infected by gastric gavage with 10<sup>8</sup> CFU C. difficile strain VPI 10463 after antibiotic treatment as described in Materials and Methods. No further deaths were observed beyond 5 d postinfection. *p = 0.0002. B and C, The overall histology (B) and epithelial damage (C) scores are based on the analysis of 12 WT control and 13 Nod1<sup>−/−</sup> mice at day 2 postinfection.

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Histological features of mouse cecum infected with C. difficile. Histology of the cecum from representative Nod1<sup>−/−</sup> (B, D) and WT mice (A, C) that were intragastrically infected with 10<sup>8</sup> CFU C. difficile stained with H&E. The arrows and arrow heads show submucosal edema and epithelial damage, respectively (A and B, original magnification ×200). Polymorphonuclear neutrophils are indicated by arrows (C and D, original magnification ×400).
CXCL1 in the serum after *C. difficile* infection compared with those in WT mice (Supplemental Fig. 1B). Unlike CXCL1, the CCL2 levels in serum were not increased significantly after *C. difficile* infection (data not shown). In addition, we observed an increase in the levels of CXCL1, IL-1β, and IL-17 in the cecum and colon, but not in the small intestine, of infected mice (Fig. 7B–D), which is consistent with known localization of *C. difficile* in humans (1). Importantly, the amounts of CXCL1 in the cecum and colon were reduced significantly in infected Nod1−/− mice to levels as low as those observed in uninfected WT mice (Fig. 7B). There was a trend for reduced levels of IL-1β in the intestinal tissue of infected Nod1−/− mice when compared with those of WT mice, but the difference did not reach statistical significance (Fig. 7C). Nor was there any difference in the levels of IL-17, CCL2, and IL-10 between WT and Nod1−/− mice (Fig. 7D–F). Moreover, significant levels of IFN-γ, XCL1, TNF-α, and IL-18 were detected in the small intestine, cecum, and colon independent of *C. difficile* infection at the baseline (Supplemental Fig. 4). Real-time PCR analysis also showed that the expression levels of CCL20 and CXCL13 genes were not changed before or after *C. difficile* infection (Supplemental Fig. 4). This suggests that the secretion of IFN-γ, IL-1β, IL-10, IL-17, IL-18, TNF-α, XCL1, CXCL13, and CCL20 are independent of Nod1. Collectively, these results indicate that *C. difficile* specifically induces the production of CXCL1 via Nod1.

**Discussion**

*C. difficile* resides in the human intestine and overgrows after antibiotic treatment to cause intestinal damage, inflammation, and clinical disease. Infection with *C. difficile* to mice that were treated with a mixture of antibiotics was associated with marked inflammation and epithelial damage in the cecum and colon, which is consistent with previous studies (4, 14). In this study, we provide evidence that Nod1 recognizes *C. difficile* and regulates...
susceptibility to this pathogen. Although the levels of epithelial damage and permeability as a result of *C. difficile* infection were similar in both WT and Nod1Δ/Δ mice, there were significantly increased numbers of translocating commensal bacterial in the peripheral tissues and increased serum levels of endotoxin and IL-1β in infected Nod1Δ/Δ mice. Together, these results suggest that during *C. difficile* infection Nod1 does not affect the intestinal membrane barrier itself but instead regulates the translocation of bacteria across the intestine or the survival of commensal bacteria in tissues. Because depletion of neutrophils increases the translocation of bacteria after *C. difficile* infection, one possibility is that the protective effect of Nod1 is mediated through the recruitment of neutrophils to intestinal sites induced in response to *C. difficile* infection. Another nonexclusive possibility is that Nod1 regulates the killing of commensal bacteria at peripheral tissues via innate mechanisms or that Nod1 alters the neutrophil function or engulfment of bacteria (16, 17). Regardless of the mechanisms involved, we suggest that the increased number of bacteria in the tissue of Nod1Δ/Δ mice triggers a harmful proinflammatory response including increased production of IL-1β that promotes the demise of *C. difficile*-infected Nod1Δ/Δ animals. Nod1Δ/Δ mice also had a slightly higher number of *C. difficile* in feces than WT mice. However, further investigation is required to understand the mechanism responsible for reduced clearance of *C. difficile*. The recognition of immunostimulatory molecules produced by *C. difficile* induces the secretion of neutrophil-recruiting chemo-
kines and host defense responses. These results are consistent with previous studies that showed that Nod1 stimulation preferentially induces cell-type–specific responses to bacterial components and specifically induces CXC chemokine secretion from nonimmune cells (10), although the mechanism of this specificity is not well understood. 

C. difficile was found to stimulate CXCL1 production both in vitro and in nonphagocytic mesothelial cells and in vivo after Nod1 stimulation. Nod1 is known to confer resistance against multiple bacteria including Helicobacter pylori, Listeria monocytogenes, Pseudomonas aeruginosa, and Staphylococcus aureus through the induction of chemokines, cytokines, and antimicrobial molecules (17, 27–30). Some of these bacteria are intracellular cytotoxic pathogens or have the ability to deliver immunostimulatory molecules into the host cytosol. However, previous studies demonstrated that extracellular Nod1-stimulatory molecules such as peptidoglycan-related peptides and disaccharide peptides possess a high ability to stimulate Nod1, suggesting that the intracellular localization of bacterial molecules is not required for Nod1 stimulation (5, 6). Although the mechanism by which extracellular microbial molecules stimulate Nod1 remains poorly understood, it is clear that Nod1 agonists are released from proliferating bacteria, including C. difficile (this study) and E. coli (5, 31). Because the production of Nod1 agonists does not require the engulfment of bacteria by phagocytic cells, our results suggest a role for nonphagocytic cells such as epithelial, mesothelial, endothelial cells and fibroblasts in innate immune responses against pathogenic bacteria via Nod1 stimulation. Although Nod1 was found to be a dominant sensor to induce chemokine secretion from mesothelial cells, Nod1−/− mesothelial cells were still capable of secreting CXCL1 at low levels, suggesting a role for additional host receptors in CXCL1 production. C. difficile did not possess Nod2-stimulatory activity, and Nod2−/− mice showed a similar susceptibility to infection as that of WT mice, suggesting that Nod2 is dispensable for host defense against the pathogen. A previous study, however, revealed a role for MyD88-mediated signaling in C. difficile infection, but the effect was observed relatively late (9 d postinfection) in mice pretreated with antibiotics (4). This suggests that TLRs and/or IL-1/IL-18 signaling mediate host responses against C. difficile at a later stage of infection than that mediated via Nod1.

TcdA and/or TcdB produced by C. difficile compromise the epithelial cell barrier by inducing disaggregation of actin microfilaments in colonocytes via glucosylation of the Rh family of proteins, causing epithelial cell destruction and opening of tight junctions (1). Several studies have shown that TcdA and TcdB activate intracellular mediators NF-kB and MAPK leading to cytokine secretion in vitro (32). However, our study shows comparable induction of CXCL1 secretion by toxin- and non-toxin-producing C. difficile strains. Therefore, the primary function of these intracellular mediators is to induce immune responses via Nod1 but not to mediate cell-damaging effects of the toxins. Our results suggest that pharmaceutical inhibition of these mediators may not provide therapeutic benefit because of the inhibitory effect on bacteria elimination by neutrophils.

In this study, we demonstrate that local Nod1 stimulation induces a host defense response against C. difficile. Because Nod1 stimulation is also protective against several types of bacteria (17, 27–30) and prestimulation by synthetic Nod1-stimulating compounds before infection confers resistance against multiple pathogens (33), it will be interesting to test whether local administration of Nod1 agonists such as KF1B will be beneficial in the prevention or treatment of C. difficile-induced pseudomembranous colitis. Nod1 stimulation is known to predominantly stimulate nonimmune cells that lack the ability to secrete potentially harmful cytokines such as TNF and IL-1, which is consistent with the observation that Nod1 stimulation induces chemokines and antimicrobial proteins without causing severe inflammation (10). Severe C. difficile disease in humans causes multiple organ dysfunction, sepsis, and even death (1). In this study, we demonstrate that C. difficile infection commensal bacteria translocate to MLNs, spleen, lung, and kidney, with greater bacterial loads detected in the organs of Nod1−/− mice compared with those of WT mice. Therefore, Nod1 stimulation may help to reduce the incidence of multiple organ dysfunction as a result of C. difficile infection in humans.

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Disclosures

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References


