MAJOR ARTICLE

Nucleotide-Binding Oligomerization Domain–Containing Protein 2 Controls Host Response to *Campylobacter jejuni* in *Il10*$^{-/-}$ Mice

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Innate signaling–induced antimicrobial response represents a key protective host feature against infectious microorganisms such as *Campylobacter* species. In this study, we investigated the role of nucleotide-binding oligomerization domain-containing protein 2 (NOD2) in *Campylobacter jejuni*–induced intestinal inflammation. Specific-pathogen-free *Il10*$^{-/-}$, *Nod2*$^{-/-}$, and *Il10*$^{-/-}$; *Nod2*$^{-/-}$ mice were infected with *C. jejuni* (10^9 colony-forming units/mouse) 24 hours after a 7-day course of antibiotic treatment. Three weeks later, host responses were determined. The nitric oxide (NO) donor sodium nitroprusside was injected intraperitoneally (2 mg/kg daily) to supplement NO. Although healthy in specific-pathogen-free conditions, *Il10*$^{-/-}$; *Nod2*$^{-/-}$ mice developed severe intestinal inflammation following *C. jejuni* infection, compared with *Nod2*$^{-/-}$ and *Il10*$^{-/-}$ mice. The onset of colitis was associated with elevated neutrophil accumulation, crypt abscesses, and expression of the endogenous proinflammatory mediators *Il-1β*, *Tnfα*, and *Cxcl1*. Fluorescence in situ hybridization and culture assay showed enhanced *C. jejuni* invasion into the colon and mesenteric lymph nodes in *Il10*$^{-/-}$; *Nod2*$^{-/-}$ mice, compared with *Il10*$^{-/-}$ mice. *C. jejuni*–induced bactericidal NO production was reduced in peritoneal macrophages from *Il10*$^{-/-}$; *Nod2*$^{-/-}$ mice, compared with *Il10*$^{-/-}$ mice. Importantly, sodium nitroprusside attenuated *C. jejuni*–induced colitis in *Il10*$^{-/-}$; *Nod2*$^{-/-}$ mice. Our findings suggest that NOD2 signaling is critical to control campylobacteriosis in *Il10*$^{-/-}$ mice, a process involving NOD2-mediated bactericidal responses.

Keywords. innate immunity; intestinal inflammation; campylobacteriosis; bactericide; *Il10*$^{-/-}$mice.

Inflammatory bowel diseases (IBDs), including ulcerative colitis and Crohn disease, afflict 1.4 million people in the United States alone. The exact etiology of IBD remains to be defined, but the host genetic susceptibility, microbiota, and environmental factors play critical roles in the development of the diseases [1]. Nucleotide-binding oligomerization domain (NOD) proteins are members of a large family of proteins named "NOD-like receptors" whose functions are essential in innate/adaptive host responses to various commensal and pathogenic bacteria [2–5]. NOD2 senses the bacterial cell wall component peptidoglycan and its derivative byproduct, muramyl dipeptide [4, 5]. In addition, NOD2 senses live intracellular pathogenic microorganisms such as *Salmonella enterica*, *Listeria monocytogenes*, *Mycobacterium tuberculosis,* and *Streptococcus pneumoniae* [6–9]. This host response is central to the elimination of the damaging agents and to the reestablishment of homeostasis. For example, defective NOD2 signaling impaired intestinal epithelial cells in their clearance of *Salmonella* Typhimurium in vitro and decreased host responses to *L. monocytogenes* infection in vivo [7, 8]. Similarly, NOD2-deficient mice are more susceptible than wild-type mice to *S.* Typhimurium infection [10]. The molecular mechanism by which NOD2 controls host response to bacteria is unclear, but its role in autophagy and microbial killing has recently been documented in vitro [11, 12]. For example, NOD2-mediated autophagy is required for bacterial handling and antigen presentation in dendritic cells

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[11]. Importantly, patients with IBD are often subject to a relapsing episode following infection with enteric bacterial pathogens such as *Salmonella* or *Campylobacter* species [13].

Campylobacter jejuni is one of the most prevalent food-borne bacterial pathogens in the world. Millions of people are infected with *C. jejuni* every year in the United States [14]. Clinical features of acute campylobacteriosis include bloody diarrhea, abdominal cramps, and severe intestinal inflammation. Despite the prevalence of campylobacteriosis, its pathogenesis remains largely unknown because of the limited availability of experimental animal models mimicking human disease.

Interleukin 10 (IL-10) is secreted by a variety of immune cells, including monocytes/macrophages and T cells, upon stimulation with various agents, such as lipopolysaccharide and bacteria [15]. IL-10 plays an essential role in maintaining intestinal homeostasis by regulating expression of proinflammatory cytokines, chemokines, and costimulatory molecules [16]. Indeed, 129SvEv *Il10*$^{-/-}$ mice born and raised in specific-pathogen-free (SPF) conditions developed intestinal inflammation after a couple of months but remained healthy when raised in germ-free conditions, showing the key role of bacteria in intestinal inflammation. Interestingly, C57BL/6 *Il10*$^{-/-}$ mice raised in SPF conditions and infected with *C. jejuni* strain 11168 displayed mild intestinal inflammation at day 35 after infection [17]. More recently, we reported that germ-free C57BL6/129SvEv and SPF 129 *Il10*$^{-/-}$ mice infected with *C. jejuni* 84–176 showed acute campylobacteriosis [18–20]. In addition, Haag et al showed that *C. jejuni* induces acute intestinal inflammation in C57BL/10 mice pretreated with antibiotics [21]. Using germ-free 129SvEv *Il10*$^{-/-}$; *Rag2*$^{-/-}$ mice, we recently showed that innate immune cells are essential in mediating the early phase of campylobacteriosis [20], suggesting an important role for innate immunity in controlling *C. jejuni* infection. These findings illustrate the benefit of using *Il10*$^{-/-}$ mice for studying mechanisms implicated in campylobacteriosis.

Reactive nitrogen species (RNS) such as nitric oxide (·NO) and superoxide ($O_2^{\cdot-}$) are a group of antimicrobial intermediate molecules produced by macrophages and are involved in eliminating intercellular and intracellular pathogens [22, 23]. The formation of RNS molecules is mediated by inducible nitric oxide synthase 2 (iNOS) and nicotinamide adenine dinucleotide phosphate oxidase. Interestingly, *C. jejuni* survival in vitro can be reduced by chemically generated RNS, using an acidified nitrite solution [24]. However, the role of host-generated NO in defense against *C. jejuni* infection remains unknown. Furthermore, no studies have directly assessed the relationship between NOD2 and RNS in the host response to bacterial infection in vivo.

In this study, we investigated the role of NOD2 in *C. jejuni*–induced intestinal inflammation. Using genetic manipulation, we showed that NOD2 enhances myeloid cell–induced bactericidal capacity and attenuates *C. jejuni*–induced intestinal inflammation through RNS generation. These findings provide a basis for developing novel approaches with the potential to control campylobacteriosis by targeting NOD2-mediated RNS.

METHODS

Mice and Tissue Processing

All animal protocols were approved by the institutional animal care and use committees of the University of North Carolina at Chapel Hill and the University of Florida. SPF 8–12-week-old C57BL/6 *Nod2*$^{-/-}$, *Il10*$^{-/-}$; *Nod2*$^{-/-}$, and *Il10*$^{-/-}$ mice received a single dose of 10^9 colony-forming units of *C. jejuni* (strain 81–176) by gavage [25] 24 hours after conclusion of a 7-day course of treatment with an antibiotic cocktail (streptomycin 2 g/L, bacitracin 1 g/L, gentamicin 0.5 g/L, and ciprofloxacin 0.125 g/L) [19]. All mice were euthanized 21 days after infection. To supplement NO in vivo, mice were injected intraperitoneally daily from days 0 to 21 after infection with sodium nitroprusside (2 mg/kg). Tissue samples from the colon, spleen, and mesenteric lymph nodes (MLNs) were collected for protein, RNA analysis, and histological analysis as described previously [19]. *C. jejuni* culture assays were performed as described previously [19]. Histological images were acquired, and intestinal inflammation was scored on a scale of 0 to 4 as described before [18, 19]. Crypt abscesses in colonic tissues were identified on the basis of morphological features, using hematoxylin-eosin–stained sections, and were counted as the number of abscesses per 100 crypts, using a microscope.

Peritoneal Macrophage Isolation and Nitrite Assay

Il10$^{-/-}$ and *Il10*$^{-/-}$; *Nod2*$^{-/-}$ mice were injected intraperitoneally with 2 mL of 3% fluid thioglycollate medium (Difco Laboratories, Detroit, MI) previously autoclaved for 15 minutes under 104 kPa. After 4 days, mice were euthanized by CO_2 intoxication, and macrophages in the peritoneal cavity were retrieved by lavaging 3 times with 3 mL of ice-cold HBSS supplemented with 0.15 mM ethylenediaminetetraacetic acid. The macrophages were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2% fetal bovine serum (FBS), and cell viability (>95%) was determined by trypan blue exclusion. Cells were infected with *C. jejuni* (multiplicity of infection [MOI], 50) for 4 hours, collected by centrifugation, and lysed in TRIzol (Invitrogen) for RNA extraction.

To measure NO production, macrophages were plated in triplicate in 24-well plates and then infected with *C. jejuni* (MOI, 50) for 12 hours. Cells were washed 3 times and incubated with 100 μg/mL gentamicin for 1 hour, and then medium was replaced with RPMI 1640 medium supplemented with 2% FBS, and 10 μg/mL gentamicin was added. After 12 hours, NO production in the supernatant was determined by the Griess assay [23].

Gentamicin Protection Assay to Determine *C. jejuni* Invasion of Peritoneal Macrophages

The *C. jejuni* gentamicin protection assay was performed by infecting 10^6 macrophages at an MOI of 50 in 12-well plates as described previously [19]. Briefly, macrophages were plated in triplicate in 12-well plates and infected with *C. jejuni* for 4 hours. After incubation for 4 hours, the macrophages were washed 3 times with phosphate-buffered saline (PBS) and incubated with fresh RPMI 1640 medium containing gentamicin (100 μg/mL) for an additional 1 hour. For sample collection at 0 hours, cells were washed 3 times before lysis in 0.1% Triton X-100. For sample collection at 1 and 2 hours, cells were incubated in fresh RPMI 1640 medium with gentamicin (10 μg/mL) for an additional 1 and 2 hours, respectively. Samples were then washed 3 times and lysed as described above. The lysates were plated on Remel plates to recover live *C. jejuni* from infected macrophages.

Confocal Microscopy to Determine *C. jejuni* Invasion

Macrophages (10^4) were plated in triplicate in 8-well chamber plates and infected for 4 hours with *C. jejuni* preincubated with 5 mM 5-Cyano-2,3-di-(p-tolyl)tetrazolium chloride (CTC). A gentamicin protection assay was then performed as described above, and the cells were fixed with 4% paraformaldehyde. The fixed cells were stained with DAPI medium (Vector Laboratory) and were visualized using confocal microscopy (Zeiss LSM710). Acquired images were analyzed using BioimageXD [26].

Fluorescence In Situ Hybridization (FISH)

Cy3-tagged 5′-AGCTAACCACACCTTATACCG-3′ was used to probe the presence of *C. jejuni* in the intestinal tissue sections as previously described [19]. Briefly, tissues were deparaffinized, hybridized with the probe, washed, stained with DAPI, and imaged using a Zeiss LSM710 Spectral Confocal Laser Scanning Microscope system with ZEN 2008 software. Acquired images were analyzed using BioimageXD.

Immunohistochemical (IHC) Analysis

Neutrophils in intestinal tissues were detected using anti-myeloperoxidase (MPO) IHC analysis as described previously [19]. Briefly, intestinal tissue sections were deparaffinized, blocked, and incubated with an anti-MPO antibody (1:400; Thermo Scientific) overnight. After incubation with anti-rabbit biotinylated antibody, avidin/biotin complex (Vectastain ABC Elite Kit, Vector Laboratories), diaminobenzidine (Dako), and hematoxylin-eosin (Fisher Scientific), the sections were imaged.

C. jejuni Quantification in Tissues

Colon, MLNs, and spleen were aseptically resected. Colon tissue was opened and washed 3 times in sterile PBS. The tissues were weighed, homogenized in PBS, serially diluted, and plated on *Campylobacter*-selective blood plates (Remel) for 48 hours at 37°C, using the GasPak system (BD). *C. jejuni* colonies were counted, and data are presented as CFU per gram of tissue.

Real-Time Reverse-Transcription Polymerase Chain Reaction (PCR)

Total RNA from intestinal tissues or peritoneal macrophages was extracted using TRIzol (Invitrogen) following the manufacture's protocol, and RNA was reverse transcribed using M-MLV (Invitrogen). Messenger RNA (mRNA) expression of the proinflammatory mediators *Il1β*, *Cxcl1*, *iNos* and *Tnfα* was measured using SYBR Green PCR Master mix (Applied Biosystems) on an ABI 7900HT Fast Real-Time PCR System and normalized to *Gapdh*. The PCR primer sequences were previously described [19]; *iNos*_forward is GTGGTGACAAGCACATTTGG, and *iNos*_reverse is GGCTGGACTTTTCACTCTGC. The PCR reactions were performed for 40 cycles according to the manufacturer's recommendation, and relative RNA fold-changes were calculated using the ΔΔCt method.

Statistical Analysis

Values are shown as mean ± standard error of the mean as indicated. Differences between groups were analyzed using the nonparametric Mann–Whitney *U* test or the *t* test. Experiments were considered statistically significant if *P* values were <.05. All calculations were performed using Prism 5.0 software.

RESULTS

NOD2 Deficiency Exacerbates *C. jejuni*–Induced Intestinal Inflammation

We previously showed that *C. jejuni*–infected $Il10^{-/-}$ mice but not wild-type mice developed colitis [18]. In addition, in our housing conditions and unlike 129SvEv $Il10^{-/-}$ mice, C57BL/6 $Il10^{-/-}$ mice did not develop inflammation, making them a valuable tool to investigate the contribution of susceptibility alleles such as NOD2 to *C. jejuni*–induced intestinal inflammation. To examine the role of NOD2 in controlling the bacteria/host interaction, we generated $Il10^{-/-}$; $Nod2^{-/-}$ mice. Interestingly, over a period of 12 months in our housing facility and husbandry conditions, none of the $Il10^{-/-}$ and $Il10^{-/-}$; $Nod2^{-/-}$ mice showed evidence of spontaneous intestinal inflammation, as evaluated by histological analysis (Supplementary Figure 1). This suggests that absence of the *Il10* gene or *Nod2* gene on a C57BL/6 background does not disrupt the host's innate responses to the commensal microbiota. To determine the function of NOD2 following infection with the human relevant clinical isolate, $Il10^{-/-}$, $Nod2^{-/-}$, and $Il10^{-/-}$; $Nod2^{-/-}$ mice were treated for 7 days with an antibiotic cocktail regimen. After a 24-hour washout period, mice received a single dose of *C. jejuni* (10^9 CFU/mouse) by gavage and were housed for an additional 21 days. The mice were euthanized, and intestinal samples were obtained for histological analysis. Interestingly,

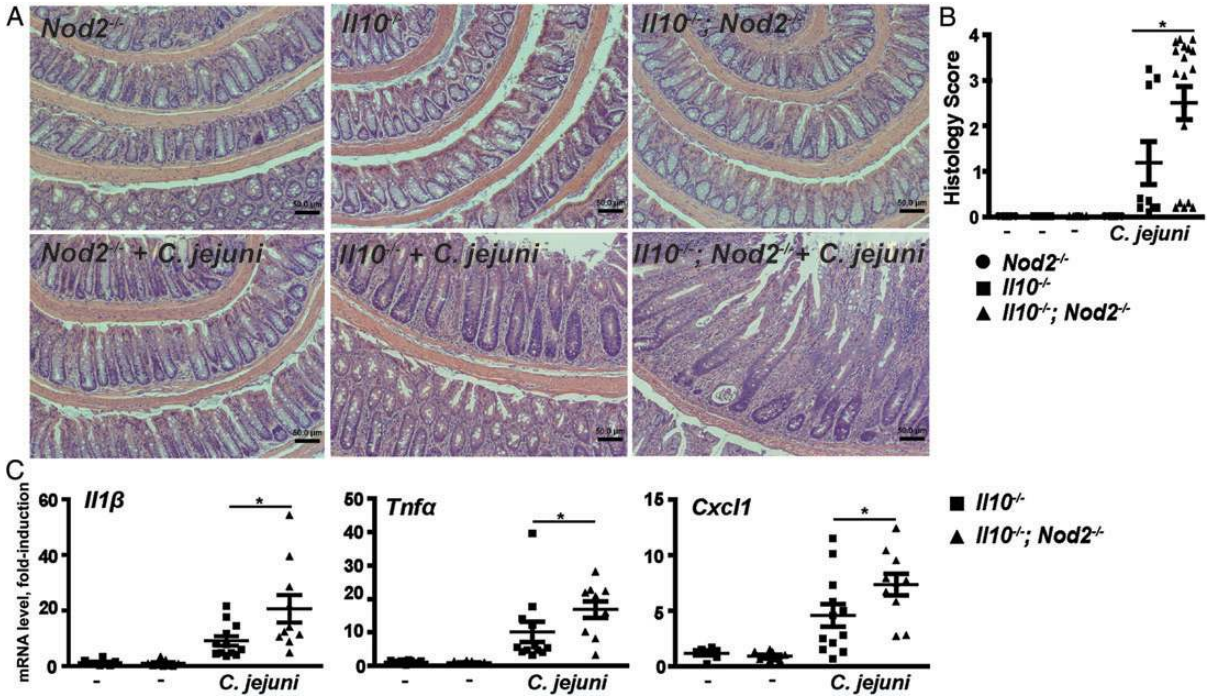

Figure 1. Nucleotide-binding oligomerization domain 2 (NOD2) attenuates *Campylobacter jejuni*–induced intestinal inflammation. Cohorts of 6–18 $Nod2^{-/-}$, $Il10^{-/-}$, and $Il10^{-/-}$; $Nod2^{-/-}$ mice raised in specific-pathogen-free conditions received a single dose of 10^9 *C. jejuni*/mouse by gavage. After 21 days of infection, colons were resected for hematoxylin-eosin staining or for RNA extraction for gene expression analysis. *A*, Representative intestinal histological images of *C. jejuni*–induced inflammation in $Nod2^{-/-}$, $Il10^{-/-}$, and $Il10^{-/-}$; $Nod2^{-/-}$ mice. *B*, Quantification of histological intestinal damage score mediated by *C. jejuni* infection. *C*, *Il1β*, *Tnfα*, and *Cxcl1* messenger RNA accumulation was quantified using an ABI 7900HT Fast Real-Time PCR System and specific primers, and data were normalized to *Gapdh*. All graphs depict means ± standard errors of the mean. $^*P < .05$. Scale bar is 200 μm. Results are representative of 3 independent experiments.

C. jejuni–infected $Nod2^{-/-}$ mice failed to develop intestinal inflammation (Figure 1*A* and 1*B*). This result suggests that NOD2 deficiency alone is not sufficient to promote *C. jejuni*–induced colitis. However, as previously reported [18], $Il10^{-/-}$ mice showed evidence of inflammation, compared with uninfected mice, as seen by immune cell infiltration, goblet cell depletion, and epithelial cell hyperplasia. Most importantly, *C. jejuni*–induced intestinal inflammation was exacerbated in $Il10^{-/-}$; $Nod2^{-/-}$ mice, compared with $Il10^{-/-}$ mice, as measured by histological scoring (2.51 vs 1.18 [$P = .03$]; Figure 1*A* and 1*B*). Therefore, we focused our attention on the differential response of $Il10^{-/-}$ mice and $Il10^{-/-}$; $Nod2^{-/-}$ mice to *C. jejuni* infection. At the molecular level, mRNA expression of the *C. jejuni*–induced proinflammatory mediators *Il1β*, *Tnfα*, and *Cxcl1* increased by 47%, 66%, and 39%, respectively, in *C. jejuni*–infected $Il10^{-/-}$; $Nod2^{-/-}$ mice, compared with levels in $Il10^{-/-}$ mice (Figure 1*C*).

C. jejuni–induced crypt abscesses are an important clinical feature of the $Il10^{-/-}$ murine model [19]. Remarkably, crypt abscesses were elevated 9-fold in *C. jejuni*–infected $Il10^{-/-}$; $Nod2^{-/-}$ mice, compared with $Il10^{-/-}$ mice (Figure 2*A*). In accordance with this finding, MPO staining revealed that *C. jejuni*–induced neutrophil infiltration into colonic tissues was strongly enhanced in $Il10^{-/-}$; $Nod2^{-/-}$ mice, compared with $Il10^{-/-}$ mice (Figure 2*B*).

NOD2 Alleviates *C. jejuni* Colonic Invasion

Since *C. jejuni* is an invasive intestinal pathogenic bacterium, we next investigated the impact of NOD2 signaling on *C. jejuni* invasion into intraintestinal and extraintestinal tissues. Following infection, *C. jejuni* DNA was visualized in the colon of $Il10^{-/-}$ and $Il10^{-/-}$; $Nod2^{-/-}$ mice, using FISH and confocal microscopy. The presence of *C. jejuni* was enhanced in inflamed crypts and lamina propria of $Il10^{-/-}$; $Nod2^{-/-}$ mice, compared with infected $Il10^{-/-}$ mice (Figure 3*A*). To quantify viable *C. jejuni* in intestinal and extraintestinal tissues, we aseptically collected samples from the colon and MLNs and enumerated the bacteria on Remel *Campylobacter*-selective plates. Consistent with the FISH results, counts of viable *C. jejuni* increased by 132% in colon samples and by 200% in MLNs, respectively, from of $Il10^{-/-}$; $Nod2^{-/-}$ mice, compared with $Il10^{-/-}$ mice (Figure 3*B*).

NOD2 Mediates Bactericidal Capacity and Antiinflammatory Responses

Since NOD2 deficiency elevated *C. jejuni* invasion into gastrointestinal tissues and is an indispensable cellular component of

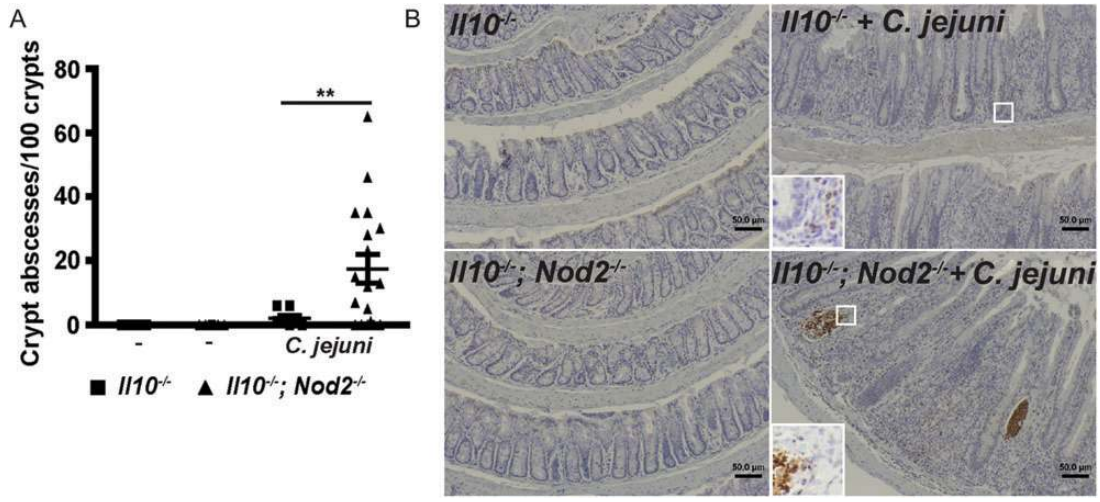

Figure 2. Nucleotide-binding oligomerization domain 2 (NOD2) deficiency exacerbates neutrophil infiltration–induced crypt abscesses in *Campylobacter jejuni*–infected mice. Cohorts of 9–18 $Il10^{-/-}$ and $Il10^{-/-}$; $Nod2^{-/-}$ mice raised in specific-pathogen-free conditions were infected as indicated in Figure 1. *A*, Number of crypt abscesses in *C. jejuni*–infected mice. *B*, Representative images of immunohistochemical analysis of myeloperoxidase (MPO) expression (brown dots), showing neutrophil infiltration. All graphs depict means ± standard errors of the mean. $^{**}P < .01$. Scale bar is 50 μm. Results are representative of 3 independent experiments.

host recognition on *S. pneumoniae* [6] and *S. enterica* [11], we next investigated the role of this innate sensor on *C. jejuni* clearance. NOD2-derived macrophage and dendritic cell signaling is a critical event in bacterial clearance [11, 27]. We then investigated the contribution of NOD2 in *C. jejuni* clearance, using primary thioglycollate-elicited peritoneal macrophages. As shown in Figure 4*A*, *C. jejuni* invasion into macrophages isolated from $Il10^{-/-}$; $Nod2^{-/-}$ mice was comparable to findings observed for macrophages from $Il10^{-/-}$ mice at 0 hours. After 2 hours of incubation, *C. jejuni* survival was 69% higher in $Il10^{-/-}$; $Nod2^{-/-}$ macrophages, compared with $Il10^{-/-}$ cells (Figure 4*A*). Fluorescence microscopy showed the persistence of *C. jejuni* in $Il10^{-/-}$; $Nod2^{-/-}$ macrophages, compared with infected $Il10^{-/-}$ macrophages (Figure 4*B*). These results suggest that NOD2 deficiency impaired bacterial killing capacity in macrophages.

NOD2-Mediated NO Expression Correlates With Bactericidal Capacity

Since NO production plays an essential role in macrophage-mediated bacterial killing [28, 29], we examine expression of inducible NO. Primary peritoneal macrophages were isolated from $Il10^{-/-}$ and $Il10^{-/-}$; $Nod2^{-/-}$ mice and infected with *C. jejuni*. Interestingly, *C. jejuni*–induced *iNos* mRNA expression was reduced by 67% in $Il10^{-/-}$; $Nod2^{-/-}$ macrophages,

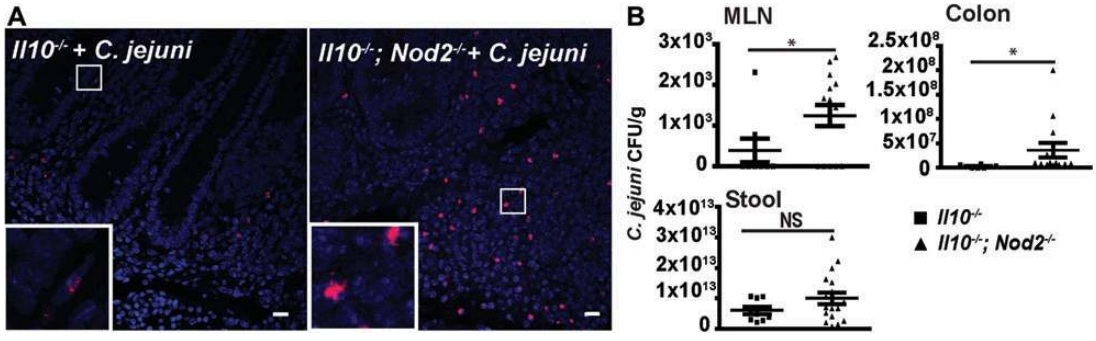

Figure 3. Nucleotide-binding oligomerization domain 2 (NOD2) prevents *Campylobacter jejuni* invasion into the colon and mesenteric lymph nodes. Cohorts of 9–18 $Il10^{-/-}$ and $Il10^{-/-}$; $Nod2^{-/-}$ mice raised in specific-pathogen-free conditions were infected as indicated in Figure 1. *A, C. jejuni* (red dots) in colonic sections of infected mice was detected using fluorescence in situ hybridization. Scale bar represents 10 μm. *B, C. jejuni* bacterial count in the colon, mesenteric lymph nodes (MLNs), and stool of mice. Data represent means ± standard errors of the mean. $^{*}P < .05$. Results are representative of 3 independent experiments. Abbreviations: CFU, colony-forming units; NS, not significant.

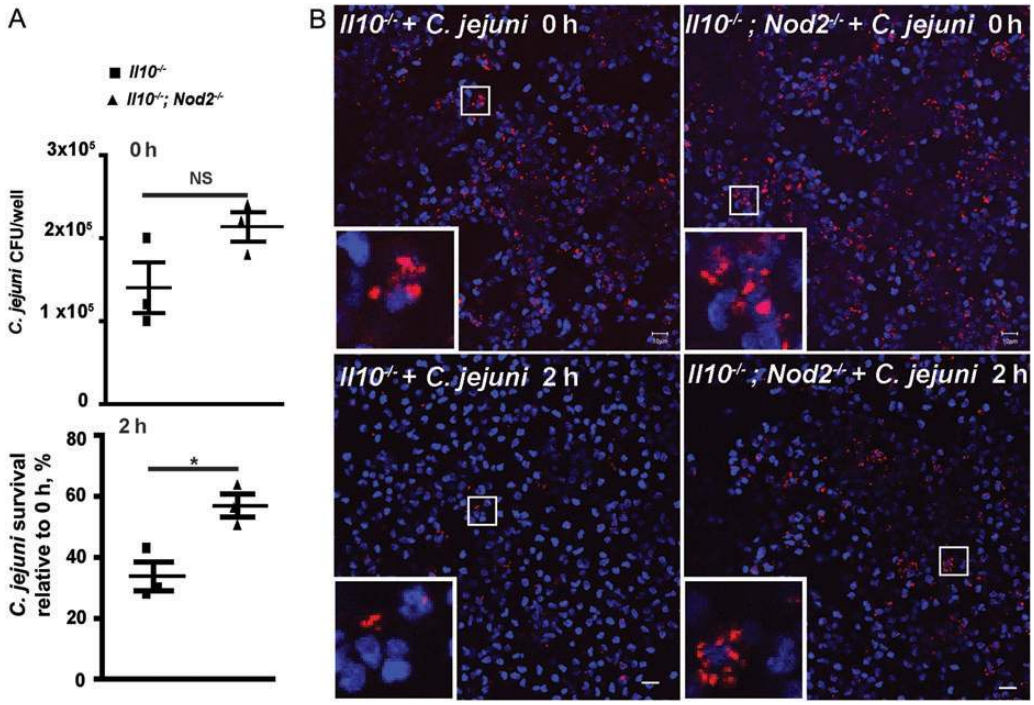

Figure 4. Nucleotide-binding oligomerization domain 2 (NOD2) enhances *Campylobacter jejuni* clearance in macrophages. Elicited peritoneal macrophages from *Il10*$^{-/-}$ and *Il10*$^{-/-}$; *Nod2*$^{-/-}$ mice were infected with *C. jejuni*, and bacterial survival was determined using a gentamicin assay. *A, C. jejuni* invasion in macrophages at 0 and 2 hours was enumerated by plating. *B,* Representative images of *C. jejuni* (red dots) invasion into macrophages, using 5-Cyano-2,3-di-(p-tolyl) tetrazolium chloride-labelled *C. jejuni*. Scale bar is 10 μm. Data represent means ± standard errors of the mean. Results are representative of 3 independent experiments. **P* < .05. Abbreviations: CFU, colony-forming units; NS, not significant.

compared with *Il10*$^{-/-}$ cells, whereas *Tnfα* expression was not affected (Figure 5*A*). These findings indicate that NOD2 controls expression of specific proinflammatory genes, including the bactericidal mediator *iNos*.

Since NOD2 deficiency impaired *C. jejuni*–induced *iNos* mRNA expression, we next investigated the level of NO production, using the Griess assay. Notably, *C. jejuni* infection strongly induced nitrite production in *Il10*$^{-/-}$ macrophages, an effect attenuated by 35% in *Il10*$^{-/-}$; *Nod2*$^{-/-}$ cells (Figure 6*A*). To evaluate the impact of NO on *C. jejuni* killing, we infected peritoneal macrophages with *C. jejuni* in the presence of sodium nitroprusside and assessed bacterial survival. *C. jejuni* survival was 33% higher in macrophages isolated from *Il10*$^{-/-}$; *Nod2*$^{-/-}$ mice, compared with *Il10*$^{-/-}$ cells, at 2 hours (Figure 6*B*). Interestingly, supplementation with sodium nitroprusside significantly reduced *C. jejuni* survival in macrophages isolated from *Il10*$^{-/-}$; *Nod2*$^{-/-}$ mice.

NO-Enhanced Bactericidal Capacity Attenuates *C. jejuni*–Induced Colitis in *Il10*$^{-/-}$; *Nod2*$^{-/-}$ Mice

To further evaluate the role of NO in *C. jejuni*–induced intestinal inflammation, *Il10*$^{-/-}$ and *Il10*$^{-/-}$; *Nod2*$^{-/-}$ mice were infected as described above and injected intraperitoneally with PBS or sodium nitroprusside (2 mg/kg in PBS, daily) for 21 days. As shown above, *C. jejuni* induced stronger intestinal inflammation in *Il10*$^{-/-}$; *Nod2*$^{-/-}$ mice, as demonstrated by increased immune cell infiltration, goblet cell depletion, and crypt hyperplasia/abscesses, compared with *Il10*$^{-/-}$ mice (2.89 vs 1.91 [*P* = .047]; Figure 7*A* and 7*B*). Notably, sodium nitroprusside treatment attenuated *C. jejuni*–induced intestinal inflammation (by approximately 60%) in *Il10*$^{-/-}$; *Nod2*$^{-/-}$ mice, compared with untreated mice (1.15 vs 2.89; *P* = .026). Interestingly, sodium nitroprusside treatment did not significantly attenuate *C. jejuni*–induced colitis in *Il10*$^{-/-}$ mice.

To assess the contribution of NO in bacterial clearance in intestinal tissue, we evaluated *C. jejuni* DNA in colonic tissues, using FISH. Sodium nitroprusside strongly diminished *C. jejuni* translocation into colonic tissues of both *Il10*$^{-/-}$; *Nod2*$^{-/-}$ and *Il10*$^{-/-}$ mice (Figure 7*C*). Overall, these findings suggest that NOD2 deficiency impairs *C. jejuni* clearance and exacerbates colitis, an effect rescued by restoring bactericidal capacity through NO supplementation.

DISCUSSION

The importance of the innate sensor NOD2 in regulating innate response and intestinal homeostasis has long been recognized, which is evident by increased susceptibility to IBD (eg, Crohn

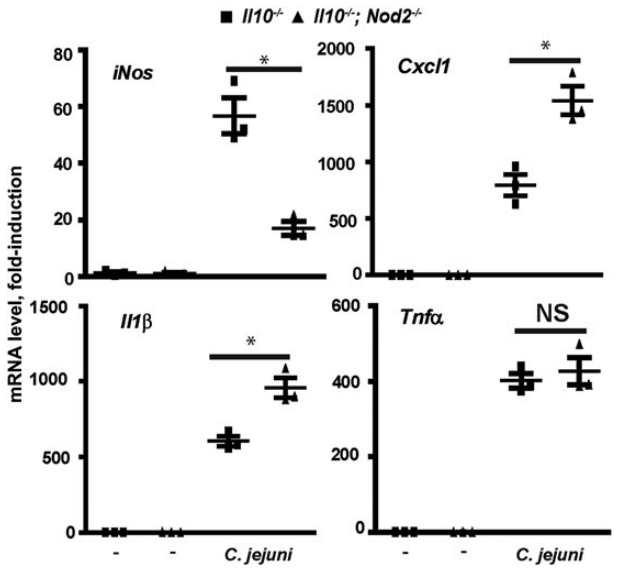

Figure 5. Nucleotide-binding oligomerization domain 2 (NOD2) promotes *Campylobacter jejuni*–induced *iNos* but attenuates *Cxcl1* and *Il1β* messenger RNA (mRNA) expression in macrophages. Elicited peritoneal macrophages from *Il10*$^{-/-}$ and *Il10*$^{-/-}$; *Nod2*$^{-/-}$ mice were infected with *C. jejuni,* and mRNA accumulation was determined using real-time polymerase chain reaction. Data represent means ± standard errors of the mean. **P* < .05. Results are representative of 3 independent experiments.

disease) in individuals carrying NOD2 polymorphisms (eg, R702W, G908R, and 1007fs) [30]. The monocyte/macrophage lineage in patients with IBD displayed an increased inflammatory response following stimulation with commensal bacteria [31] and inferior *Escherichia coli* clearance in the gastrointestinal tract [32]. The impact of NOD2 on bacterial host responses could involve the regulation of proinflammatory molecule

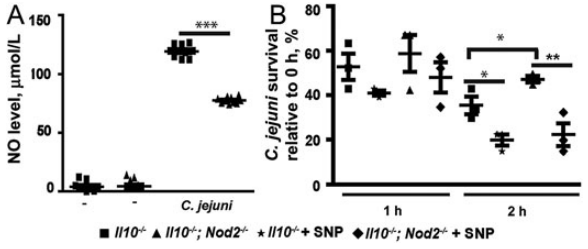

Figure 6. Nucleotide-binding oligomerization domain 2 (NOD2) enhances nitric oxide (NO) production and promotes *Campylobacter jejuni* clearance in macrophages. Elicited peritoneal macrophages from *Il10*$^{-/-}$ and *Il10*$^{-/-}$; *Nod2*$^{-/-}$ mice were infected with *C. jejuni. A,* Production of NO as measured by the Griess assay. *B, C. jejuni* survival in the presence of NO donor sodium nitroprusside as determined by culture assay. Data represent means ± standard errors of the mean. ****P* < .001, ***P* < .01, **P* < .05. Results are representative of 3 independent experiments.

expression, control of Toll-like receptor signaling intensity, regulation of antimicrobial peptide production, and control of microbial composition [8, 33–37]. Whether host-derived NOD2 signaling utilizes some or all of these mechanisms to respond to enteric pathogen, especially *C. jejuni,* is unknown. Our study showed that NOD2 deficiency exacerbates *C. jejuni*–induced colitis in *Il10*$^{-/-}$ mice, as demonstrated by increased hyperplasia, immune cell infiltration and crypt abscesses. Interestingly, *C. jejuni*-infected *Nod2*$^{-/-}$ mice failed to develop colitis. Therefore, this defective innate response to *C. jejuni* infection is insufficient to promote colitis, and a more global immune alteration, such as the one afforded by absence of IL-10 signaling in *Il10*$^{-/-}$ mice is necessary to reveal NOD2 contribution. This two hit model is compatible with the limited risk factor confer by NOD2 polymorphisms in humans, which highlights the polygenic nature of IBD [38, 39].

The increased colitis among *Il10*$^{-/-}$; *Nod2*$^{-/-}$ mice was coupled with elevated expression of the inflammatory mediators *Il-1β* and *Cxcl1*, as well as with increased *C. jejuni* invasion into the colon and MLN, compared with *Il10*$^{-/-}$ mice. Interestingly, the inability to eliminate *C. jejuni* from *Il10*$^{-/-}$; *Nod2*$^{-/-}$ macrophages was associated with reduced *C. jejuni*–induced bactericidal NO production. Supplementation with the NO-donor sodium nitroprusside promoted *C. jejuni* eradication and attenuated *C. jejuni*–induced colitis in *Il10*$^{-/-}$; *Nod2*$^{-/-}$ mice. Therefore, we have found a unique role for NOD2 in protecting *Il10*$^{-/-}$ mice from enteric bacterial pathogen *C. jejuni*–induced colitis, likely through NOD2-induced bactericidal responses.

Interestingly, Jamontt et al recently showed spontaneous colitis is attenuated in *Il10*$^{-/-}$*; Nod2*$^{-/-}$ mice, compared with *Il10*$^{-/-}$ mice, at 9 weeks of age [40], suggesting a deleterious role for NOD2 signaling in the intestine. In contrast, both *Il10*$^{-/-}$ and *Il10*$^{-/-}$*; Nod2*$^{-/-}$ mice raised in our SPF facility remained healthy for >56 weeks, suggesting a different environmental trigger (eg, microbial composition) between our animal facility and that of Jamontt et al. Further investigation would be necessary to resolve this phenotypic discrepancy.

Using FISH and culture assays, we found that NOD2 deficiency impairs *C. jejuni* clearance in the colon and in peritoneal macrophages in vitro, leading to increased bacterial invasion/survival. Mammalian cells (eg, epithelial cells and macrophages) possess a variety of tools to eliminate invading bacterial pathogens, including bactericidal and autophagy responses [6, 7, 11]. We previously found that rapamycin-induced autophagy enhances *C. jejuni* clearance in *Il10*$^{-/-}$ splenocytes [19]. Interestingly, generation of the *C. jejuni*–induced autophagy protein LC3 II is reduced in *Il10*$^{-/-}$*; Nod2*$^{-/-}$ splenocytes, compared with *Il10*$^{-/-}$ cells (data not shown). The diminished bacterial clearance observed in *C. jejuni*–infected *Il10*$^{-/-}$*; Nod2*$^{-/-}$ mice may involve an impaired autophagic response. Further investigation would be required to demonstrate the role of NOD2/autophagy in *C. jejuni* clearance.

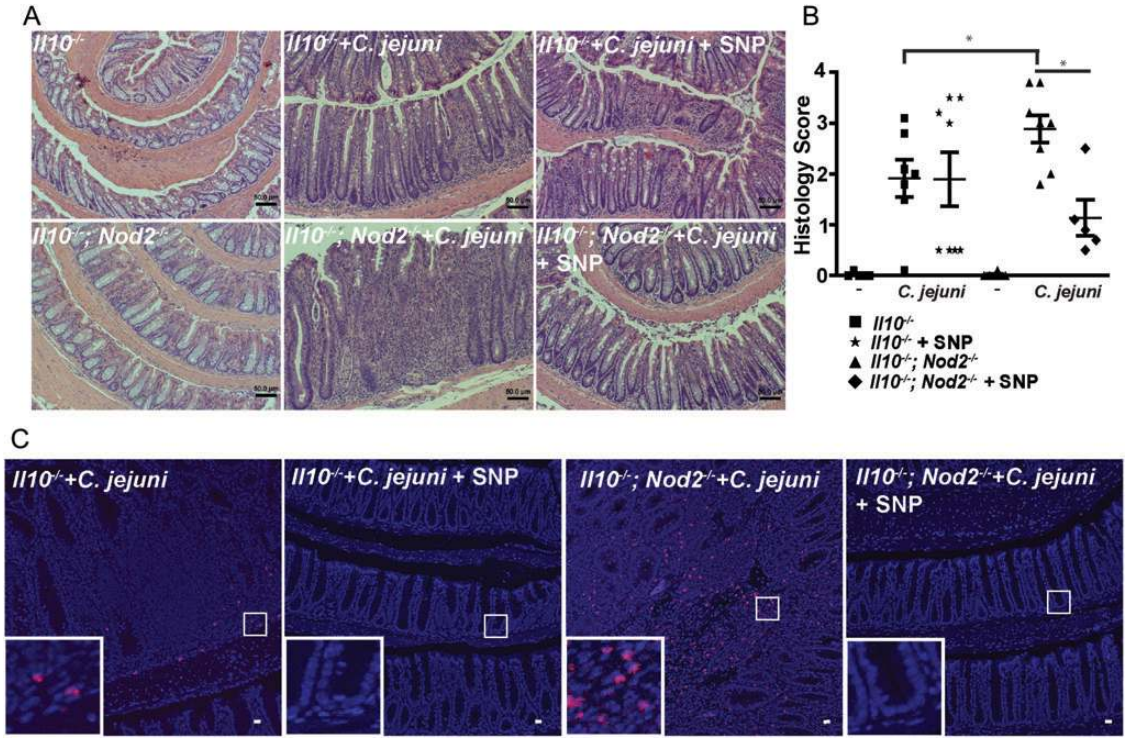

Figure 7. Supplementation with nitric oxide (NO) attenuates *Campylobacter jejuni*–induced intestinal inflammation. Cohorts of 5–8 $Il10^{-/-}$ and $Il10^{-/-}$; $Nod2^{-/-}$ mice raised in specific-pathogen-free conditions received a single dose of 10^9 *C. jejuni*/mouse by gavage and daily intraperitoneal injection of phosphate-buffered saline or sodium nitroprusside (SNP). After 21 days, colons were resected for hematoxylin-eosin staining or fluorescence in situ hybridization (FISH). *A*, Representative intestinal histological images of *C. jejuni*–induced inflammation in $Il10^{-/-}$ and $Il10^{-/-}$; $Nod2^{-/-}$ mice in the presence of SNP. *B*, Quantification of histological intestinal damage score mediated by *C. jejuni* infection. *C*, *C. jejuni* (red dots) in colonic sections of infected mice was detected using FISH. Scale bars are 50 μm (*A*) and 20 μm (*C*). Data represent means ± standard errors of the mean. Results are representative of 3 independent experiments. ***$P < .001$, **$P < .01$, *$P < .05$.

In addition to autophagy, host-derived RNS such as the bactericidal agent NO actively participate in the elimination of intercellular and intracellular bacteria and suppresses the growth of *E. coli* and *Salmonella* in vitro [22, 41]. *C. jejuni* survival in vitro is reduced in the presence of chemically generated NO [24]. Here, we found that *C. jejuni*–induced *iNos* expression and NO generation is attenuated in $Il10^{-/-}$; $Nod2^{-/-}$ macrophages, compared with $Il10^{-/-}$ cells. Impaired NO generation by $Il10^{-/-}$; $Nod2^{-/-}$ macrophages is associated with increased *C. jejuni* survival in these cells, whereas the NO-donor sodium nitroprusside attenuated bacterial survival. Furthermore, sodium nitroprusside attenuated *C. jejuni* invasion and intestinal inflammation in $Il10^{-/-}$; $Nod2^{-/-}$ mice. Interestingly, sodium nitroprusside did not significantly diminish intestinal inflammation in $Il10^{-/-}$ mice, which correlated with lower levels of bacterial invasion, compared with $Il10^{-/-}$; $Nod2^{-/-}$ mice. These findings indicate that iNOS expression and NO production are dependent on functional NOD2 signaling and likely represent an important mechanism for controlling campylobacteriosis.

NOD2 deficiency is associated with defective intestinal antimicrobial peptide expression and microbial dysbiosis [8, 42, 43]. Interestingly, microbial composition is an important environmental factor influencing the susceptibility to *C. jejuni* infection in experimental models [44]. However, recent findings by Shanahan et al showed that $Nod2^{-/-}$ mice and cohoused wild-type littermates showed comparable antimicrobial peptide expression [45]. Moreover, microbial composition was not influenced by NOD2 status [45, 46]. These findings suggest that increased *C. jejuni* translocation and intestinal inflammation in $Il10^{-/-}$; $Nod2^{-/-}$ mice is mostly driven by defective bactericidal activity rather than by impaired antimicrobial peptide production and microbial dysbiosis. Further experiments would be necessary to fully assess the contribution of microbial composition to *C. jejuni* infection.

Taken together, our findings highlight an essential role for NOD2 in defending the host against enteropathogenic *C. jejuni*. The protective function of NOD2 seems to depend on enhancing bactericidal capacity through NO production. Targeting NOD2-mediated NO production may represent an alternative therapeutic approach to treating campylobacteriosis.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of

data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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C. J. and X. S. conceived and designed the experiments. X. S. performed the experiments. X. S. and C. J. analyzed the data. X. S. and C. J. wrote the manuscript.

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