

TOLL-LIKE RECEPTOR-4 IS ESSENTIAL FOR *ARCOBACTER BUTZLERI*-INDUCED COLONIC AND SYSTEMIC IMMUNE RESPONSES IN GNOTOBIOTIC IL-10^{-/-} MICE

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Arcobacter butzleri causes sporadic cases of gastroenteritis, but the underlying immunopathological mechanisms of infection are unknown. We have recently demonstrated that *A. butzleri*-infected gnotobiotic IL-10^{-/-} mice were clinically unaffected but exhibited intestinal and systemic inflammatory immune responses. For the first time, we here investigated the role of Toll-like receptor (TLR)-4, the main receptor for lipopolysaccharide and lipooligosaccharide of Gram-negative bacteria, in murine arcobacteriosis. Gnotobiotic TLR-4/IL-10-double deficient (TLR-4^{-/-} IL-10^{-/-}) and IL-10^{-/-} control mice generated by broad-spectrum antibiotics were perorally infected with *A. butzleri*. Until day 16 postinfection, mice of either genotype were stably colonized with the pathogen, but fecal bacterial loads were approximately 0.5–2.0 log lower in TLR-4^{-/-} IL-10^{-/-} as compared to IL-10^{-/-} mice. *A. butzleri*-infected TLR-4^{-/-} IL-10^{-/-} mice displayed less pronounced colonic apoptosis accompanied by lower numbers of macrophages and monocytes, T lymphocytes, regulatory T-cells, and B lymphocytes within the colonic mucosa and lamina propria as compared to IL-10^{-/-} mice. Furthermore, colonic concentrations of nitric oxide, TNF, IL-6, MCP-1, and, remarkably, IFN- γ and IL-12p70 serum levels were lower in *A. butzleri*-infected TLR-4^{-/-} IL-10^{-/-} versus IL-10^{-/-} mice. In conclusion, TLR-4 is involved in mediating murine *A. butzleri* infection. Further studies are needed to investigate the molecular mechanisms underlying *Arcobacter*–host interactions in more detail.

Keywords: *Arcobacter butzleri*, Toll-like receptor-4, lipopolysaccharide, lipooligosaccharide, gnotobiotic IL-10^{-/-} mice, pro-inflammatory immune responses, systemic immune responses, colon, apoptosis, innate and adaptive immunity

Introduction

Arcobacter and *Campylobacter* are taxonomically related given that the genus *Arcobacter* belongs to the *Campylobacteraceae* family [1]. Nineteen distinct *Arcobacter* species have been isolated from a multitude of hosts and environments so far [2]. In animals, the motile Gram-negative *Arcobacter* spp. are regarded as gastrointestinal commensals. For humans, however, *Arcobacter butzleri* and *Arcobacter cryaerophilus* have been rated as potential hazards by the International Commission on Microbiological Specifications for Foods [3]. Reliable data on prevalence and incidence of *Arcobacter* related human diseases are limited given that *Arcobacter* spp. are very frequently not identified by routine diagnostic procedures applied in conventional microbiology laboratories [1]. In retrospective studies, however, *Arcobacter* spp. have

been shown to be the fourth most common *Campylobacteriales* species isolated from patients with diarrhea [4–8]. *Arcobacter*-induced disease outbreaks are most likely caused by ingestion of contaminated food or water [7, 9]. Diseased patients present with symptoms of acute gastroenteritis such as acute diarrhea with abdominal pain or prolonged watery diarrhea for up to several weeks [7, 8, 10]. So far, however, our knowledge about the underlying immunopathogenic mechanisms of arcobacteriosis is limited. Phenotypic assays revealed that *A. butzleri* is able to adhere to and invade into several cell lines. However, adhesive and invasive properties of *A. butzleri* isolates could not be correlated with distinct virulence gene patterns [11, 12]. *In vitro* studies revealed cytotoxic effects of several *A. butzleri* strains, but defined toxins have not been identified so far [5, 13–18].

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Data regarding the immunopathological mechanisms underlying *A. butzleri* infection and the corresponding host responses are scarce and conflicting. *In vivo* studies revealed discrepant results so far depending on the animal species, the breed, and on the respective *A. butzleri* strain. For instance, whereas *A. butzleri* was unable to infect conventional chicken, distinct turkey strains could be colonized at different loads and displayed variable mortality rates [19]. Furthermore, *A. butzleri* exhibited invasive properties in neonatal piglets and could also be isolated from extra-intestinal compartments such as kidney, liver, and brain [20]. Whereas neonatal albino rats developed self-limiting diarrhea and small intestinal and hepatic necrosis upon *A. butzleri* infection, adult rats presented with watery diarrhea and disturbed serum electrolyte balance [21, 22].

Conventionally colonized mice are protected against enteric pathogens such as *Campylobacter jejuni* due to their specific microbiota composition [23]. In previous studies, we have shown that colonization resistance of IL-10^{-/-} mice could be overcome by depletion of the murine intestinal microbiota following broad-spectrum antibiotic treatment. Subsequently, *C. jejuni* infection was thus facilitated and resulted in immunopathological sequelae such as acute enterocolitis, mimicking human campylobacteriosis [24–26]. Given that *C. jejuni* and *A. butzleri* are taxonomically related, we very recently applied the gnotobiotic IL-10^{-/-} mouse model to determine the pathogenic potential of *A. butzleri* and to investigate bacterial–host interactions *in vivo*. The results indicate that, upon peroral *A. butzleri* infection, mice could be stably colonized by the respective strains and displayed significant small and large intestinal as well as extraintestinal and systemic inflammatory responses [27, 28].

Toll-like receptors (TLRs) mediate essential signaling pathways involved in innate and adaptive host immune responses to commensal and pathogenic bacteria [29]. Given that bacterial lipopolysaccharides (LPS) as well as lipooligosaccharides (LOS) derived from Gram-negative bacterial cell walls are sensed by TLR-4 [29], we investigated the role of TLR-4 in *Arcobacter* infection *in vivo*.

Materials and methods

Mice

IL-10^{-/-} mice (in C57BL/10 background, B10) were bred and maintained in the facilities of the “Forschungseinrichtungen für Experimentelle Medizin” (FEM, Charité — Universitätsmedizin, Berlin, Germany) under specific pathogen-free (SPF) conditions. In order to generate TLR-4 IL-10 double deficient (TLR-4^{-/-} IL-10^{-/-}) mice, TLR-4^{-/-} mice (in B10 background) were crossed to IL-10^{-/-} mice and backcrossed more than nine generations before use.

To overcome physiological colonization resistance and assure stable pathogenic colonization, gnotobiotic (i.e., secondary abiotic) TLR-4^{-/-} IL-10^{-/-} and IL-10^{-/-}

mice with a depleted gastrointestinal microbiota were generated following broad-spectrum antibiotic treatment as described earlier [24, 30]. In brief, mice were transferred to sterile cages and treated by adding ampicillin–sulbactam (1 g/l; Pfizer, Berlin, Germany), vancomycin (500 mg/l; Hexal, Holzkirchen, Germany), ciprofloxacin (200 mg/l; Hexal), imipenem (250 mg/l; Fresenius Kabi, Graz, Austria), and metronidazole (1 g/l; Braun, Melsungen, Germany) to the drinking water *ad libitum* starting at 3 weeks of age immediately after weaning and continued for approximately 3 months before the infection experiment [26]. Three days prior infection, the antibiotic cocktail was replaced by sterile tap water (*ad libitum*). Germfree status of gnotobiotic mice was confirmed as described previously [23, 30].

A. butzleri infection of mice

Age-matched female gnotobiotic TLR-4^{-/-} IL-10^{-/-} and IL-10^{-/-} mice were perorally infected with approximately 10⁹ viable colony forming units (CFU) of two different *A. butzleri* strains either (CCUG 30485 or C1 strain, respectively) by gavage in a total volume of 0.3 ml phosphate buffered saline (PBS) on two consecutive days (day 0 and day 1). Naive age- and sex-matched gnotobiotic IL-10^{-/-} mice served as uninfected controls. The *A. butzleri* reference strain CCUG 30485 was initially isolated from a fecal sample derived from a diarrheal patient [31], whereas the C1 strain was isolated from fresh chicken meat [11]. Both *A. butzleri* strains were grown on Karmali-agar (Oxoid, Wesel, Germany) for 2 days at 37 °C under microaerobic conditions using CampyGen gas packs (Oxoid) as described earlier [23, 27, 28].

Clinical score

To assess clinical signs of *A. butzleri*-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 Haemocult, Beckman Coulter/PCD, Krefeld, Germany; 4: overt 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 [24].

Sampling procedures

Mice were sacrificed by isofluran treatment (Abbott, Greifswald, Germany) on days 6 or 16 postinfection (p.i.). Tissue samples from liver, kidney, and colon were removed under sterile conditions. Absolute large intestinal lengths were determined by measuring the distances from the ascending colon leaving the cecum to the rectum by

a ruler. Colonic *ex vivo* biopsies from each mouse were collected in parallel for immunohistochemical, microbiological, and immunological analyses. Immunohistopathological changes were determined in colonic samples immediately fixed in 5% formalin and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (H&E) or respective antibodies for *in situ* immunohistochemistry as described earlier [26].

Histopathological grading intestinal lesions

To evaluate the severity of small intestinal histopathological lesions, an established scoring scheme [32] with minor modifications was applied. In detail, the character of immune cell infiltration (0: none; 1: mononuclear cells; 2: mononuclear cell dominated, fewer neutrophils; 3: neutrophil dominated, fewer mononuclear cells), quantity of immune cell infiltration (0: none; 1: mild; 2: moderate; 3: severe), vertical extent of inflammation (0: none; 1: mucosa; 2: mucosa and muscularis; 3: transmural), and horizontal extent of inflammation (0: no; 1: focal; 2: multifocal; 3: multifocal-coalescent; 4: diffuse) was assessed. The cumulative histologic scores ranged from 0 to 16 for colonic tissue.

Immunohistochemistry

In situ immunohistochemical analysis of colonic paraffin sections was performed as described previously [23, 24, 33–35]. Primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, Beverly, MA, USA, 1:200), F4/80 (no. 14-4801, clone BM8, eBioscience, 1:50), CD3 (no. N1580, Dako, 1:10), FOXP3 (FJK-16s, eBioscience, San Diego, CA, USA, 1:100), and B220 (eBioscience, 1:200) were used. The average number of positively stained cells within at least six high power fields (HPF, 0.287 mm^2 , 400 \times magnification) was determined for each mouse microscopically by a double-blinded investigator.

Quantitative analysis of A. butzleri

Viable *A. butzleri* was detected in fecal samples until day 16 p.i., dissolved in sterile phosphate buffered saline (PBS) and cultured in serial dilutions on Karmali and Columbia agar supplemented with 5% sheep blood (Oxoid) in parallel for 2 days at 37 $^{\circ}\text{C}$ under microaerobic conditions using CampyGen gas packs (Oxoid). To quantify bacterial translocation, *ex vivo* biopsies derived from liver and kidney were homogenized in 1 ml sterile PBS, whereas cardiac blood (≈ 200 μl) was directly streaked onto Karmali agar and cultivated accordingly. 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 gram sample.

Measurement of colonic and systemic pro-inflammatory mediators

Colonic *ex vivo* biopsies were cut longitudinally and washed in PBS. Approximately 1 cm^2 colonic tissue was placed in 24-flat-bottom well culture plates (Nunc, Wiesbaden, Germany), containing 500 μl serum-free RPMI 1640 medium (Gibco, life technologies, Paisley, UK) supplemented with penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$; PAA Laboratories). After 18 h at 37 $^{\circ}\text{C}$, culture supernatants and serum samples were tested for TNF, IL-6, MCP-1, IFN- γ , and IL-12p70 by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences, San Jose, CA, USA) on a BD FACSCanto II flow cytometer (BD Biosciences). Nitric oxide (NO) was determined by Griess reaction as described earlier [30].

Statistical analysis

Medians and levels of significance were determined using Mann–Whitney *U* test (GraphPad Prism v5, La Jolla, CA, USA). Two-sided probability (*P*) values ≤ 0.05 were considered significant. Experiments were reproduced twice.

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 G0184/12). Animal welfare was monitored twice daily by assessment of clinical conditions.

Results

Fecal shedding of A. butzleri in gnotobiotic IL-10^{-/-} mice lacking TLR-4

Gnotobiotic IL-10^{-/-} mice lacking TLR-4 (TLR-4^{-/-} IL-10^{-/-}) and IL-10^{-/-} control mice were generated by broad-spectrum antibiotic treatment and perorally infected with 10⁹ CFU *A. butzleri* strains CCUG 30485 or C1 on two consecutive days (days 0 and 1). Kinetic survey of intestinal colonization densities following infection revealed that gnotobiotic mice of either genotype could be stably colonized by the respective *A. butzleri* strain with high loads between 10⁶ to 10⁹ CFU per gram feces (Fig. 1). *A. butzleri* loads were lower in TLR-4^{-/-} IL-10^{-/-} mice at defined time points as compared to infected IL-10^{-/-} control mice (Fig. 1A). At days of necropsy (namely days 6 and 16 p.i.), fecal loads in TLR-4^{-/-} IL-10^{-/-} mice were approximately one and two orders of magnitude, respectively, lower as compared to those observed in IL-10^{-/-} mice ($p < 0.005$; Fig. 1A). Following *A. butzleri* C1 strain infection, differ-

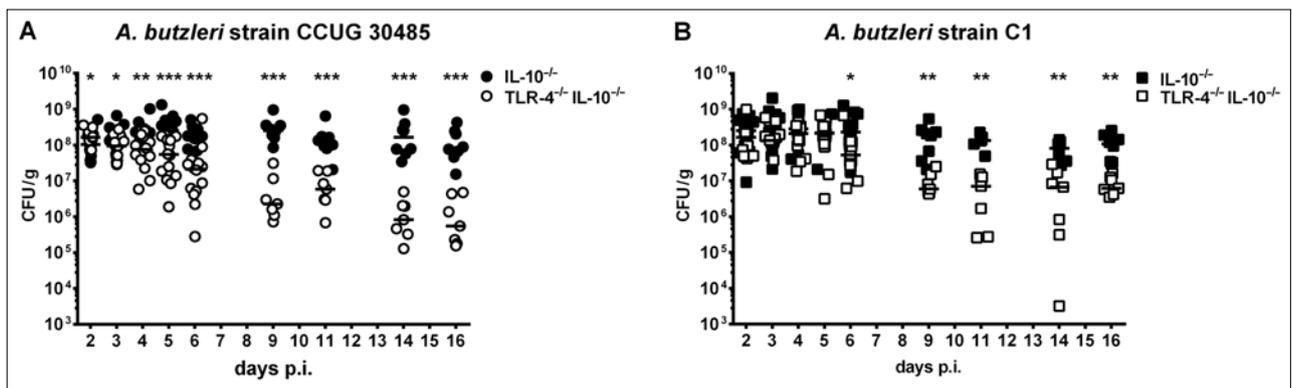


Fig. 1. Colonization kinetics of *A. butzleri* strains in orally infected gnotobiotic IL-10^{-/-} mice lacking TLR-4. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (open symbols) and IL-10^{-/-} control mice (closed symbols) were generated by antibiotic treatment and perorally infected either with (A) *A. butzleri* strain CCUG 30485 (circles) or (B) strain C1 (squares). *A. butzleri* loads were determined in fecal samples (CFU, colony forming units per g, gram) over 16 days postinfection (p.i.) by culture. Medians (black bars) and significance levels (*p*-value; **p* < 0.05; ***p* < 0.01; ****p* < 0.005) determined by Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments

ences in fecal bacterial loads were even less distinct with approximately one order of magnitude lower loads in TLR-4^{-/-} IL-10^{-/-} mice at days 6 and 16 p.i. (*p* < 0.05 and *p* < 0.01, respectively; Fig. 1B). Interestingly, in IL-10^{-/-} mice, fecal loads of the respective *A. butzleri* strains did not

differ at defined time points p.i. (Fig. 2A). In fecal samples derived from TLR-4^{-/-} IL-10^{-/-} mice *A. butzleri* strain C1 concentrations were slightly (i.e., up to one order of magnitude) higher as compared to strain CCUG 30485 between days 3 and 6 p.i. as well as at day 16 p.i. (*p* < 0.05; Fig. 2B).

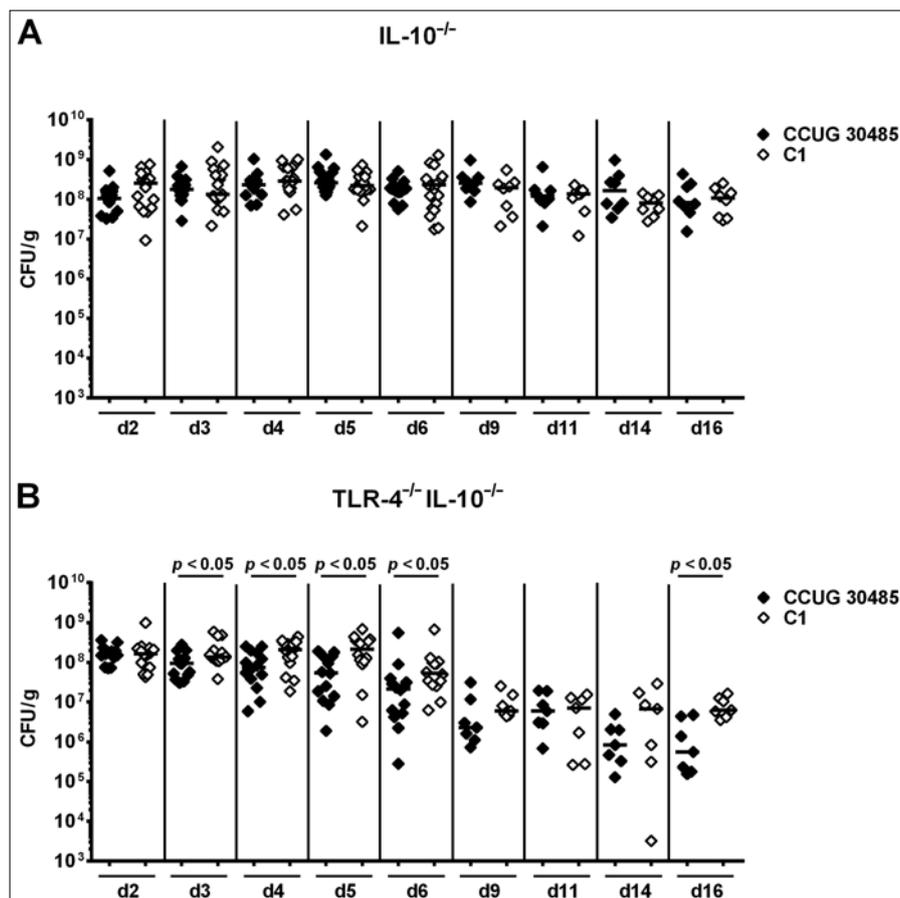


Fig. 2. Comparative kinetic survey of fecal *A. butzleri* densities in orally infected gnotobiotic IL-10^{-/-} mice lacking TLR-4 and IL-10^{-/-} mice. Gnotobiotic (A) IL-10^{-/-} and (B) TLR-4^{-/-} IL-10^{-/-} mice were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (black diamonds) or strain C1 (white diamonds). *A. butzleri* loads were determined in fecal samples (CFU, colony forming units per g, gram) over 16 days (d) postinfection by culture. Medians (black bars) and significance levels determined by Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments

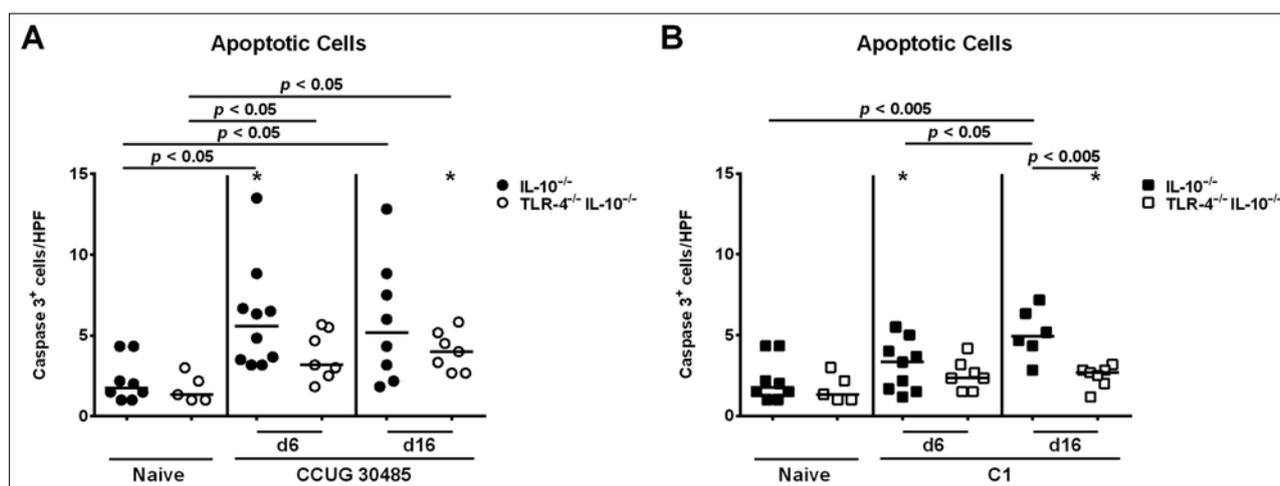


Fig. 3. Apoptotic cells in colonic epithelium of gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (open symbols) and IL-10^{-/-} mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* (A) strain CCUG 30485 (circles) or (B) strain C1 (squares). Uninfected mice served as negative controls (naive). The average numbers of apoptotic cells (positive for caspase-3, Casp3) from at least six high power fields (HPF, 400× magnification) per mouse were determined microscopically in immunohistochemically stained colon sections at days (d) 6 or 16 p.i. Medians (black bars) and levels of significance (*p*-values) determined by Mann–Whitney *U* test are indicated. Significant differences between *A. butzleri* strains are indicated by stars within the graph (**p* < 0.05). Data shown were pooled from three independent experiments

Clinical and microscopic aspects of large intestinal inflammatory sequelae in A. butzleri-infected gnotobiotic IL-10^{-/-} mice lacking TLR-4

Despite stable and high intestinal colonization, infected gnotobiotic mice of either genotype did not exhibit any clinical signs of enteric disease such as wasting or bloody diarrhea in the course of *A. butzleri* infection with either strain (not shown). Given that intestinal inflammation results in significant shortening of the intestinal tract [24, 30], we measured colonic lengths at days of necropsy. Neither at day 6 nor at day 16 following *A. butzleri* infec-

tion with either strain, shortening of the large intestines in infected gnotobiotic TLR-4^{-/-} IL-10^{-/-} or IL-10^{-/-} mice could be observed when compared to uninfected control animals (not shown), thus further supporting absence of macroscopic disease.

We next surveyed microscopic intestinal sequelae of infection in H&E stained colonic paraffin sections applying a standardized histopathological scoring system. Histopathological scores did not differ between naive and infected TLR-4^{-/-} IL-10^{-/-} and IL-10^{-/-} mice at days 6 and 16 p.i., irrespective of the bacterial strain (not shown).

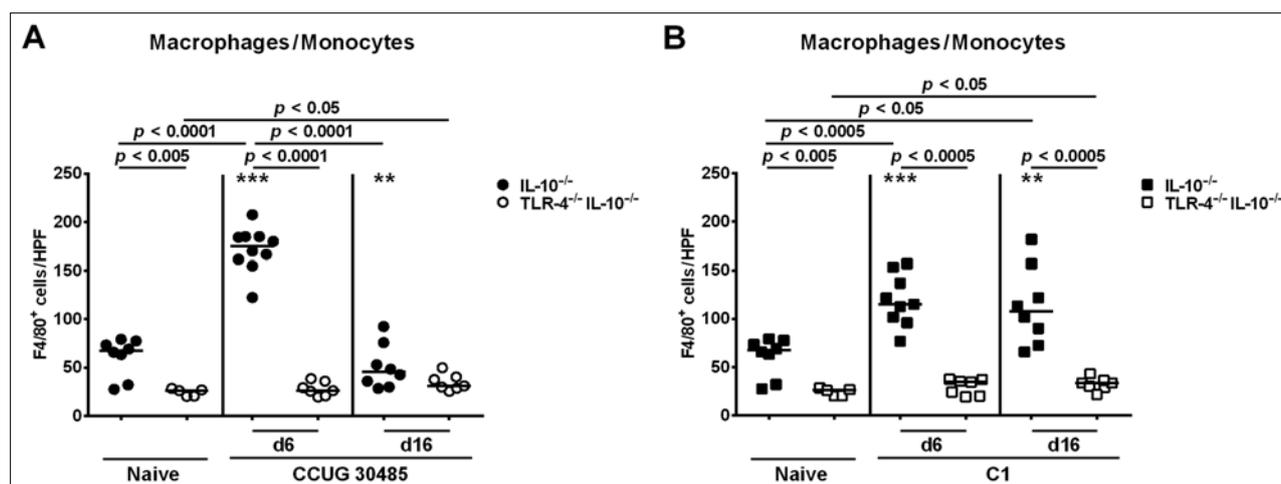


Fig. 4. Innate immune cells in colonic mucosa of gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (open symbols) and IL-10^{-/-} mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* (A) strain CCUG 30485 (circles) or (B) strain C1 (squares). Uninfected mice served as negative controls (naive). The average numbers of macrophages and monocytes (positive for F4/80) from at least six high power fields (HPF, 400× magnification) per mouse were determined microscopically in immunohistochemically stained colon sections at days (d) 6 or 16 p.i. Medians (black bars) and levels of significance (*p*-values) determined by Mann–Whitney *U* test are indicated. Significant differences between *A. butzleri* strains are indicated by stars within the graph (***p* < 0.01; ****p* < 0.005). Data shown were pooled from three independent experiments

Apoptosis is a commonly used diagnostic marker in the histopathological evaluation and grading of intestinal disease and a hallmark of *C. jejuni*-induced enterocolitis in gnotobiotic IL-10^{-/-} mice [24]. We therefore determined numbers of caspase-3⁺ cells within the colonic mucosa of infected mice. Upon *A. butzleri* CCUG 30485 strain infection, numbers of apoptotic cells increased in the colonic epithelium of mice of either genotype until days 6 and 16 p.i. ($p < 0.05$ – 0.005 ; Fig. 3A) with a trend towards lower apoptotic cell numbers in TLR-4^{-/-} IL-10^{-/-} as compared to IL-10^{-/-} control mice (n.s.; Fig. 3A) and less distinct relative increase when compared to naive mice of the respective phenotype (Fig. 3IA). Sixteen days following *A. butzleri* C1 strain infection, apoptotic cell numbers significantly increased in IL-10^{-/-}, but not TLR-4^{-/-} IL-10^{-/-} mice, as compared to naive animals ($p < 0.0005$; Fig. 3B). Moreover, large intestinal apoptotic cells were higher in *A. butzleri* CCUG 30485 strain- versus C1 strain-infected IL-10^{-/-} mice at day 6 p.i. ($p < 0.05$; Fig. 3). Taken together, TLR-4 gene deficiency was associated with low-

er abundances of colonic apoptotic cells during the late course of *A. butzleri* C1 strain infection.

Distinct immune cell populations in the colon of *A. butzleri*-infected gnotobiotic IL-10^{-/-} mice lacking TLR-4

Recruitment of innate and adaptive immune cells as well as of effector cells to sites of inflammation is a hallmark of enteric infection including campylobacteriosis. Therefore, we next quantitatively determined distinct immune cell populations by *in situ* immunohistochemical staining of large intestinal paraffin sections. Interestingly, already in the naive state, TLR-4^{-/-} IL-10^{-/-} mice exhibited lower numbers of F4/80⁺ macrophages and monocytes ($p < 0.005$; Fig. 4), CD3⁺ T lymphocytes ($p < 0.05$; Fig. 5A), and B220⁺ B lymphocytes ($p < 0.05$; Fig. 5C) in their large intestines as compared to IL-10^{-/-} mice. Six days following *A. butzleri* CCUG 30485 strain infection,

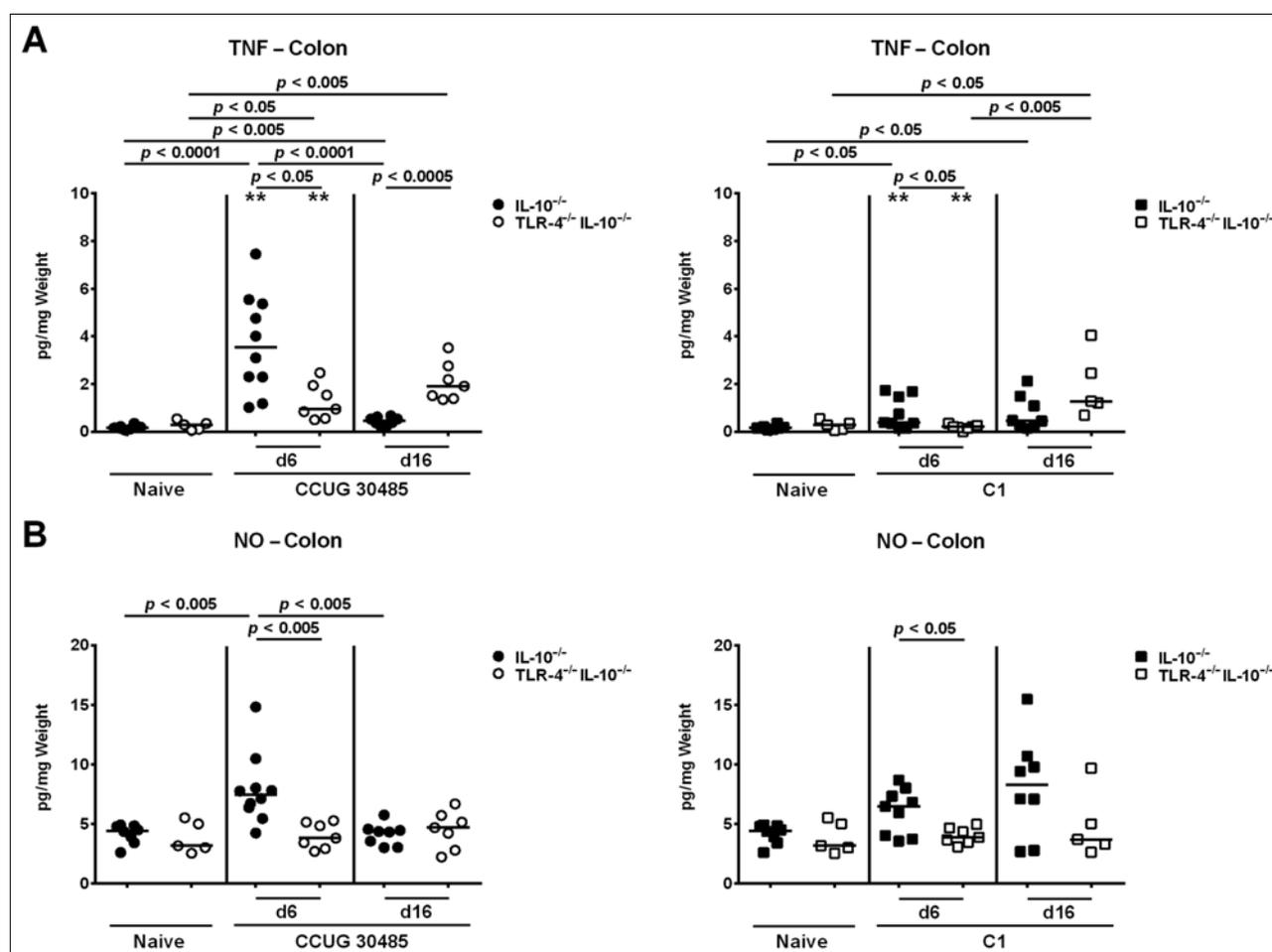


Fig. 6. Colonic TNF and nitric oxide concentrations in gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (open symbols) and IL-10^{-/-} mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (circles; left panel) or strain C1 (squares; right panel). Uninfected mice served as negative controls (naive). (A) TNF and (B) nitric oxide (NO) concentrations (pg per mg colonic tissue) were determined in supernatants of *ex vivo* colonic biopsies taken at days (d) 6 or 16 p.i. Medians (black bars) and significance levels as determined by the Mann–Whitney *U* test are indicated. Significant differences between *A. butzleri* strains are indicated by stars within the graph (** $p < 0.01$). Data shown were pooled from three independent experiments

however, numbers of macrophages and monocytes increase multifold in the colon of IL-10^{-/-}, but not TLR-4^{-/-} IL-10^{-/-} mice ($p < 0.0001$; Fig. 4A; Fig. S1B), and declined back to naive values until day 16 p.i. (Fig. 4A). In TLR-4^{-/-} IL-10^{-/-} mice, a slight increase of colonic F4/80+ cells could be observed until day 16 following infection with either *A. butzleri* strain ($p < 0.05$; Fig. 4, Fig. S1B). Following C1 strain infection, IL-10^{-/-} mice displayed elevated numbers of colonic macrophages and monocytes at either time point ($p < 0.0005$ and $p < 0.05$, respectively; Fig. 4B), which were significantly higher as compared to those observed in respective TLR-4^{-/-} IL-10^{-/-} mice ($p < 0.0005$; Fig. 4B). As compared to uninfected mice, numbers of CD3⁺ T lymphocytes were higher in large intestines of IL-10^{-/-}, but not TLR-4^{-/-} IL-10^{-/-} mice at day 6 following CCUG 30485 strain ($p < 0.0001$; Fig. 5A: left panel, Fig. S1C). In gnotobiotic IL-10^{-/-} mice, colonic T cell numbers were higher at either time point following C1 strain infection as compared to TLR-4^{-/-} IL-10^{-/-} mice ($p < 0.05$ and $p < 0.005$, respectively) and naive animals ($p < 0.05$; Fig. 5A: right panel). *A. butzleri* CCUG 30485 strain-infected IL-10^{-/-} mice exhibited increased num-

bers of colonic FOXP3⁺ Tregs and B220⁺ B lymphocytes at days 6 and 16 p.i. ($p < 0.05$ – 0.0001 ; Fig. 5B, and C: left panels) that were significantly higher as compared to those determined in respective TLR-4^{-/-} IL-10^{-/-} mice ($p < 0.05$ – 0.0001 ; Fig. 5B, and C: left panels). At day 6 following *A. butzleri* C1 strain infection, IL-10^{-/-} but not TLR-4^{-/-} IL-10^{-/-} mice displayed elevated Treg numbers in their colon that further increased until day 16 p.i. ($p < 0.05$ and $p < 0.0005$, respectively; Fig. 5B: right panel, Fig. S2D). In IL-10^{-/-} mice, colonic B lymphocytes increased rather late following C1 strain infection until day 16 p.i. ($p < 0.005$; Fig. 5C: right panel, Fig. S1E). As for Tregs, B lymphocyte numbers were lower in colons of TLR-4^{-/-} IL-10^{-/-} versus IL-10^{-/-} mice at either time point ($p < 0.005$; Fig. 5B, and C: right panels). Overall, *A. butzleri* C1 strain-infected IL-10^{-/-} mice harbored lower numbers of innate (macrophages and monocytes) and adaptive immune cells (T and B lymphocytes, Tregs) in the colon at day 6 p.i. as compared to *A. butzleri* CCUG 30485 strain-infected animals (Figs 4 and 5). Taken together, upon *A. butzleri* infection, TLR-4^{-/-} IL-10^{-/-} mice displayed less distinct influx of innate and adaptive immune cells

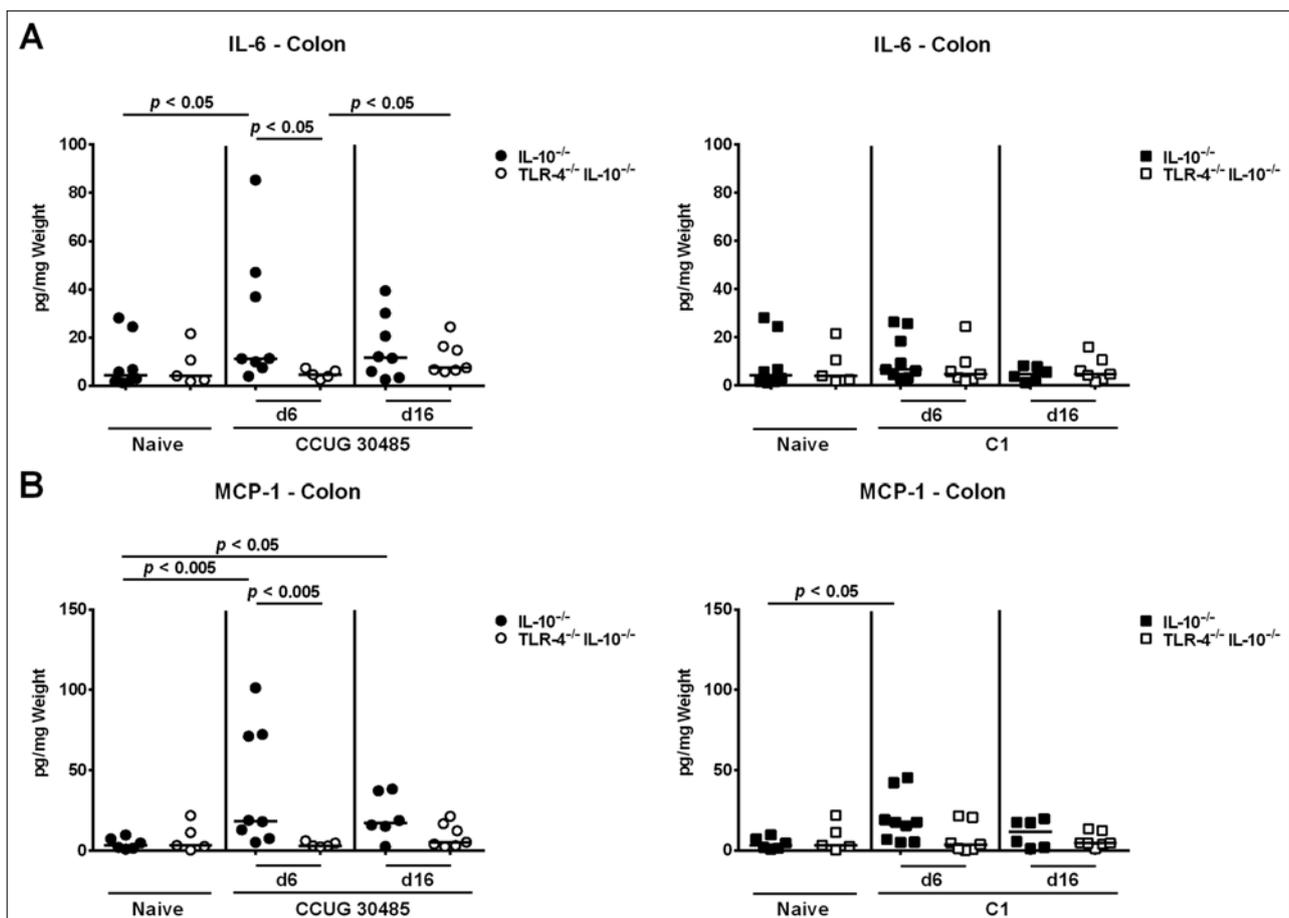


Fig. 7. Colonic IL-6 and MCP-1 concentrations in gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (open symbols) and IL-10^{-/-} mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (circles; left panel) or strain C1 (squares; right panel). Uninfected mice served as negative controls (naive). (A) IL-6 and (B) MCP-1 concentrations (pg per mg colonic tissue) were determined in supernatants of *ex vivo* colonic biopsies taken at days (d) 6 or 16 p.i. Medians (black bars) and significance levels as determined by the Mann–Whitney *U* test are indicated. Data shown were pooled from three independent experiments

into the colonic mucosa and lamina propria as compared to IL-10^{-/-} control animals.

Colonic inflammatory mediators in *A. butzleri*-infected gnotobiotic IL-10^{-/-} mice lacking TLR-4

We next measured colonic pro-inflammatory cytokines in *A. butzleri*-infected gnotobiotic mice. As early as 6 days following *A. butzleri* CCUG 30485 strain infection, colonic concentrations of NO, IL-6, and MCP-1 increased in IL-10^{-/-}, but not TLR-4^{-/-} IL-10^{-/-} mice ($p < 0.05$ – 0.0001 ; Figs 6, and 7: left panels, Fig. S2), and remained elevated until day 16 p.i. for MCP-1 only ($p < 0.05$; Fig. 7B: left panel). In CCUG 30485 strain-infected TLR-4^{-/-} IL-10^{-/-} mice, higher colonic TNF concentrations could be determined at days 6 and 16 p.i. as compared to uninfected mice ($p < 0.005$; Fig. 6A: left panel). Following C1 strain infection of IL-10^{-/-} animals, colonic TNF concentrations were elevated at days 6 and 16 p.i. ($p < 0.05$, Fig. 6A: right

panel), and MCP-1 at day 6 p.i. only ($p < 0.05$, Fig. 7B: right panel). At day 6 p.i., large intestinal TNF levels were lower in C1 strain as compared to CCUG 30485 strain-infected mice of either genotype ($p < 0.01$; Fig. 6A). In strain C1-infected TLR-4^{-/-} IL-10^{-/-} mice, however, only colonic TNF levels slightly increased until day 16 p.i. ($p < 0.05$, Fig. 6A: right panel, Fig. S2A). Taken together, *A. butzleri*-induced colonic inflammatory responses were less pronounced in gnotobiotic IL-10^{-/-} mice lacking TLR-4 as compared to gnotobiotic IL-10^{-/-} control animals.

Systemic pro-inflammatory cytokine responses in *A. butzleri*-infected gnotobiotic IL-10^{-/-} mice lacking TLR-4

We next determined whether systemic pro-inflammatory cytokine responses could be observed upon *A. butzleri* infection. Six days following CCUG 30485 strain infection, serum IFN- γ levels increased more than two-fold in IL-10^{-/-}

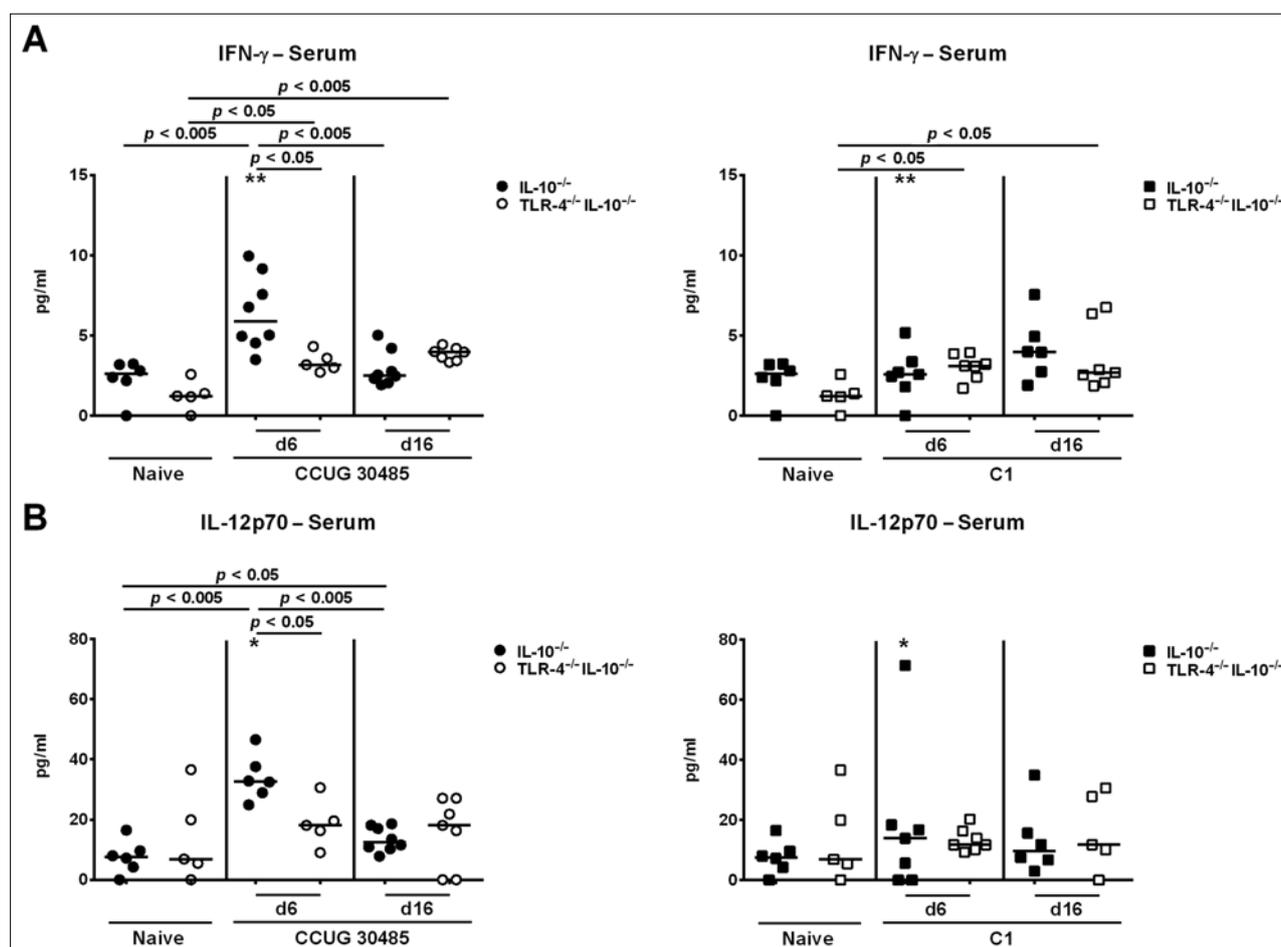


Fig. 8. Systemic pro-inflammatory cytokine concentrations in gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (open symbols) and IL-10^{-/-} mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (circles; left panel) or strain C1 (squares; right panel). Uninfected mice served as negative controls (naive). (A) IFN- γ and (B) IL-12p70 concentrations were determined in serum samples taken at days (d) 6 or 16 p.i. Medians (black bars) and significance levels as determined by the Mann-Whitney *U* test are indicated. Significant differences between *A. butzleri* strains are indicated by stars within the graph (* $p < 0.05$; ** $p < 0.01$). Data shown were pooled from three independent experiments

mice ($p < 0.005$ vs. naive; Fig. 8A: left panel) but, remarkably, were lower in TLR-4^{-/-} IL-10^{-/-} mice ($p < 0.05$; Fig. 8A: left panel, Fig. S3A). In TLR-4^{-/-} IL-10^{-/-} mice, increased IFN- γ concentrations could be measured in sera at days 6 and 16 following infection with either *A. butzleri* strain ($p < 0.05$ – 0.005 , Fig. 8A, Fig. S3A). Furthermore, serum IL-12p70 concentrations were significantly higher in IL-10^{-/-} as compared to TLR-4^{-/-} IL-10^{-/-} mice at days 6 and 16 following CCUG 30485 strain infection ($p < 0.005$ and 0.05 , respectively; Fig. 8B: left panel). When compared to respective naive control animals, the relative increase of IL-12p70 serum concentrations was more pronounced in IL-10^{-/-} than TLR-4^{-/-} IL-10^{-/-} mice at day 6 following CCUG 30485 strain infection (Fig. S3B). Hence, *A. butzleri* induced systemic pro-inflammatory cytokine responses that were less pronounced in IL-10^{-/-} mice lacking TLR-4 as compared to IL-10^{-/-} control animals.

Discussion

The Gram-negative *A. butzleri* bacteria have been shown to be responsible for sporadic cases of human gastroenteritis [4–8]. We have recently shown that following *A. butzleri* infection, gnotobiotic IL-10^{-/-} mice exhibited intestinal and systemic pro-inflammatory immune responses [27, 28]. In the present study, we assessed the role of TLR-4, the main innate receptor for LPS and LOS of Gram-negative bacteria, in murine arcobacteriosis. To address this, we applied our murine gnotobiotic IL-10^{-/-} infection model and investigated *A. butzleri* infection in IL-10^{-/-} mice lacking TLR-4. Following peroral infection with either *A. butzleri* strain CCUG 30485 or C1, gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice were stably colonized at high loads, but harbored slightly lower intestinal bacterial burdens of approximately 0.5 and one to two orders of magnitude at days 6 and 16 p.i., respectively, as compared to IL-10^{-/-} control mice. In the present and our very recent studies [27, 28], *A. butzleri* infection with either strain did not cause overt disease such as diarrhea, occurrence of blood in feces, or even wasting in gnotobiotic IL-10^{-/-} mice, as one would have expected upon *C. jejuni* infection [24–26]. This is somewhat surprising, given that the two *A. butzleri* strains are known for their adhesive and invasive properties *in vitro* [11]. Given that *Arcobacter* strains might express variable lipopolysaccharide (LPS) or lipooligosaccharide (LOS) structures, this could determine whether the respective strains rather act as commensals or as pathogens *in vivo*. Despite lack of clinical symptoms, however, *A. butzleri* induced colonic epithelial apoptosis in IL-10^{-/-} mice as early as 6 days p.i., which is well in line with our previous *in vivo* study [27], and further supported by caspase-3 dependent apoptosis induction in intestinal epithelial cells leading to epithelial barrier dysfunction *in vitro* [36]. Interestingly, colonic epithelial apoptosis was significantly less pronounced in IL-10^{-/-} mice lacking TLR-4 at day 16 following C1 strain infection. Furthermore, *A. butzleri*-infected TLR-4^{-/-} IL-10^{-/-} mice displayed a less distinct influx of innate and

adaptive immune cells (including macrophages and monocytes, T lymphocytes, Tregs, and B lymphocytes) into the colonic mucosa and lamina propria that was accompanied by less pronounced large intestinal and, strikingly, systemic pro-inflammatory cytokine responses. One could argue that distinct TLR-4-dependent pro-inflammatory immune responses upon *A. butzleri* infection might be most likely due to differences in colonization status and can, indeed, not be excluded *per se*. At the first time point of necropsy, namely, day 6 p.i., however, the differences in bacterial loads between mice of either genotype were less than one order of magnitude and hence rather subtle. Furthermore, given the range of intestinal *A. butzleri* loads of between 10⁶ and 10⁸ CFU per g feces at day 16 p.i., one should take into consideration that two orders of magnitude differences in bacterial burdens might not have a major biologically relevant impact within this high level of colonization, since the threshold for inducing disease would be most likely far lower. Furthermore, the *A. butzleri* CCUG 30485 and C1 strain loads were comparable in the small intestines of infected TLR4^{-/-} IL-10^{-/-} and IL-10^{-/-} mice (Heimesaat et al., 2015: this issue of European J Microbiol Immunol (Bp)). These less pronounced immunopathological sequelae of *A. butzleri* infection in the absence of TLR-4 point towards an important role of TLR-4 dependent signalling of *A. butzleri* LPS or LOS during arcobacteriosis. To date, however, neither LPS nor LOS have been isolated and characterized in *A. butzleri*, whereas the structure of the carbohydrate backbone of LOS has been described in the halophilic bacterium *A. halophilus* [37]. In line with our results presented here, gnotobiotic IL-10^{-/-} mice lacking TLR-4 were protected from acute ulcerative enterocolitis following peroral *C. jejuni* infection [24]. Furthermore, *C. jejuni*-induced pro-inflammatory responses were less pronounced in infected gnotobiotic TLR2^{-/-} and TLR-9^{-/-} mice [23, 24]. Hence, TLR-2, TLR-4, and TLR-9 dependent signalling of pathogenic lipoprotein, LOS, and bacterial CpG-DNA, respectively, were shown to be essential for induction and progression of *C. jejuni*-induced immunopathology. It is therefore highly likely that, besides TLR-4, other innate immune receptors of the TLR or NOD family contribute to inflammatory and immune responses induced by *A. butzleri* infection.

We conclude that TLR-4 is involved in mediating immune responses induced by *A. butzleri* infection *in vivo*. It is tempting to speculate that pathogenic LOS or LPS (even though not yet identified) acts as a virulence factor in arcobacteriosis. Future studies are needed to gain more detailed insights into the molecular mechanisms underlying *Arcobacter*–host interactions.

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Competing interests

The authors declare that no competing interests exist.

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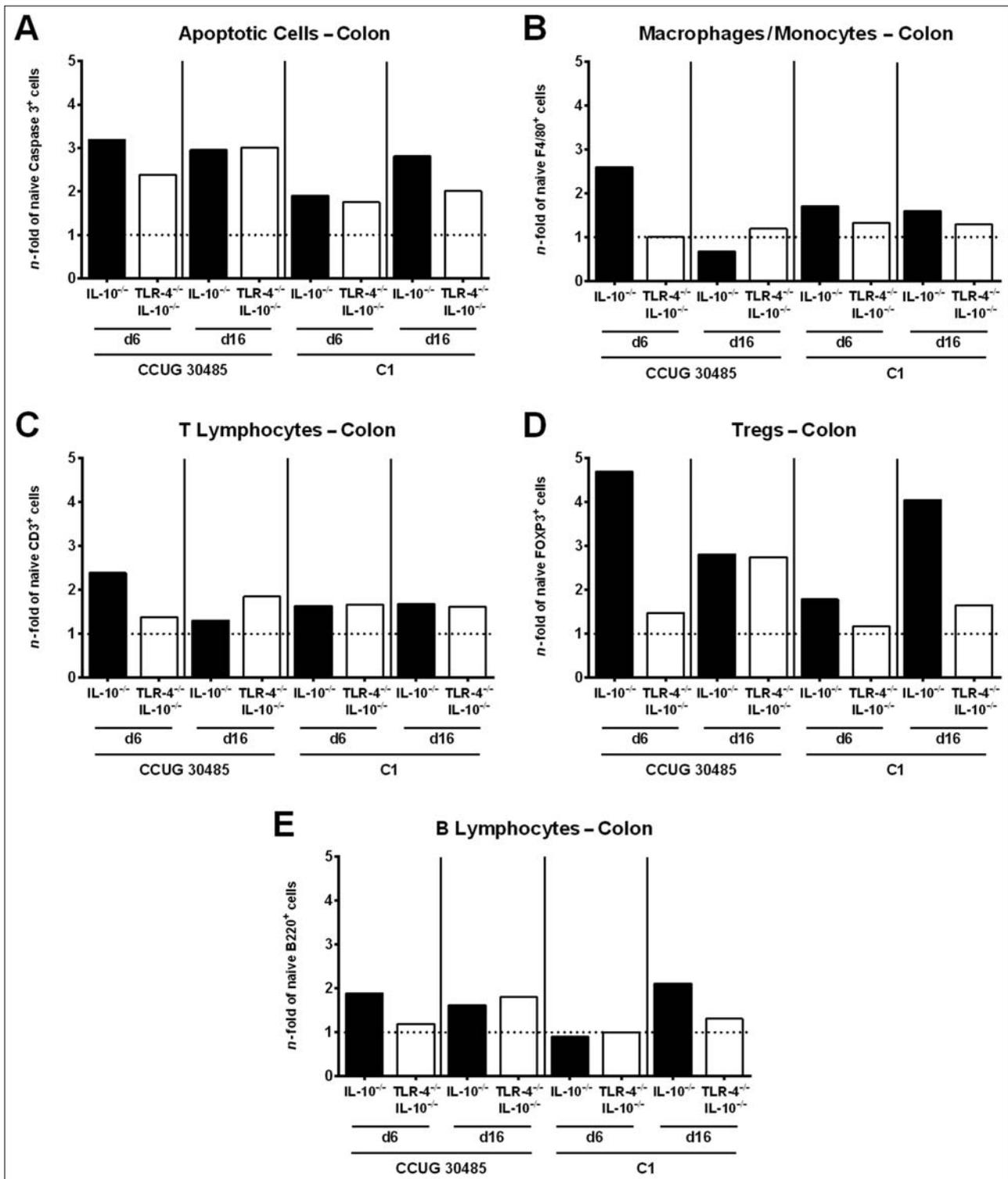


Fig. S1. Relative increases in apoptotic cells and in adaptive as well as innate immune cell populations in colonic tissue of gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (white bars) and IL-10^{-/-} mice (black bars) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 or strain C1 as indicated. Uninfected mice served as negative controls. The average numbers of (A) apoptotic cells (caspase-3+), (B) macrophages and monocytes (F4/80+), (C) T lymphocytes (CD3+), (D) regulatory T lymphocytes (Tregs, FOXP3+), and (E) B lymphocytes (B220+) per animal were determined microscopically in immunohistochemically stained colonic sections at days (d) 6 or 16 p.i. The respective bars indicate the fold infection-induced increases of the means of respective cell abundance, and the dotted line, the respective relative levels of naive controls. Data shown were pooled from three independent experiments

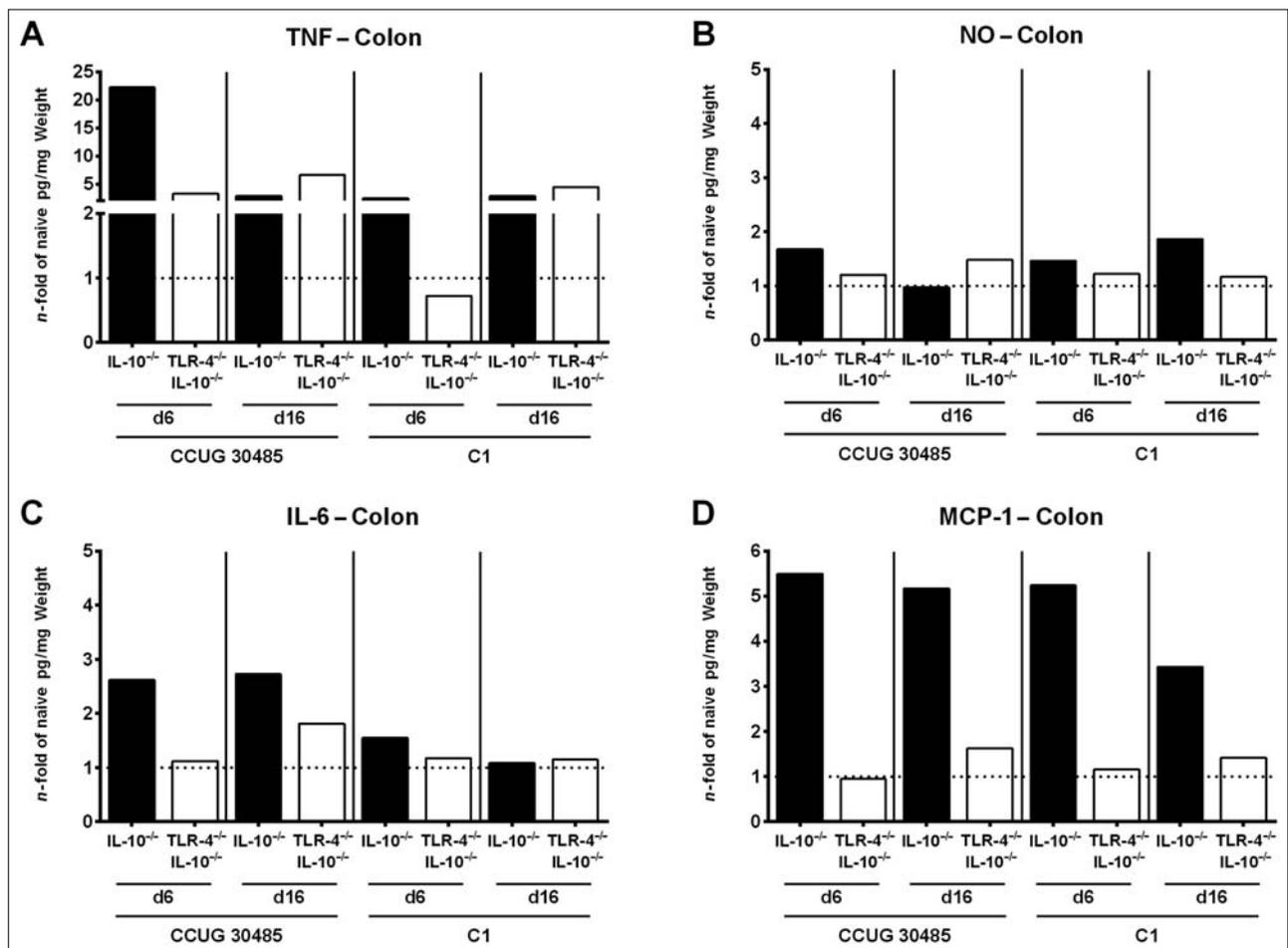


Fig. S2. Relative increases in infection-induced colonic pro-inflammatory cytokines in colonic tissue of gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (white bars) and IL-10^{-/-} mice (black bars) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 or strain C1 as indicated. Uninfected mice served as negative controls. (A) TNF, (B) nitric oxide (NO), (C) IL-6, and (D) MCP-1 concentrations were determined in supernatants of colonic *ex vivo* biopsies taken at days (d) 6 or 16 p.i. The respective bars indicate the fold infection-induced increases of the means of respective cytokines, and the dotted line, the respective relative levels of naive controls. Data shown were pooled from three independent experiments

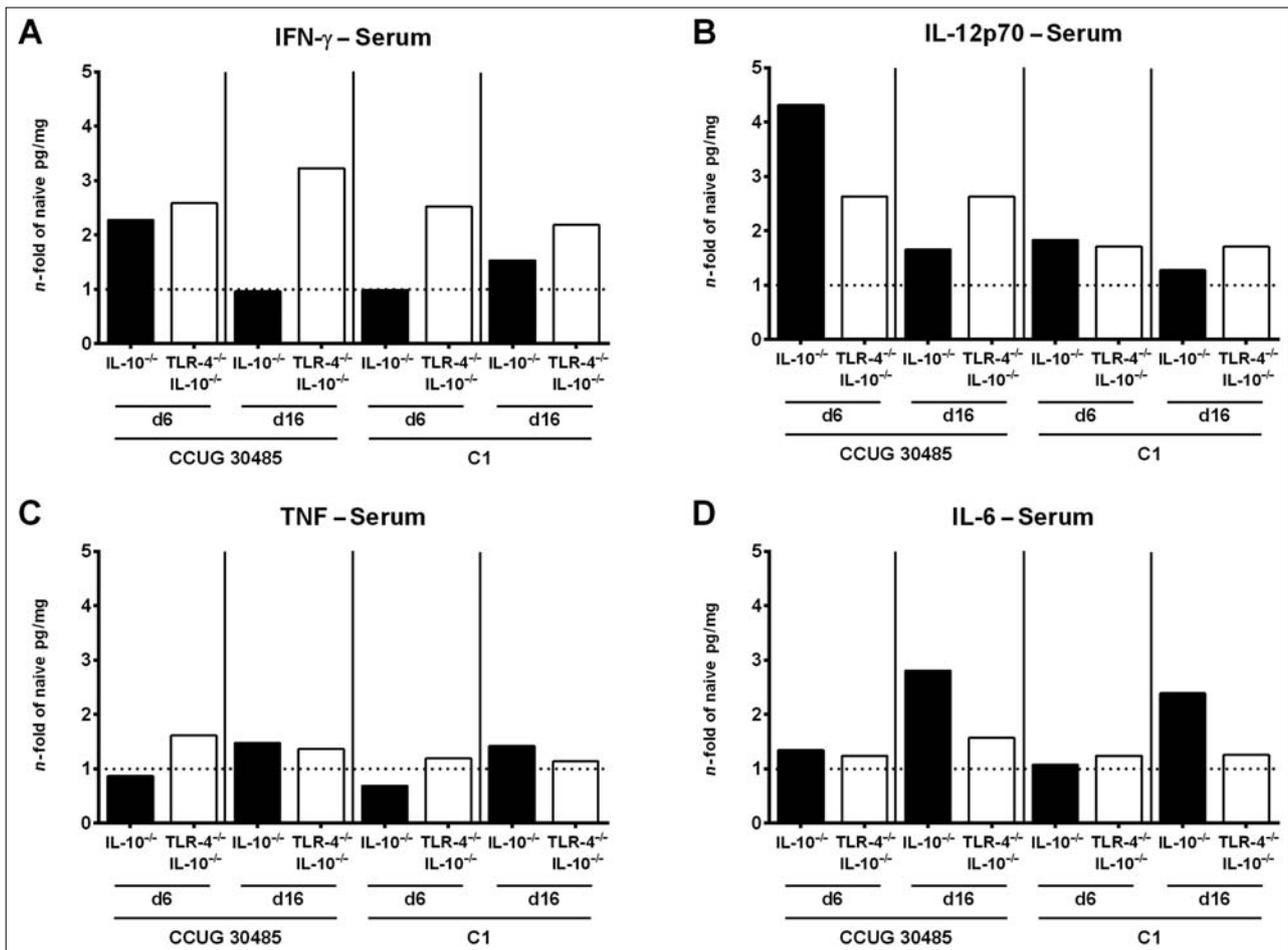


Fig. S3. Relative increases in infection-induced systemic pro-inflammatory cytokines in sera of gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (white bars) and IL-10^{-/-} mice (black bars) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 or strain C1 as indicated. Uninfected mice served as negative controls. (A) IFN- γ , (B) IL-12p70, (C) TNF, and (D) IL-6 concentrations were determined in serum samples taken at days (d) 6 or 16 p.i. The respective bars indicate the fold infection-induced increases of the means of respective cytokines, and the dotted line, the respective relative levels of naive controls. Data shown were pooled from three independent experiments