Original article

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MICROBIOTA COMPOSITION AND IMMUNE RESPONSES DURING *CAMPYLOBACTER JEJUNI* INFECTION IN CONVENTIONALLY COLONIZED IL-10$^{-/-}$ MICE LACKING NUCLEOTIDE OLIGOMERIZATION DOMAIN 2

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Host immune responses are pivotal for combating enteropathogenic infections. We here assessed the impact of the innate receptor nucleotide oligomerization domain protein 2 (NOD2) in murine *Campylobacter jejuni*-infection. Conventionally colonized IL-10$^{-/-}$ mice lacking NOD2 and IL-10$^{-/-}$ controls were perorally challenged with *C. jejuni* strain 81-176 and displayed comparable pathogenic colonization of intestines until day 14 postinfection (p.i.). Whereas overall intestinal microbiota compositions were comparable in naive mice, NOD2$^{-/-}$ IL-10$^{-/-}$ mice exhibited less fecal bifidobacteria and lactobacilli than IL-10$^{-/-}$ counterparts after infection. Interestingly, NOD2$^{-/-}$ IL-10$^{-/-}$ mice were clinically more compromised during the early phase of infection, whereas, conversely, IL-10$^{-/-}$ animals exhibited more frequently bloody feces lateron. While colonic apoptotic cell and T lymphocyte numbers were comparable in either *C. jejuni*-infected mice, B lymphocytes were lower in the colon of infected NOD2$^{-/-}$ IL-10$^{-/-}$ mice versus controls. At day 14 p.i., colonic TNF and IL-23p19 mRNA levels were upregulated in NOD2$^{-/-}$ IL-10$^{-/-}$ mice only. Translocation rates of intestinal commensals to mesenteric lymphnodes and extra-intestinal compartments including liver and kidney were comparable, whereas viable bacteria were more frequently detected in spleens derived from IL-10$^{-/-}$ as compared to NOD2$^{-/-}$ IL-10$^{-/-}$ mice. In conclusion, NOD2 is involved during *C. jejuni* infection in conventionally colonized IL-10$^{-/-}$ mice in a time-dependent manner.

Keywords: *Campylobacter jejuni,* nucleotide oligomerization domain-2, IL-10$^{-/-}$ infection model, IL-23/IL-22/IL-18 axis, pro-inflammatory immune responses, bacterial translocation, colonization resistance, intestinal microbiota, bifidobacteria, lactobacilli

Introduction

Human *Campylobacter* infections are of major importance worldwide [1–3]. Domestic and wild animals harbor *Campylobacter jejuni* as commensals in their intestinal tract. Humans, however, become infected via the food chain by consumption of contaminated products from farm animals or surface water [4, 5]. Depending on the virulence of the respective ingested *C. jejuni* strain and the immune status of the host, infected patients suffer from gastroenteritis of varying degree. Whereas some patients present with watery diarrhea and rather mild symtoms, others display severe malaise such as ulcerative colitis with inflammatory, bloody diarrhea, fever, and abdominal cramps [3, 6]. Whereas, in most cases, disease resolves spontaneously without sequelae, postinfectious complications might manifest in rare cases within the nervous system (i.e., Guillain–Barré syndrome, Miller–Fisher syndrome, and Bickerstaff encephalitis), the joints (i.e., reactive polyarthritis, Reiter's syndrome), or the intestinal tract (i.e., irritable bowel syndrome) weeks to months post infection [3, 7]. Susceptibility of the vertebrate host for *Camplylobacter* infections is highly depending on the host specific intestinal microbiota composition [8]. Conventionally colonized mice, for example, have been shown to be protected from *C. jejuni* infection [9]. This physiological colonization resistance is mediated by the distinct gut microbiota and can be overcome upon its modification by antibiotic treat-

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ment, for instance. Subsequently, stable infection results in *C. jejuni*-induced pro-inflammatory responses mimicking key features of campylobacteriosis observed in human patients [9, 10]. *C. jejuni* infection could also be facilitated by acute or chronic intestinal infection or obesity of conventionally colonized mice [11–13]. In this context, we were able to show that, following peroral *C. jejuni* challenge, conventionally colonized IL-10$^{-/-}$ mice suffering from chronic colitis could in fact be stably infected and exhibited pathogen-induced intestinal as well as extra-intestinal immune responses. Our group further unraveled that *C. jejuni*-induced immunopathology was mediated by distinct innate immune receptors such as Toll-like receptors (TLR) -2 and -4 [12, 14].

The nucleotide-binding oligomerization domain (NOD)-like receptors comprise another important family of signaling molecules that are pivotal for innate immunity. These intracellular pattern recognition receptors sense microbial products and damage-associated factors [15]. Among these, NOD2 (encoded by the *card15* gene) is expressed by Paneth cells and innate (dendritic cells, macrophages), but also adaptive (i.e., T lymphocytes), immune cell populations [16–19]. NOD2 is activated by muramyl dipeptide (MDP), a major constituent of bacterial peptidoglycan known for its immunomodulatory potency [20]. The NOD2 signaling pathway subsequently confers resistance against a plethora of bacterial pathogens including *Campylobacter* [15, 21–23]. Whether NOD2 is also able to sense other microbial structures or rather acts as a mere signaling partner is not yet fully understood [24].

In the present study, we elucidated the role of NOD2 in *C. jejuni* infection of conventionally colonized IL-10$^{-/-}$ mice with chronic colitis. We first addressed whether intestinal microbiota composition in IL-10$^{-/-}$ mice was depending on NOD2, both in the basal and infected state. We further surveyed potential NOD2-dependent intestinal and extra-intestinal pro-inflammatory sequelae of *C. jejuni* infection and determined translocation of viable commensal intestinal bacteria to extra-intestinal including systemic compartments applying conventional IL-10$^{-/-}$ mice lacking NOD2.

Methods

Mice and C. jejuni *infection*

Female IL-10$^{-/-}$ mice and IL-10$^{-/-}$ mice lacking NOD2 (NOD2$^{-/-}$ IL-10$^{-/-}$) (all in C57BL/6j background) were reared and maintained within the same specific pathogen free (SPF) unit of the Forschungseinrichtungen für Experimentelle Medizin (FEM, Charité – University Medicine Berlin). At the age of approximately 12 weeks, conventionally colonized mice were perorally infected with 10^9 colony forming units (CFU) of viable *C. jejuni* strain 81-176 in a volume of 0.3 ml phosphate buffered saline (PBS; Gibco, Life Technologies, UK) on three consecutive days (days 0, 1, and 2) by gavage as described earlier [9].

Clinical conditions

To assess clinical signs of *C. jejuni*-induced infection on a daily basis, a standardized cumulative clinical score (maximum 12 points), addressing the occurrence of blood in feces (0: no blood; 2: microscopic detection of blood by the Guajac method using Haemoccult, Beckman Coulter/PCD, Germany; 4: macroscopic blood visible), diarrhea (0: formed feces; 2: pasty feces; 4: liquid feces), and the clinical aspect (0: normal; 2: ruffled fur, less locomotion; 4: isolation, severely compromised locomotion, pre-final aspect) was used as described earlier [14, 25, 26].

Sampling procedures

Mice were sacrificed at day 14 post infection (p.i.) by isofluran treatment (Abbott, Germany). Colonic *ex vivo* biopsies were asserved under sterile conditions and collected in parallel for microbiological and immunological analyses. Immunohistopathological changes were assessed in large intestinal samples that were immediately fixed in 5% formalin and embedded in paraffin. Sections (5 µm) were stained with the respective antibodies for *in situ* immunohistochemistry as described previously [25–27].

Immunohistochemistry

In situ immunohistochemical analysis of colonic paraffin sections was performed as stated elsewhere [25–27]. Primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, USA, 1:200), Ki67 (TEC3; Dako, Denmark; 1:100), CD3 (#N1580; Dako; 1:10), FOXP3 (FJK-16s; eBioscience, Germany; 1:100), B220 (eBioscience; 1:200), and myeloperoxidase (MPO-7, # A0398; Dako; 1:500) were used. For each animal, the average number of positively stained cells within at least six high power fields (HPF, 0.287 mm^2, 400× magnification) was determined microscopically by a blinded independent investigator.

Quantitative analysis of bacterial colonization and translocation

Viable *C. jejuni* and commensal *Escherichia coli* were detected in feces over time p.i. and at time of necropsy (day 14 p.i.) in luminal samples of the gastrointestinal tract (i.e., stomach, duodenum, ileum, and colon) or homogenates of whole tissue *ex vivo* biopsies derived from mesenteric lymph nodes (MLN), spleen, liver (approximately 1 cm^3), and kidney. For *C. jejuni* quantification, serial dilutions (dissolved in sterile PBS) were cultured on Karmali- and Columbia-Agar supplemented with 5% sheep blood (Oxoid, Germany) for 2 days at 37 °C under microaerobic conditions using CampyGen gas packs (Oxoid). *E. coli* were quantitated follow-

ing incubation of Columbia-Agar supplemented with 5% sheep blood and MacConkey Agar (both Oxoid) in aerobic atmosphere for 2 days at 37 °C. Translocation of further intestinal commensals to extra-intestinal compartments was assessed in the respective organ homogenates under aerobic, microaerobic, and obligate anaerobic conditions as described earlier [28–30]. The respective weights of fecal or tissue samples were determined by the difference of the sample weights before and after asservation. The detection limit of viable pathogens was ≈100 CFU per g.

Real-time PCR

RNA was isolated from snap frozen colonic *ex vivo* biopsies, reverse transcribed, and analyzed as described previously [31]. Murine TNF, IFN-γ, IL-23p19, IL-22, IL-18, and mucin-2 (MUC2) mRNA expression were detected by real-time polymerase chain reaction (PCR) with specific primers and quantified by analysis with the Light Cycler Data Analysis Software (Roche). The mRNA of the housekeeping gene for hypoxanthine-phosphoribosyltransferase (HPRT) was used as reference, and the mRNA expression levels of the individual genes were normalized to the lowest measured value and expressed as fold expression (Arbitrary Units).

Statistical analysis

Medians and levels of significance were determined using Mann–Whitney test (GraphPad Prism v5, La Jolla, CA, USA) as indicated. Two-sided probability (p) values ≤ 0.05 were considered significant.

Ethics statement

All animal experiments were conducted according to the European Guidelines for animal welfare (2010/63/EU) with approval of the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin, registration number G0135/10). Animal welfare was monitored twice daily by assessment of clinical conditions.

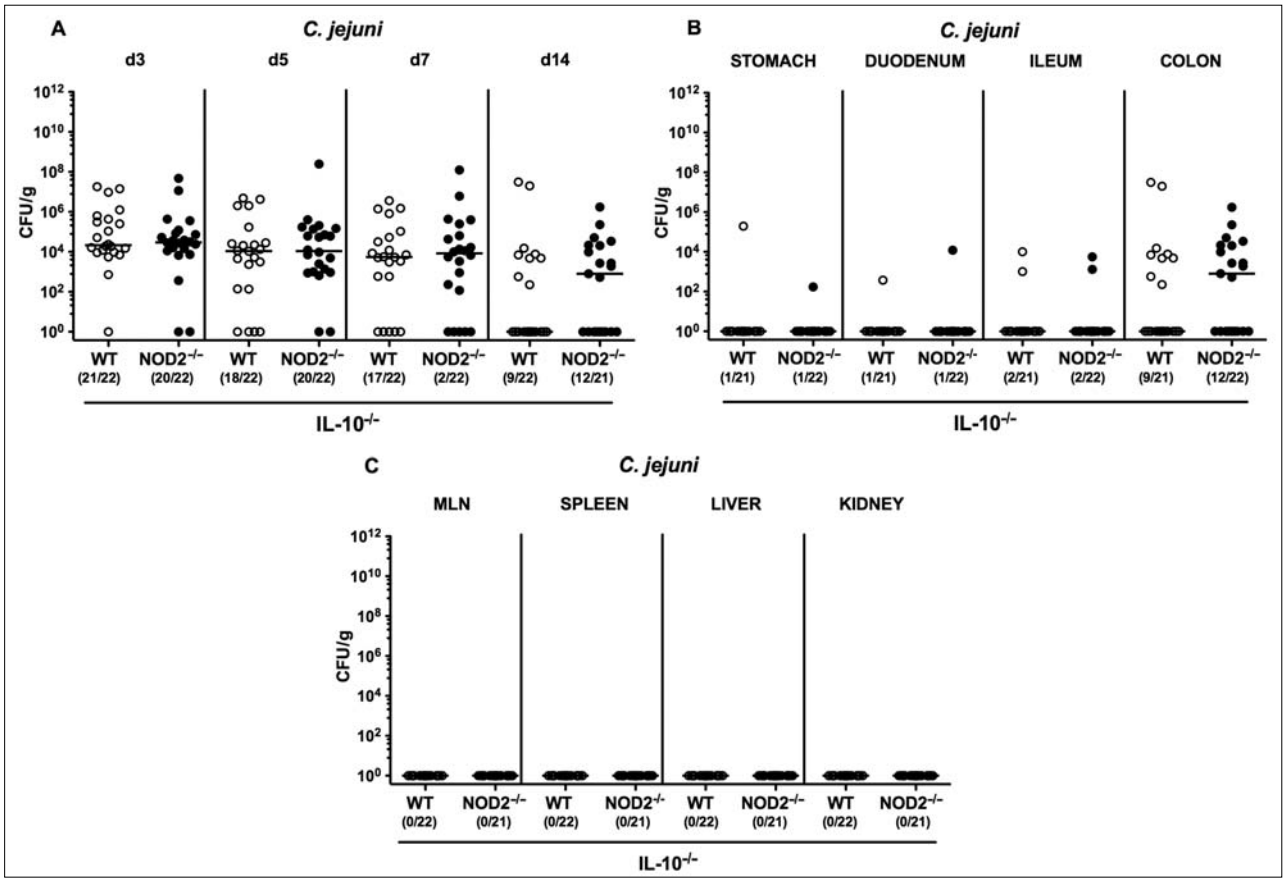

Fig. 1. Gastrointestinal and extra-intestinal *C. jejuni* strain 81-176 loads in perorally infected conventionally colonized IL-10$^{-/-}$ mice lacking NOD2. IL-10$^{-/-}$ (white circles) and IL-10$^{-/-}$ mice lacking NOD2 (NOD2$^{-/-}$ IL-10$^{-/-}$; black circles) were perorally infected with *C. jejuni* strain 81-176 by gavage at days (d) 0, 1, and 2. (A) Pathogenic loads were determined in fecal samples (CFU, colony forming units per gram) by culture over time postinfection (p.i.). At necrosy (d14 p.i.), *C. jejuni* loads were quantitated in *ex vivo* biopsies derived from (B) gastrointestinal and (C) extra-intestinal compartments. Medians (black bars) and levels of significance (p values) determined by Mann–Whitney U test are indicated. Numbers of mice harboring *C. jejuni* strain 81-176 out of the total number of analyzed animals are given in parentheses. Data were pooled from four independent experiments

Results

C. jejuni *infection and translocation in conventional IL-10$^{-/-}$ mice lacking NOD2*

Conventionally colonized IL-10$^{-/-}$ mice lacking NOD2 and IL-10$^{-/-}$ counterparts were perorally infected with 10^9 CFU *C. jejuni* strain 81-186 on three consecutive days (days 0, 1, and 2). Whereas as early as 24 h following the latest oral challenge (i.e., day 3) nearly all mice of either genotype harbored comparable but rather low median *C. jejuni* loads of 10^4 CFU per g fecal samples, approximately half of infected animals lost the pathogen until necropsy at day 14 p.i. *(Fig. 1A; Fig. S1)*. Fecal *C. jejuni* loads

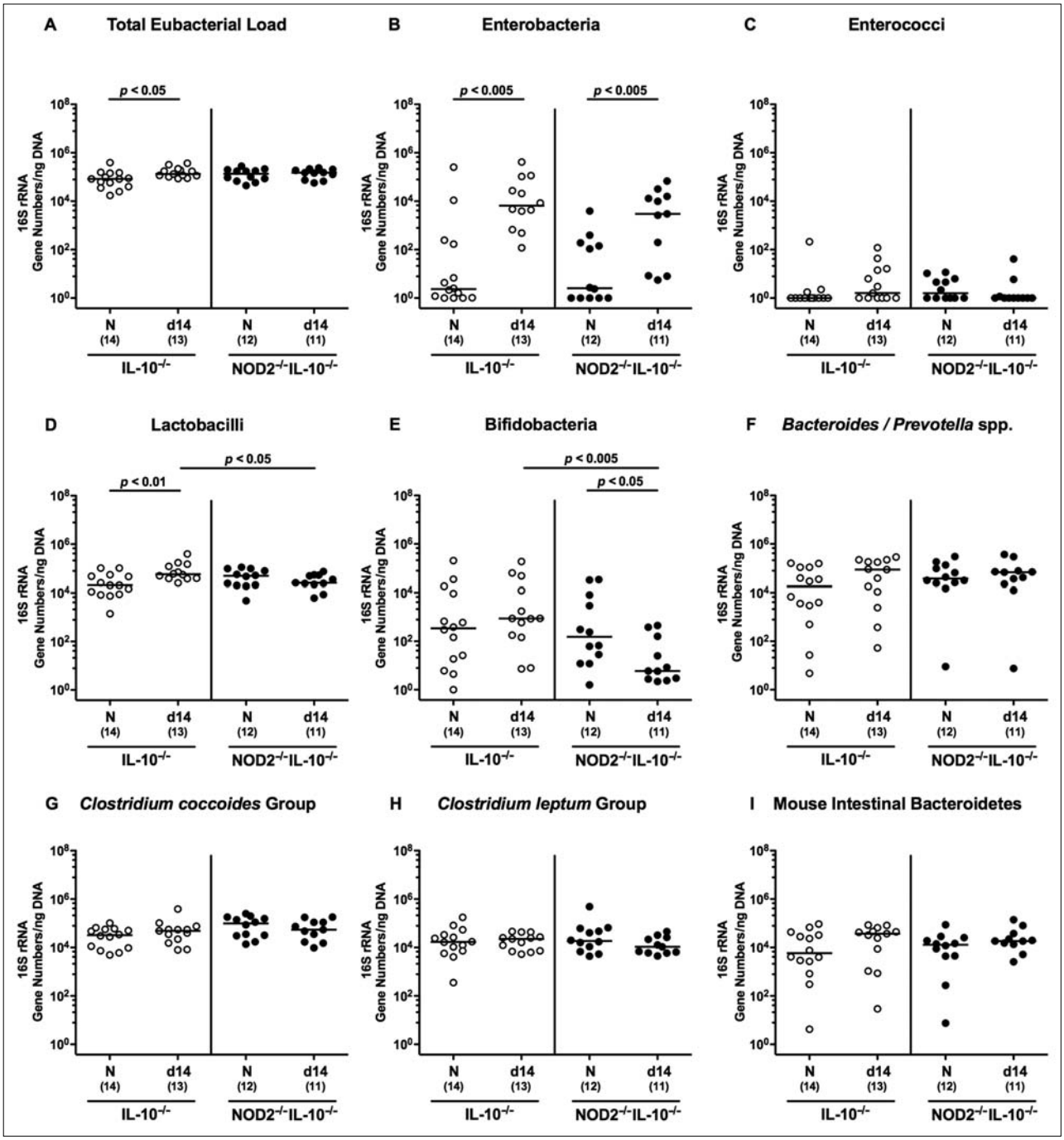

Fig. 2. Commensal intestinal microbiota composition of *C. jejuni* strain 81-176 infected conventional IL-10$^{-/-}$ mice lacking NOD2. IL-10$^{-/-}$ (white circles) and IL-10$^{-/-}$ mice lacking NOD2 (NOD2$^{-/-}$ IL-10$^{-/-}$; black circles) were perorally infected with *C. jejuni* strain 81-176 by gavage at days 0, 1, and 2. 16S rRNA of main intestinal bacterial groups were quantitated in fecal samples derived at day (d) 14 postinfection. (A) Individual total eubacterial loads and quantitative abundances of (B) enterobacteria, (C) enterococci, (D) lactobacilli, (E) bifidobacteria, (F) *Bacteroides/Prevotella* species (spp.), (G) *Clostridium coccoides* group, (H) *Clostridium leptum* group, and (I) mouse intestinal bacteroidetes are expressed as gene copy numbers per ng DNA. Naive (N) mice served as uninfected controls. Medians (black bars) and levels of significance (*p*-values) determined by Mann–Whitney *U* test are indicated. Numbers of analyzed animals are given in parentheses. Data were pooled from three independent experiments

cultured from IL-10$^{-/-}$ mice at day 14 p.i. were lower as compared to those detected in fecal sampels at days 3, 4, 5, and 7 p.i. ($p < 0.05$–0.001; *Fig. S1A*), whereas, in NOD2$^{-/-}$ IL-10$^{-/-}$ mice, intestinal pathogenic burdens were higher until day 5 versus day 14 p.i. ($p < 0.05$–0.005; *Fig. S1B*). At day of necropsy, *C. jejuni* could be isolated in 42.9% and 57.1% of IL-10$^{-/-}$ and NOD2$^{-/-}$ IL-10$^{-/-}$ mice, respectively, whereas the small intestines including ileum and duodenum as well as the stomach of infected mice were virtually pathogen-free *(Fig. 1B)*. Notably, *C. jejuni* could neither be cultured from MLN nor did the pathogen translocate from the intestines to extra-intestinal compartments such as spleen, liver, or kidney *(Fig. 1C)*. Hence, NOD2 deficiency does not impact gastrointestinal colonization properties of *C. jejuni* in IL-10$^{-/-}$ mice harboring a conventional microbiota.

Changes in intestinal microbiota composition in C. jejuni*-infected conventionally colonized IL10$^{-/-}$ mice lacking NOD2*

We next investigated whether the composition of the commensal intestinal microbiota in conventionally colonized IL-10$^{-/-}$ mice was NOD2 dependent. To address this, fecal samples from uninfected IL-10$^{-/-}$ mice lacking NOD2 and from IL-10$^{-/-}$ controls were subjected to 16S rRNA analysis of the most prevalent commensal intestinal groups by

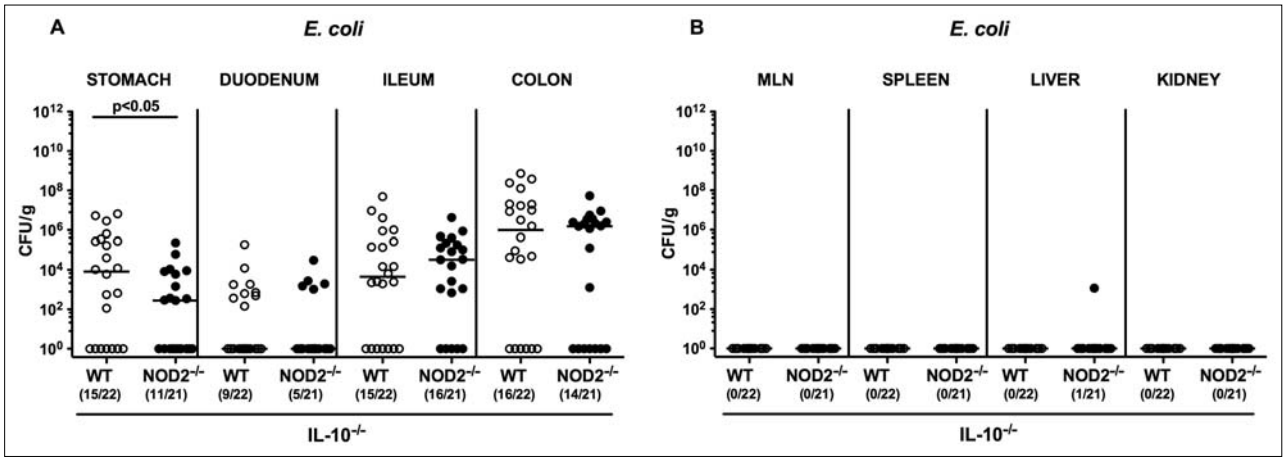

Fig. 3. Gastrointestinal and extra-intestinal commensal *E. coli* loads in *C. jejuni* strain 81-176 infected conventional IL-10$^{-/-}$ mice lacking NOD2. IL-10$^{-/-}$ (IL-10$^{-/-}$; white circles) and IL-10$^{-/-}$ mice lacking NOD2 (NOD2$^{-/-}$ IL-10$^{-/-}$; black circles) were perorally infected with *C. jejuni* strain 81-176 by gavage at days (d) 0, 1, and 2. Commensal *E. coli* loads were quantitated in *ex vivo* biopsies derived from (A) gastrointestinal and (B) extra-intestinal compartments at day 14 following *C. jejuni* infection. Medians (black bars) and levels of significance (p-values) determined by Mann–Whitney U test are indicated. Numbers of mice harboring *C. jejuni* strain 81-176 out of the total number of analyzed animals are given in parentheses. Data were pooled from four independent experiments

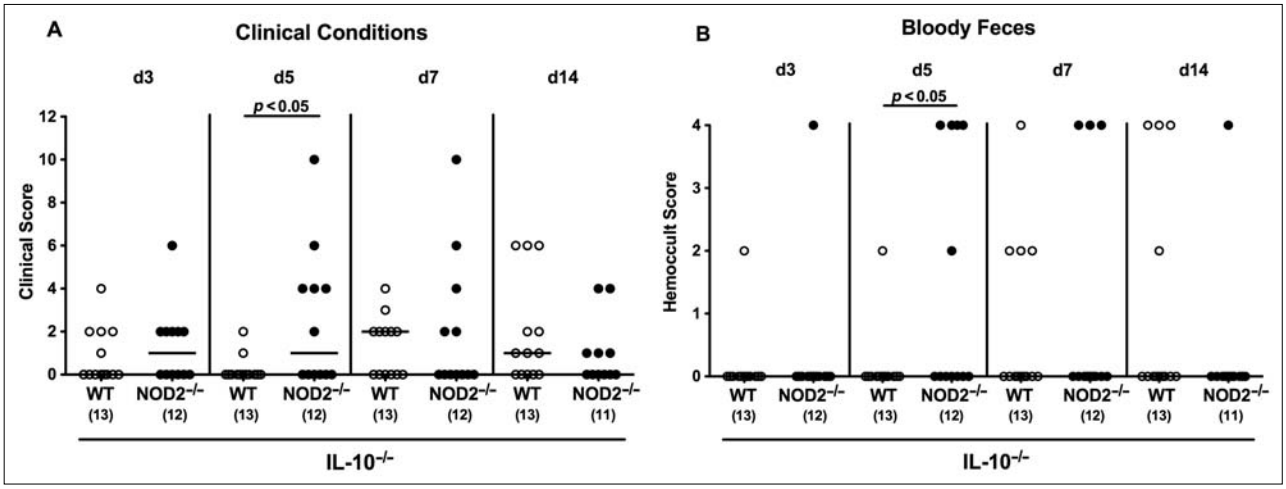

Fig. 4. Kinetic survey of clinical conditions and abundance of fecal blood in IL-10$^{-/-}$ mice lacking NOD2 following *C. jejuni* strain 81-176 infection. IL-10$^{-/-}$ (IL-10$^{-/-}$; white circles) and IL-10$^{-/-}$ mice lacking NOD2 (NOD2$^{-/-}$ IL-10$^{-/-}$; black circles) were perorally infected with *C. jejuni* strain 81-176 by gavage at days (d) 0, 1, and 2. (A) Clinical symptoms were quantitated postinfection applying a standardized clinical scoring system (see Methods). (B) Abundance of fecal blood was surveyed by microscopic (applying the Guajac method) or macroscopic detection of blood in fecal samples over time following *C. jejuni* infection (hemoccult score, see Methods). Means (black bars), levels of significance (p-values) determined by Mann–Whitney U test, and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from three independent experiments

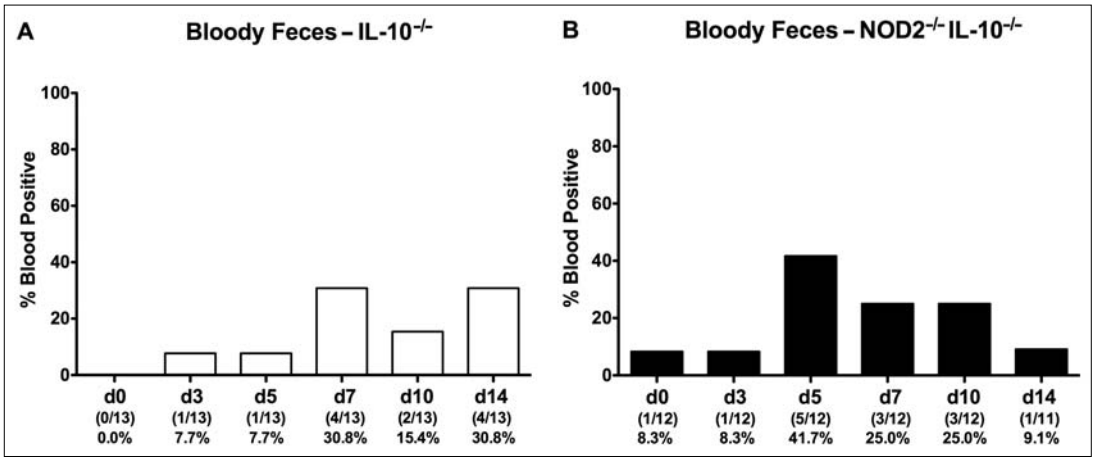

Fig. 5. Kinetic survey of bloody diarrhea in IL-10$^{-/-}$ mice lacking NOD2 following *C. jejuni* strain 81-176 infection. (A) IL-10$^{-/-}$ (white bars) and (B) IL-10$^{-/-}$ mice lacking NOD2 (NOD2$^{-/-}$ IL-10$^{-/-}$; black bars) were perorally infected with *C. jejuni* strain 81-176 by gavage at days (d) 0, 1, and 2. Microscopic or macroscopic ccurrence of blood in fecal samples before and after infection were assessed as described in Methods. Bars indicated mean relative abundances (in %) of blood-positive fecal samples. In addition, absolute numbers of animals with blood-positive fecal samples out of the total number of analyzed mice are indicated (in parentheses). Data were pooled from three independent experiments

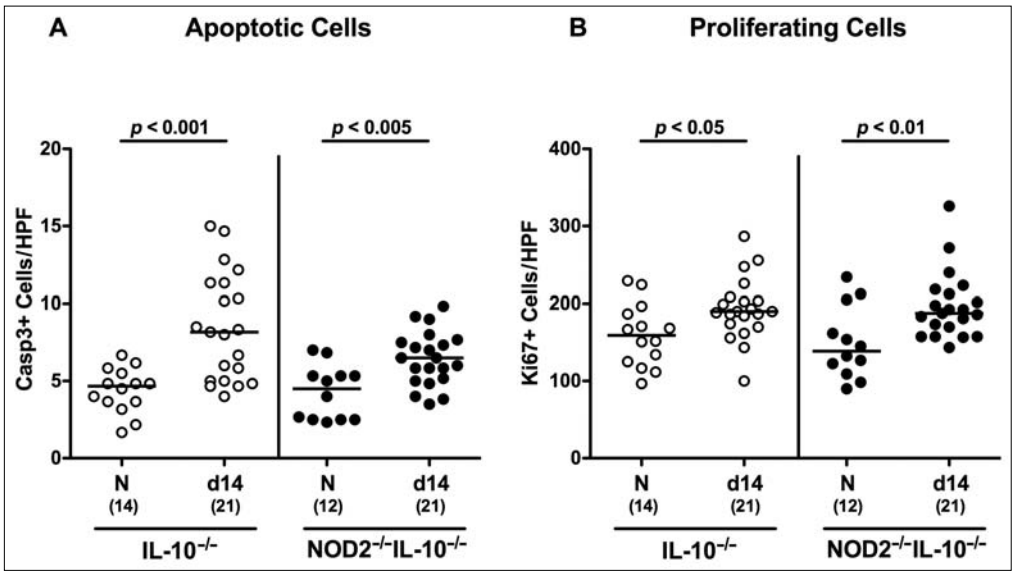

Fig. 6. Apoptotic and proliferating cells in the colonic epithelium of *C. jejuni* strain 81-176 infected conventional IL-10$^{-/-}$ mice lacking NOD2. IL-10$^{-/-}$ (IL-10$^{-/-}$; white circles) and IL-10$^{-/-}$ mice lacking NOD2 (NOD2$^{-/-}$ IL-10$^{-/-}$; black circles) were perorally infected with *C. jejuni* strain 81-176 by gavage at days 0, 1, and 2. The average number of colonic (A) apoptotic cells (positive for caspase-3, Casp3) and (B) proliferating cells (positive for Ki67) from at least six high power fields (HPF, 400x magnification) per animal was determined microscopically in immunohistochemically stained colonic paraffin sections at day (d) 14 following *C. jejuni* infection. Naive (N) mice served as uninfected controls. Medians (black bars), levels of significance (p-values) determined by Mann–Whitney U test, and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments

quantitative RT-PCR [32, 33]. Notably, the microbiota composition did not differ between naive mice of either genotype as indicated by virtually comparable eubacterial total loads and comparable gene numbers of aerobic, anaerobic, and microaerophilic bacterial groups and species in fecal samples derived from uninfected NOD2$^{-/-}$ IL-10$^{-/-}$ and IL-10$^{-/-}$ mice *(Fig. 2)*. We next addressed whether *C. jejuni* infection might induce NOD2-dependent changes in microbiota composition of IL-10$^{-/-}$ mice. Until day 14 p.i., bifidobacterial gene numbers dropped by approximately 1.5 orders of magnitude in fecal samples derived from NOD2$^{-/-}$ IL-10$^{-/-}$ ($p < 0.05$; *Fig. 2E*), but not IL-10$^{-/-}$ mice, and were more than 2 log lower as compared to infected IL-10$^{-/-}$ counterparts ($p < 0.005$; *Fig. 2E*). Fecal lactobacilli slightly increased (i.e., by less than 1 log) in IL10$^{-/-}$ mice upon *C. jejuni* infection ($p < 0.01$; *Fig. 2D*). At day 14 p.i., NOD2$^{-/-}$ IL-10$^{-/-}$ mice harbored approximately 0.5 order of magnitude lower lactobacilli gene numbers in their feces as compared to IL-10$^{-/-}$ mice. Furthermore, enterobacterial loads increased in mice of either

genotype upon *C. jejuni* challenge ($p < 0.005$; *Fig. 2B*). Hence, within 2 weeks following *C. jejuni* infection, rather subtle NOD2-dependent changes of the intestinal microbiota of IL-10$^{-/-}$ mice could be observed affecting colonic bifidobacteria and lactobacilli.

Since *C. jejuni* infection is facilitated by elevated commensal intestinal *Escherichia coli* loads [8, 10, 11, 34], we next determined gastrointestinal *E. coli* numbers in NOD2$^{-/-}$ IL-10$^{-/-}$ mice. At day 14 p.i., IL-10$^{-/-}$ mice lacking NOD2 exhibited less than 2 orders of magnitude lower *E. coli* loads in the stomach lumen as compared to infected IL-10$^{-/-}$ counterparts ($p < 0.05$; *Fig. 3A*), whereas, in the small and large intestines, the *E. coli* counts were comparable *(Fig. 3A)*. As for *C. jejuni, E. coli* could neither be cultured from MLN, nor did viable *E. coli* translocate to extra-intestinal tissue sites such as spleen, liver, or kidney *(Fig. 3B)*.

Clinical sequelae upon C. jejuni *infection of IL-10$^{-/-}$ mice lacking NOD2*

We further quantitatively assessed *C. jejuni*-induced symptoms of IL-10$^{-/-}$ mice by a standardized cumulative clinical scoring system applying clinical conditions, degree of diarrhea, and the occurrence of blood in fecal samples. At day 5 p.i., IL-10$^{-/-}$ mice lacking NOD2 were more clinically compromised as indicated by higher cumu-

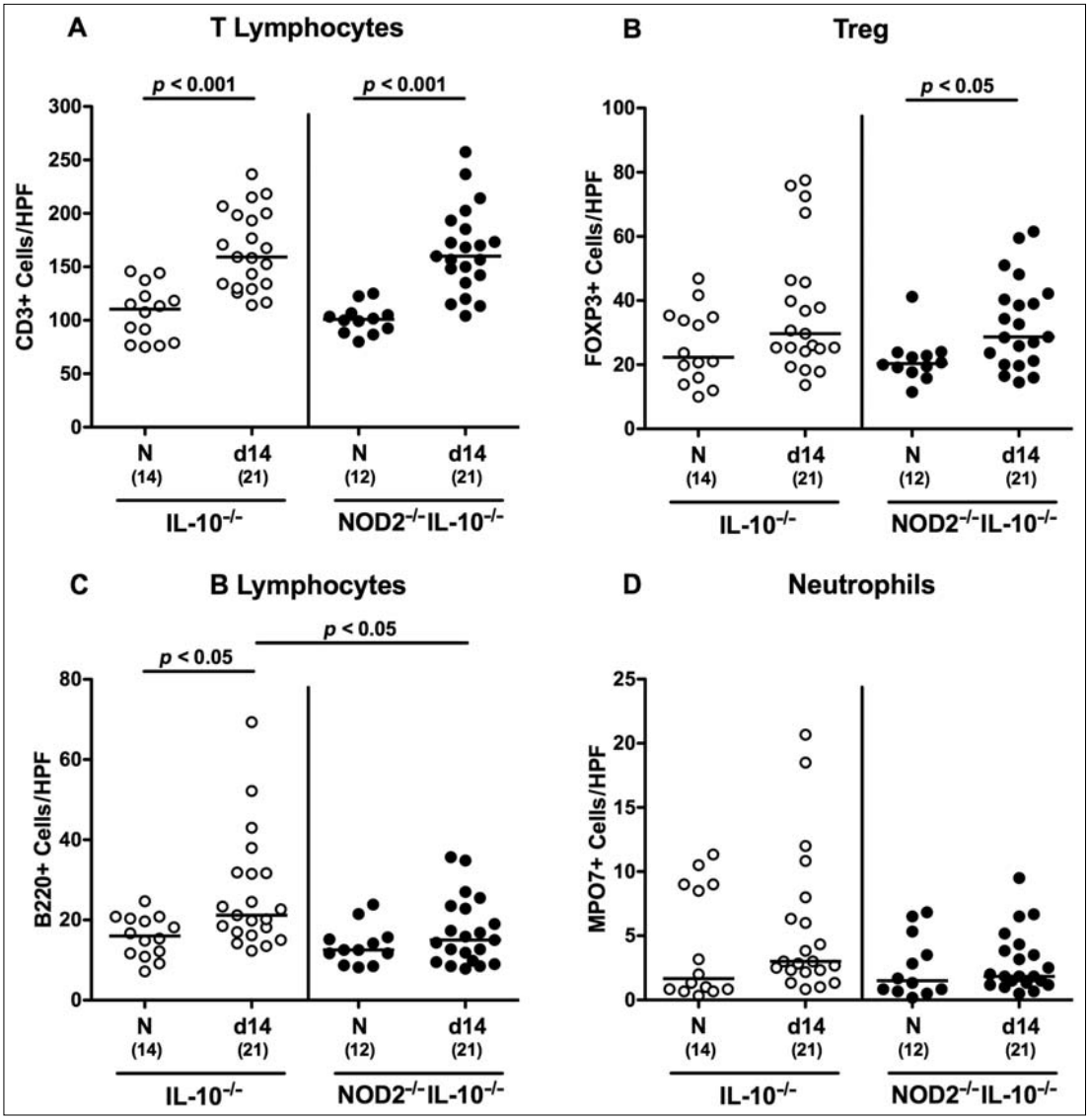

Fig. 7. Colonic immune cell responses in *C. jejuni* strain 81-176 infected conventional IL-10$^{-/-}$ mice lacking NOD2. IL-10$^{-/-}$ (IL-10$^{-/-}$; white circles) and IL-10$^{-/-}$ mice lacking NOD2 (NOD2$^{-/-}$ IL-10$^{-/-}$; black circles) were perorally infected with *C. jejuni* strain 81-176 by gavage at days 0, 1, and 2. The average number of colonic (A) T lymphocytes (positive for CD3), (B) regulatory T cells (Treg, positive for FOXP3), (C) B lymphocytes (positive for B220), and (D) neutrophils (positive for MPO7) from at least six high power fields (HPF, 400× magnification) per animal was determined microscopically in immunohistochemically stained colonic paraffin sections at day (d) 14 following *C. jejuni* infection. Naive (N) mice served as uninfected controls. Medians (black bars), levels of significance (p values) determined by Mann–Whitney U test, and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments

lative clinical and specifically by higher hemoccult scores (the latter assessing the occurrence of macroscopic or microscopic blood in feces) as compared to IL-10$^{-/-}$ controls ($p < 0.05$; *Fig. 4*). Thereafter, however, *C. jejuni*-induced symptoms were comparable until day 14 p.i. (*Figs. 4* and *5; Figs. S2* and *S3*). Interestingly, fecal blood-positivity rates were higher in NOD2$^{-/-}$ IL-10$^{-/-}$ (41.7%) as compared to IL-10$^{-/-}$ mice (7.7%) at day 5 p.i., whereas, the other way round, 30.8% of IL-10$^{-/-}$ controls but only 9.1% of NOD2$^{-/-}$ IL-10$^{-/-}$ mice exhibited fecal blood at day 14 p.i. *(Fig. 5, Fig. S3)*. Hence, NOD2 seems to mediate protective measures during the early course of *C. jejuni* infection in the IL-10$^{-/-}$ mouse model.

Microscopic sequelae upon C. jejuni *infection of IL-10$^{-/-}$ mice lacking NOD2*

We next assessed microscopic sequelae of *C. jejuni* infection. Given that apoptosis is regarded as diagnostic marker for the histopathological evaluation and grading of intestinal disease including murine campylobacteriosis [9, 11], we stained colonic paraffin sections against caspase-3 by *in situ* immunohistochemistry to quantitate apoptotic cells within the colonic epithelium. At day 14 p.i., both NOD2$^{-/-}$ IL-10$^{-/-}$ mice and IL-10$^{-/-}$ controls displayed approximately two-fold higher numbers of caspase-3+ cells as compared to uninfected controls in their colonic epithelium *(Fig. 6A)*. These increases in apoptotic cells were accompanied by elevated numbers of regenerating cells as indicated by up to 50% increases in colonic epithelial Ki67+ cells in both NOD2$^{-/-}$ IL-10$^{-/-}$ and IL-10$^{-/-}$ mice at day 14 p.i. *(Fig. 6B)*. Hence, NOD2 does neither impact apoptotic nor proliferative (i.e., regenerative) responses upon *C. jejuni* infection of IL-10$^{-/-}$ mice.

Colonic pro-inflammatory immune responses upon C. jejuni *infection of IL-10$^{-/-}$ mice lacking NOD2*

Recruitment of pro-inflammatory immune cell population to the site of infection is one of the hallmarks of campylobacteriosis [9]. We therefore quantitatively assessed the numbers of distinct immune cell populations by *in situ* immunohistochemical staining of colonic paraffin sections at day 14 following *C. jejuni* infection. *C. jejuni*-induced increases in colonic T lymphocytes (approximately 50%) were similar in NOD2$^{-/-}$ IL-10$^{-/-}$ and IL-10$^{-/-}$ mice ($p < 0.001$; *Fig. 7A*). Whereas large intestinal Tregs increased in NOD2$^{-/-}$ IL-10$^{-/-}$ mice only until day 14 p.i. ($p < 0.05$; *Fig. 7B*), B lymphocytes were elevated in the colon of infected IL-10$^{-/-}$ mice, but not NOD2 deficient counterparts, as compared to naive controls ($p < 0.05$; *Fig. 7C*), and thus higher as compared to infected NOD2$^{-/-}$ IL-10$^{-/-}$ mice ($p < 0.05$; *Fig. 7C*). Unexpectedly, only a trend towards increased neutrophil numbers within the large intestines at day 14 p.i. could be observed (n.s.; *Fig. 7D*).

We next measured pro-inflammatory cytokine expression in colonic *ex vivo* biopsies. At day 14 p.i., TNF mRNA levels were elevated in the large intestines of NOD2$^{-/-}$ IL-10$^{-/-}$ mice only ($p < 0.05$; *Fig. 8A*), whereas colonic IFN-γ expression levels did not differ in infected and naive mice of either genotype (n.s.; *Fig. 8B*).

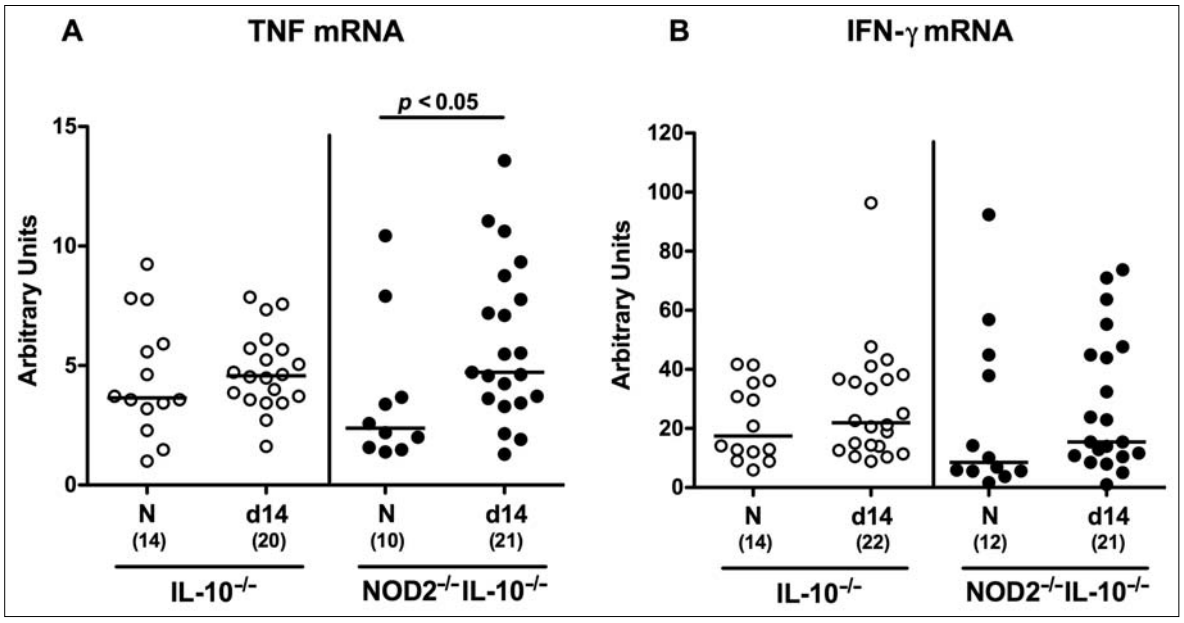

Fig. 8. Colonic expression of pro-inflammatory cytokines in *C. jejuni* strain 81-176 infected conventional IL-10$^{-/-}$ mice lacking NOD2. IL-10$^{-/-}$ (IL-10$^{-/-}$; white circles) and IL-10$^{-/-}$ mice lacking NOD2 (NOD2$^{-/-}$ IL-10$^{-/-}$; black circles) were perorally infected with *C. jejuni* strain 81-176 by gavage at days 0, 1, and 2. (A) TNF and (B) IFN-γ mRNA expression levels were determined in colonic *ex vivo* biopsies at day (d) 14 postinfection by real-time PCR and expressed in arbitrary units (fold expression). Naive (N) mice served as uninfected controls. Medians (black bars), level of significance (p value) determined by Mann–Whitney U test, and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments

We have recently shown that cytokines belonging to the IL-23/IL-22/IL-18 axis are involved in mediating murine campylobacteriosis [35–37] and, therefore, assessed expression levels of the respective cytokines in colonic *ex vivo* biopsies. As for TNF mRNA, expression of IL-23p19 was upregulated 14 days following *C. jejuni* infection of NOD2$^{-/-}$ IL-10$^{-/-}$, but not IL-10$^{-/-}$ mice ($p < 0.05$; *Fig. 9A*), whereas IL-22 or IL-18 mRNA expression did not change upon infection of mice of either genotype (n.s.; *Fig. 9B*,*C*). Hence, NOD2-dependent differences of *C. jejuni*-induced colonic pro-inflammatory cytokine expression were, if at all, only minor.

Commensal bacterial translocation and colonic mucin expression upon C. jejuni *infection of IL-10$^{-/-}$ mice lacking NOD2*

We next addressed whether NOD2 deficiency might facilitate translocation of commensal bacteria derived from the intestinal microbiota to extra-intestinal compartments. In approximately one third of MLN derived from *C. jejuni*-infected NOD2$^{-/-}$ IL-10$^{-/-}$ and IL-10$^{-/-}$ mice, commensal intestinal species such as *E. coli, Enterococcus* spp., and *Lactobacillus* spp. could be cultured at day 14 p.i. by direct plating. Viable commensal bacteria could be detect-

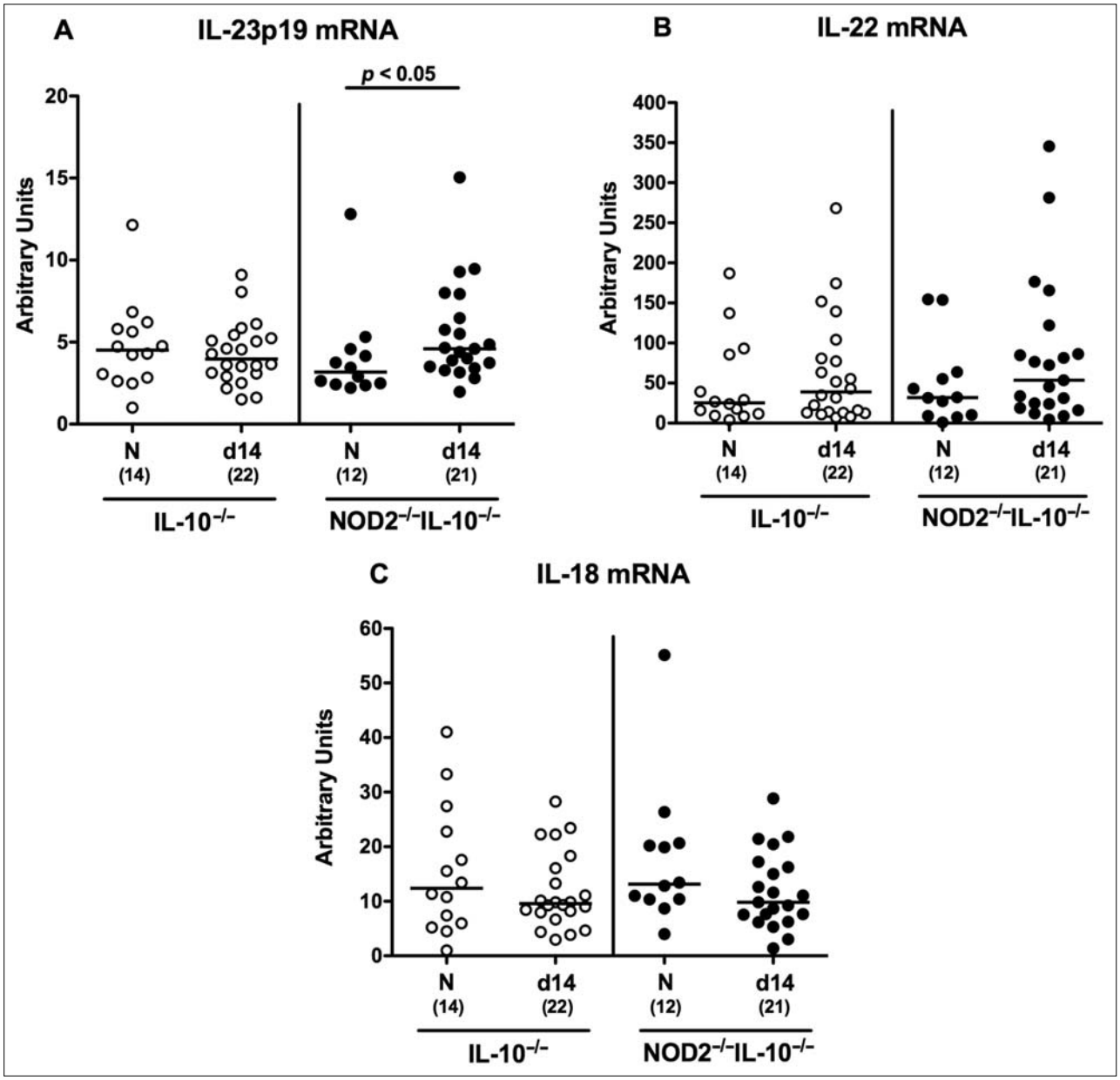

Fig. 9. Colonic expression of IL-23p19, IL-22, and IL-18 mRNA in *C. jejuni* strain 81-176 infected conventional IL-10$^{-/-}$ mice lacking NOD2. IL-10$^{-/-}$ (IL-10$^{-/-}$; white circles) and IL-10$^{-/-}$ mice lacking NOD2 (NOD2$^{-/-}$ IL-10$^{-/-}$; black circles) were perorally infected with *C. jejuni* strain 81-176 by gavage at days 0, 1, and 2. (A) IL-23p19, (B) IL22, and (C) IL-18 mRNA expression levels were determined in colonic *ex vivo* biopsies at day (d) 14 postinfection by real-time PCR and expressed in arbitrary units (fold expression). Naive (N) mice served as uninfected controls. Medians (black bars), level of significance (p value) determined by Mann–Whitney U test, and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments

ed in 13.6% of spleens taken from IL-10$^{-/-}$, but none of NOD2$^{-/-}$ IL-10$^{-/-}$ animals. Bacterial translocation rates into liver were 14.3% and 9.1% for NOD2$^{-/-}$ IL-10$^{-/-}$ and IL-10$^{-/-}$ mice, respectively, whereas viable commensal intestinal bacteria could be isolated in 9.5% and 13.6% of kidneys derived from NOD2$^{-/-}$ IL-10$^{-/-}$ and IL-10$^{-/-}$ mice, respectively *(Fig. 10)*.

Given that mucin-2 (MUC2) is expressed within the mucus layer of the intestinal tract and contributes significanty to combating bacterial infections and maintaining epithelial barrier function [38, 39], we determined MUC2 mRNA expression levels in colonic *ex vivo* biopsies of IL-10$^{-/-}$ mice lacking NOD2. Irrespective of the genotype of mice and their infection status, however, colonic MUC2 mRNA expression levels were comparable *(Fig. 11)*. Hence, neither colonic mucin-2 expression nor bacterial translocation following *C. jejuni* infection of IL-10$^{-/-}$ mice is NOD2 dependent.

Discussion

In the present study we shed further light onto the role of NOD2, a pivotal innate immune sensor of bacterial MDP, in *C. jejuni* infection of conventionally colonized IL-10$^{-/-}$ mice with and without additional NOD2 deficiency. Following peroral challenge, mice of either genotype displayed comparable intestinal *C. jejuni* loads over time until day 14 p.i., indicating that *C. jejuni* colonization in IL-10$^{-/-}$ mice harboring a conventional microbiota occurs NOD2 independently. Our results are contrasted by previous infection studies applying other intracellular pathogens indicating that NOD2$^{-/-}$ mice are more susceptible to infection with Salmonella Typhimurium [40] or *Listeria monocytogenes* [41], for instance.

Many recent reports highlighted the pivotal role of the distinct intestinal microbiota composition on initiating, mediating, and perpetuating immunopathological conditions in mice and men [29, 42–45]. Given that NOD2 among other factors is shaping the commensal intestinal microbiota [46, 47], that in turn, determines host susceptibility to *C. jejuni* infection [8–10], we performed a comprehensive quantitative molecular survey of the conventional intestinal microbiota in NOD2$^{-/-}$ IL-10$^{-/-}$ mice and IL-10$^{-/-}$ counterparts, before and after pathogenic infection. Our results revealed that, under naive conditions, NOD2 deficiency did not impact intestinal microbiota composition and is supported by previous studies with NOD2$^{-/-}$ and co-housed WT mice [48, 49]. These results might appear somewhat surprising, since NOD2 deficiency has been shown to lead to altered expression of antimicrobial peptides including defensins [41, 50], which might result not only in compromised pathogenic clearance by the host but also in different shaping of the intestinal microbiota in health and disease.

In our study, at the first glance rather minor differences in intestinal microbiota composition became evident at day 14 p.i. as indicated by lower bifidobacteria and lactobacilli counts (approximately 2.0 and 0.5 orders of magnitude, respectively) in feces taken from *C. jejuni*-infected NOD2$^{-/-}$ IL-10$^{-/-}$ mice versus IL-10$^{-/-}$ controls. Our data are at least in part supported by our previous study indicat-

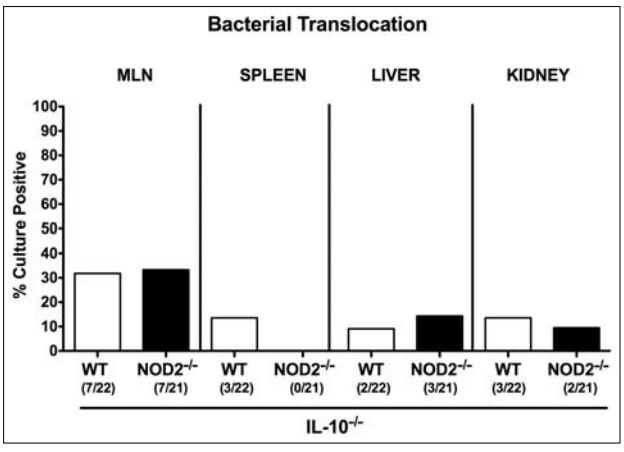

Fig. 10. Pathogenic translocation in *C. jejuni* strain 81-176 infected conventional IL-10$^{-/-}$ mice lacking NOD2. IL-10$^{-/-}$ (IL-10$^{-/-}$; white bars) and IL-10$^{-/-}$ mice lacking NOD2 (NOD2$^{-/-}$ IL-10$^{-/-}$; black bars) were perorally infected with *C. jejuni* strain 81-176 by gavage at days 0, 1, and 2. Translocation of viable pathogens was assessed by culture in *ex vivo* biopsies derived from mesenteric lymphnodes (MLN), spleen, liver, and kidney at day (d) 14 postinfection. Mean relative abundances of viable pathogens in the respective compartments are shown (bars; in %), and numbers of mice harboring *C. jejuni* out of the total number of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments

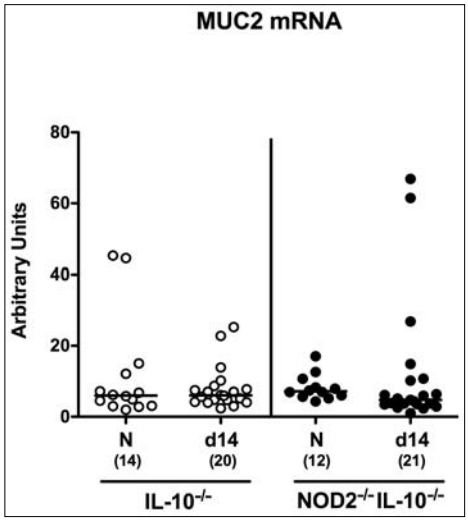

Fig. 11. Colonic mucin-2 mRNA expression levels in *C. jejuni* strain 81-176 infected conventional IL-10$^{-/-}$ mice lacking NOD2. IL-10$^{-/-}$ (IL-10$^{-/-}$; white circles) and IL-10$^{-/-}$ mice lacking NOD2 (NOD2$^{-/-}$ IL-10$^{-/-}$; black circles) were perorally infected with *C. jejuni* strain 81-176 by gavage at days 0, 1, and 2. Mucin-2 (MUC2) mRNA expression levels were determined in colonic *ex vivo* biopsies at day (d) 14 postinfection by real-time PCR and expressed in arbitrary units (fold expression). Naive (N) mice served as uninfected controls. Medians (black bars) and numbers of analyzed animals (in parentheses) are indicated. Data were pooled from four independent experiments

ing that bifidobacteria were also less abundant in the ilea of naive conventionally colonized NOD2$^{-/-}$ mice (IL-10$^{+/+}$), whereas distal small intestinal lactobacilli loads were even slightly higher as compared to WT controls [29]. Particularly bifidobacterial species constitute beneficial, "probiotic" microbes with anti-inflammatory properties that are also involved in conferring host resistance against pathogens [51]. It is, however, questionable, whether the observed minor changes in intestinal lactobacilli loads would have a biologically relevant impact. Notably, enterobacterial loads known to facilitate *C. jejuni* infection [11] were similar in naive and infected mice, irrespective of their genotype.

NOD2 seems to be involved in mediating inflammatory responses upon enteropathogenic infection in a time-dependent manner given that NOD2$^{-/-}$ IL-10$^{-/-}$ mice were clinically more compromised during the early phase of *C. jejuni* infection (i.e., day 5 p.i.), whereas, later on, at day 14 p.i., IL-10$^{-/-}$ counterparts were more frequently exhibiting blood in their feces as compared to NOD2$^{-/-}$ IL-10$^{-/-}$ mice. It is hence tempting to speculate that NOD2 could mediate protective measures during the early course of *C. jejuni* infection in the conventional IL-10$^{-/-}$ mouse model. A protective role of NOD2 in campylobacteriosis would be well in line with recent findings in antibiotics-treated NOD2$^{-/-}$ IL-10$^{-/-}$ mice [52]. In the respective study, conventional NOD2$^{-/-}$ IL-10$^{-/-}$ mice were subjected to broad-spectrum antibiotic treatment for 1 week before pathogenic challenge and exhibited an *C. jejuni*-induced exacerbation of colitis. However, the differences in murine disease outcomes in comparison to the experimental models used herein can be attributed to fundamental differences in the respective study designs [52].

C. jejuni-induced microscopic changes in the large intestines in our study, however, were not NOD2 dependent as indicated by similar apoptotic as well as proliferating/regenerating cell numbers in large intestinal epithelia that were accompanied by a comparable infux of neutrophils and T lymphocytes into the colonic mucoasa and lamina propria, whereas colonic B lymphocytes were slightly less abundant in infected NOD2 deficient IL-10$^{-/-}$ mice as compared to IL-10$^{-/-}$ counterparts. In line with this, NOD2-dependent differences of *C. jejuni*-induced colonic pro-inflammatory cytokine expression were, if at all, only minor. Only in NOD2$^{-/-}$ IL-10$^{-/-}$ mice, colonic TNF and IL-23p19 were upregulated upon *C. jejuni* infection.

To date, there are conflicting data regarding the distinct role of NOD2 in large intestinal inflammation, given that, depending of the applied model system, NOD2 deficiency might either accelerate or prevent from colitis development. For instance, adoptive transfer of NOD2$^{-/-}$ T cells into immunocompromised mice resulted in less severe chronic colitis, indicating that NOD2 signaling exacerbates colonic inflammation [15]. MDP application, however, was sufficient to prevent from 2,4,6-trinitrobenzenesulphonic acid (TNBS) induced colitis, whereas MDP-mediated protection from disease was abrogated in NOD2$^{-/-}$ mice pointing towards a protective role of NOD2 signaling [53]. NOD2 was further shown to promote IL-10$^{-/-}$ colitis, given that NOD2$^{-/-}$ IL-10$^{-/-}$ mice were prevented from severe colitis development [54]. In contrast, NOD2 was capable of mediating anti-inflammatory responses in murine IL-10$^{-/-}$ colitis [52]. As already mentioned, in the study by Sun and Jobin, conventional NOD2$^{-/-}$ IL-10$^{-/-}$ mice were pretreated with antibiotics before pathogenic challenge and exhibited an *C. jejuni*-induced exacerbation of colitis [52]. The differences in disease outcomes are most likely due to distinct differences in experimental setups [52, 54].

An intact epithelial barrier function constitutes a pivotal prerequisite for successfully limiting bacterial including pathogenic translocation from the intestines to extra-intestinal including systemic sites [55]. In the present study, translocation of viable bacterial commensals could not be observed under basal conditions in mice of either genotype. Following *C. jejuni* infection of conventional NOD2$^{-/-}$ IL-10$^{-/-}$ and IL-10$^{-/-}$ control mice, however, commensals such as *E. coli, Enterococcus* spp., and/or *Lactobacillus* spp. could be isolated from MLN, liver, and kidneys at comparable frequencies, whereas systemic bacterial translocation (namely, into the spleen) could be observed in IL-10$^{-/-}$ only. Comparable bacterial translocation rates were accompanied by similar expression levels of mucin-2 (MUC2), constituting a secreted glycoprotein and pivotal component of the mucous layer protecting the underlying mucosal epithelial layer not only from invading pathogens but also from translocating intestinal commensals [56]. Hence, neither colonic mucin-2 expression nor commensal bacterial translocation following *C. jejuni* infection of IL-10$^{-/-}$ mice was NOD2 dependent.

Taken together, NOD2 does not impact the overall intestinal microbiota composition in conventionally colonized IL-10$^{-/-}$ mice under basal conditions. Following *C. jejuni* infection, however, lower colonic fecal loads of bifidobacteria could be determined in IL-10$^{-/-}$ mice lacking NOD2. Whereas NOD2 suppresses bloody diarrhea upon *C. jejuni* infection of conventionally colonized IL-10$^{-/-}$ mice in the early phase of infection, the overall intestinal and extra-intestinal pro-inflammatory immune responses as well as commensal bacterial translocation were found to be NOD2 independent.

In conclusion, further studies are needed to unravel the molecular mechanisms of NOD2 signaling in *C. jejuni*–host interactions in more detail.

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Competing Interests

Stefan Bereswill and Markus M. Heimesaat are Editorial Board members.

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Supplemental Material

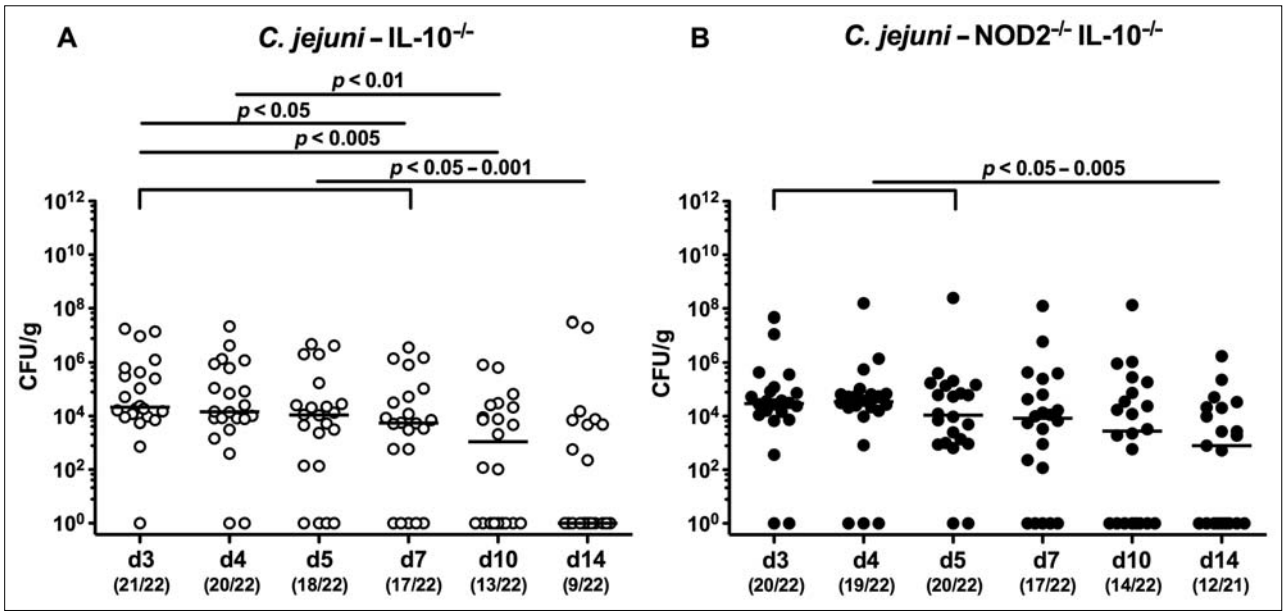

Fig. S1. Intestinal *C. jejuni* strain 81-176 loads over time in perorally infected conventionally colonized IL-10$^{-/-}$ mice lacking NOD2. (A) IL-10$^{-/-}$ (white circles) and (B) IL-10$^{-/-}$ mice lacking NOD2 (NOD2$^{-/-}$ IL-10$^{-/-}$; black circles) were perorally infected with *C. jejuni* strain 81-176 by gavage at days (d) 0, 1, and 2. Pathogenic loads were determined in fecal samples (CFU, colony forming units per gram) by culture over time postinfection as indicated. Medians (black bars) and levels of significance (p values) determined by Mann–Whitney U test are indicated. Numbers of mice harboring *C. jejuni* strain 81-176 out of the total number of analyzed animals are given in parentheses. Data were pooled from four independent experiments

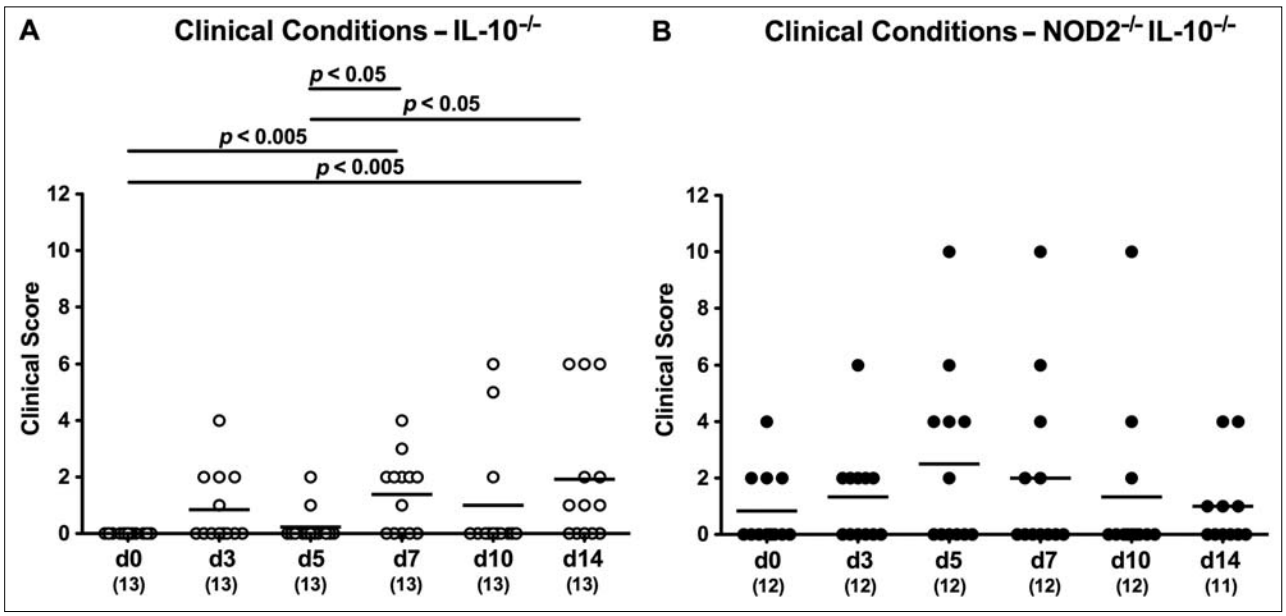

Fig. S2. Kinetic survey of clinical conditions of IL-10$^{-/-}$ mice lacking NOD2 following *C. jejuni* strain 81-176 infection. (A) IL-10$^{-/-}$ (white circles) and (B) IL-10$^{-/-}$ mice lacking NOD2 (NOD2$^{-/-}$ IL-10$^{-/-}$; black circles) were perorally infected with *C. jejuni* strain 81-176 by gavage at days (d) 0, 1, and 2. Severities of clinical symptoms before and after infection were quantitatively assessed applying a standardized clinical scoring system (see Methods). Means (black bars) and levels of significance (p values) determined by Mann–Whitney U test are indicated. Numbers of analyzed mice are given in parentheses. Data were pooled from three independent experiments

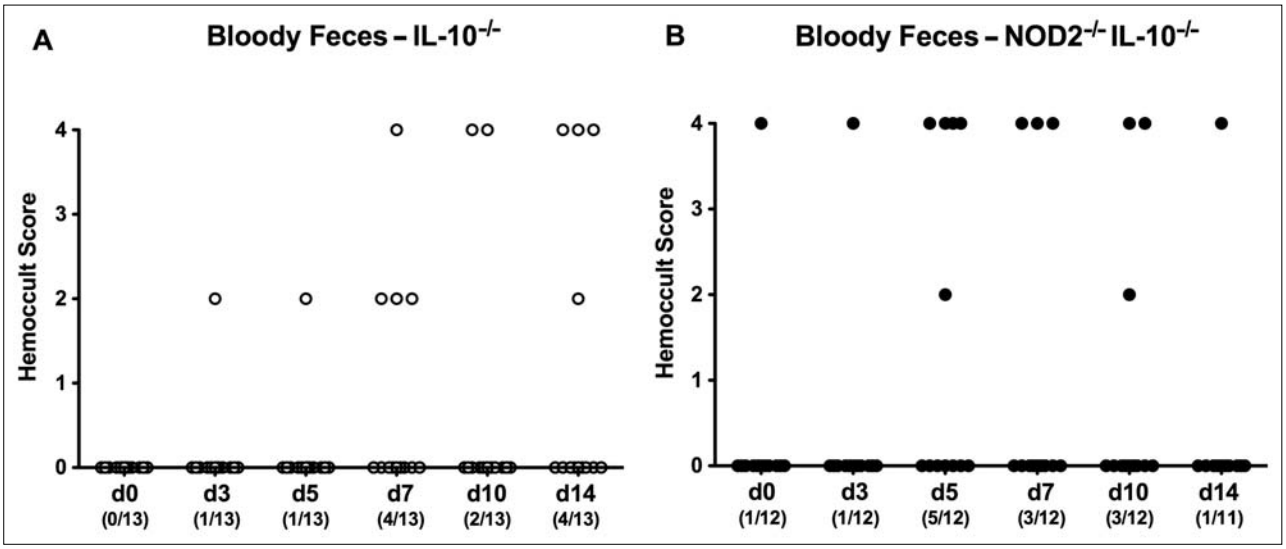

Fig. S3. Kinetic survey of bloody feces in IL-10$^{-/-}$ mice lacking NOD2 following *C. jejuni* strain 81-176 infection. (A) IL-10$^{-/-}$ (white circles) and (B) IL-10$^{-/-}$ mice lacking NOD2 (NOD2$^{-/-}$ IL-10$^{-/-}$; black circles) were perorally infected with *C. jejuni* strain 81-176 by gavage at days (d) 0, 1, and 2. Microscopic or macroscopic abundance of blood in fecal samples before and after infection was quantitatively assessed applying a standardized hemoccult score (see Methods). Means (black bars) and levels of significance (*p* values) determined by Mann–Whitney *U* test are indicated. Absolute numbers of animals with blood-positive fecal samples out of the total number of analyzed mice (in parentheses) and relative abundances (in %) are given. Data were pooled from three independent experiments