TOLL-LIKE RECEPTOR-4 DEPENDENT SMALL INTESTINAL IMMUNE RESPONSES FOLLOWING MURINE *ARCOBACTER BUTZLERI* INFECTION

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Sporadic cases of gastroenteritis have been attributed to *Arcobacter butzleri* infection, but information about the underlying immunopathological mechanisms is scarce. We have recently shown that experimental *A. butzleri* infection induces intestinal, extraintestinal and systemic immune responses in gnotobiotic IL-10$^{-/-}$ mice. The aim of the present study was to investigate the immunopathological role of Toll-like Receptor-4, the receptor for lipopolysaccharide and lipooligosaccharide of Gram-negative bacteria, during murine *A. butzleri* infection. To address this, gnotobiotic IL-10$^{-/-}$ mice lacking TLR-4 were generated by broad-spectrum antibiotic treatment and perorally infected with two different *A. butzleri* strains isolated from a patient (CCUG 30485) or fresh chicken meat (C1), respectively. Bacteria of either strain stably colonized the ilea of mice irrespective of their genotype at days 6 and 16 postinfection. As compared to IL-10$^{-/-}$ control animals, TLR-4$^{-/-}$ IL-10$^{-/-}$ mice were protected from *A. butzleri*-induced ileal apoptosis, from ileal influx of adaptive immune cells including T lymphocytes, regulatory T-cells and B lymphocytes, and from increased ileal IFN-γ secretion. Given that TLR-4-signaling is essential for *A. butzleri*-induced intestinal inflammation, we conclude that bacterial lipooligosaccharide or lipopolysaccharide compounds aggravate intestinal inflammation and may thus represent major virulence factors of *Arcobacter*. Future studies need to further unravel the molecular mechanisms of TLR-4-mediated *A. butzleri*-host interactions.

Keywords: *Arcobacter butzleri,* Toll-like Receptor-4, lipopolysaccharide, lipooligosaccharide, pro-inflammatory immune responses, small intestine, spleen, apoptosis, innate immunity, host-pathogen interactions

Introduction

The motile, spiral-shaped Gram-negative *Arcobacter* species belong to the *Campylobacteraceae* family and can be isolated from a plethora of environmental habitats, including surface water. In animals, for instance, *Arcobacter* spp. form part of the commensal gastrointestinal microbiota [1]. Among the 19 so far described *Arcobacter* spp., *A. butzleri* and *A. cryaerophilus* are regarded as hazards for human health by the International Commission on Microbiological Specifications for Foods [2]. Notably, detection of *Arcobacter* spp. may fail in applied microbacteriological routine procedures [3]. Hence, prevalence and incidence of *Arcobacter* associated human diseases are not known yet and the reported cases rather underestimating the epidemiological situation. Nevertheless, several single clinical cases and outbreaks of arcobacteriosis are documented in the literature to date, hence providing evidence for an important role of *Arcobacter* spp. in causing intestinal disease [4, 5]. Retrospective studies revealed that *Arcobacter* spp. are the fourth most common Campylobacterales that could be isolated from diarrheal patients [6–8]. Patients are mostly likely infected by contaminated food or water [5, 9] and present with symptoms of acute gastroenteritis such as abdominal pain, acute diarrhea, or prolonged watery diarrhea for up to 2 months [6, 7]. However, information about the underlying mechanisms of infection and induced host immune responses is scarce. Phenotypic assays revealed adhesive, invasive, and cytotoxic effects of *A. butzleri in vitro* [10–17]. *A. butzleri* has been further shown to compromise barrier function in monolayers of the human colon cell line HT-29/B6 pointing towards potential mechanisms by which diarrhea might be induced in humans [18].

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We have recently shown that, upon peroral *A. butzleri* infection, gnotobiotic IL-10$^{-/-}$ mice with a depleted commensal microbiota following broad-spectrum antibiotic treatment [19] could be stably infected by the bacterium and displayed significant small and large intestinal pro-inflammatory immune responses [20, 21]. Remarkably, infection-induced sequelae were not restricted to the intestinal tract, given that even systemic inflammatory responses could be observed in infected mice.

Toll-like receptors (TLRs) are essentially involved in mediating innate immune responses of the host to commensals and pathogens [22]. Since bacterial lipopoly-saccharides (LPS) and lipooligosaccharides (LOS) from Gram-negative bacteria are sensed by TLR-4 [22], we investigated the role of TLR-4 in mediating arcobacteriosis *in vivo* in the present study. To address this, IL-10 deficient mice lacking TLR-4 (TLR-4$^{-/-}$ IL-10$^{-/-}$) were perorally infected either with *A. butzleri* reference strain CCUG 30485 (initially isolated from a diseased patient) [23] or C1 (derived from fresh chicken meat) [10], and the colonization and immunopathological properties of the respective bacterial strains were surveyed over time.

Materials and methods

Mice

IL-10$^{-/-}$ mice (in C57BL/10 background, B10) were bred and maintained in the facilities of the "Forschungsein-richtungen 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 TLR-4$^{-/-}$ IL-10$^{-/-}$ and IL-10$^{-/-}$ mice (with a virtually depleted gastrointestinal microbiota) were generated following broad-spectrum antibiotic treatment as described earlier [19, 24]. 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 [25]. 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 [24].

Arcobacter butzleri *infection of mice*

Age-matched female gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ and IL-10$^{-/-}$ mice were perorally infected with approximately 10^9 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 TLR-4$^{-/-}$ IL-10$^{-/-}$ and 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 [23], whereas the C1 strain was isolated from fresh chicken meat [10]. 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 [20, 21, 26].

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 Haemoccult, 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, prefinal aspect), was used [19].

Sampling procedures

Mice were sacrificed by isofluran treatment (Abbott, Greifswald, Germany) on day six or day 16 postinfection (p.i.). Tissue samples from mesenteric lymphnodes (MLNs), spleen, and ileum were removed under sterile conditions. Absolute small intestinal lengths were determined by measuring the distances from the transition of the stomach to the duodenum to the very distal terminal ileum by a ruler. Ileal *ex vivo* biopsies from each mouse were collected in parallel for immunohistochemical, microbiological, and immunological analyses. Immunohisto-pathological changes were determined in ileal 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* immunohisto-chemistry as described earlier [25].

Histopathological grading of small intestinal lesions

To evaluate the severity of small intestinal histopathological lesions, an established scoring scheme with minor modifications was applied [27]. 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 scoring ranged from 0 to 13 for ileal samples.

Immunohistochemistry

In situ immunohistochemical analysis of ileal paraffin sections was performed as described previously [19, 26, 28–30]. 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× magnification) was determined for each mouse microscopically by a double-blinded investigator.

Quantitative analysis of A. butzleri

Viable *A. butzleri* was detected in luminal samples derived from the proximal (duodenum) and distal (ileum) part of the small intestines at days 6 or 16 p.i., dissolved in sterile PBS and cultured in serial dilutions on Karmali- and Columbia-Agar supplemented with 5% sheep blood (Oxoid) in parallel for 2 days at 37 °C under microaerobic conditions using CampyGen gas packs (Oxoid). To quantify bacterial translocation, MLNs and spleen (≈1 cm^2) were homogenized in 1 ml sterile PBS, 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 colony forming units (CFU) per g.

Cytokine detection in culture supernatants of ex vivo biopsies taken from ileum, mesenteric lymphnodes, and spleen

Ileal *ex vivo* biopsies were cut longitudinally and washed in PBS. Spleen, MLNs, or strips of approximately 1 cm^2 ileum were 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 μg/ml; PAA Laboratories). After 18 h at 37 °C, culture supernatants were tested for IFN-γ by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences, San Jose, CA, USA) on a BD FACSCanto II flow cytometer (BD Biosciences).

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"
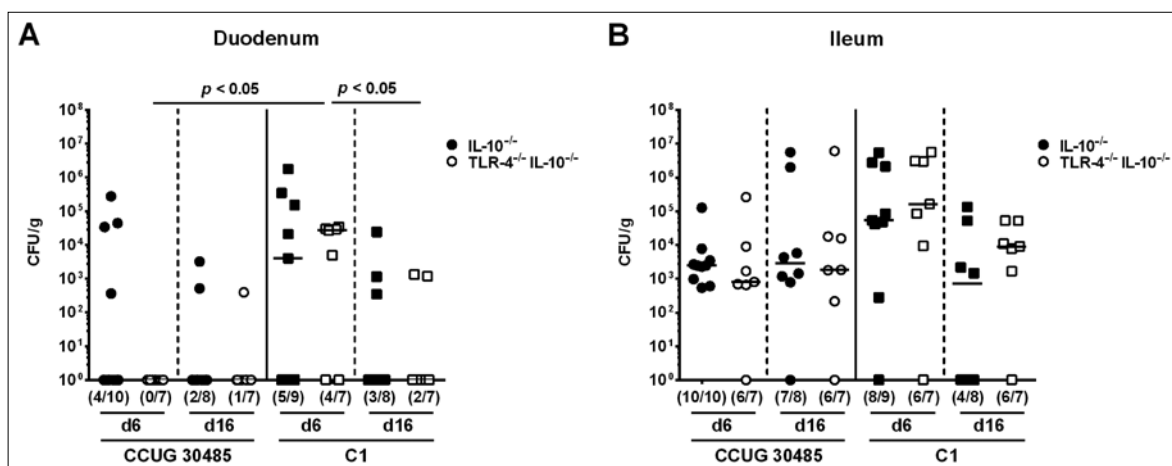

Fig. 1. Colonization of *Arcobacter butzleri* in the small intestines of gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ mice following peroral infection. Gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ (open symbols) and IL-10$^{-/-}$ control mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (circles) or strain C1 (squares). *A. butzleri* loads were determined in luminal samples derived from the (A) duodenum and (B) ileum at days 6 or 16 p.i. and expressed as colony forming units (CFU) per gram (g) sample. Medians (black bars), numbers of mice harboring the pathogen out of the total number of analyzed animals (in parentheses), and levels of significance (*p*-value) determined by Mann–Whitney *U* test are indicated. Data shown were pooled from three independent experiments

(LaGeSo, Berlin, registration number G0184/12). Animal welfare was monitored twice daily by assessment of clinical conditions.

Results

Colonization properties of A. butzleri strains in small intestines of gnotobiotic IL-10$^{-/-}$ mice lacking TLR-4

Gnotobiotic IL-10$^{-/-}$ mice lacking TLR-4 (TLR-4$^{-/-}$ IL-10$^{-/-}$) and gnotobiotic IL-10$^{-/-}$ control mice with a virtually depleted intestinal microbiota were generated by broad-spectrum antibiotic treatment and perorally infected with *A. butzleri* strains CCUG 30485 or C1. Six and 16 days following *A. butzleri* infection bacterial concentrations of either strain did not differ in the proximal (i.e., duodenum) and distal (i.e., ileum) part of the small intestines of TLR-4$^{-/-}$ IL-10$^{-/-}$ and IL-10$^{-/-}$ mice *(Fig. 1)*. Irrespective of the bacterial strain and time point p.i., mice of either genotype harbored approximately 10^3 to 10^5 CFU per gram *A. butzleri* in their ileal lumen *(Fig. 1B)*. Notably, neither in MLN nor in spleen, viable *A. butzleri* could be detected in infected mice of either genotype at days 6 or 16 p.i. by direct plating (not shown).

Clinical aspects and intestinal apoptosis induced by A. butzleri in gnotobiotic IL-10$^{-/-}$ mice lacking TLR-4

Despite intestinal colonization, however, infected mice of either genotype did not exhibit any clinical signs of enteric disease such as wasting, diarrhea, or occurence of blood in stool, neither early (i.e., at day 6 p.i.) nor later (i.e., day 16 p.i.) in the course of *A. butzleri* infection with either strain (not shown).

Since intestinal inflammation leads to significant shortening of the intestinal tract [19, 24], we assessed small intestinal lengths at days of necropsy. Neither at day 6 nor at day 16 following *A. butzleri* infection with either strain, shortening of the small 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 potential microscopic intestinal sequelae of infection in H&E stained small intestinal 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, irrespective of the applied strain and time point p.i. (not shown).

Given that apoptosis is a commonly used diagnostic marker in the histopathological evaluation and grading of intestinal inflammation and a hallmark of *C. jejuni*-induced enterocolitis in gnotobiotic IL-10$^{-/-}$ mice [19], we next assessed numbers of caspase-3+ cells within the small intestinal mucosa of infected mice. Numbers of apoptotic ileal epithelial cells increased in IL-10$^{-/-}$ mice until day 16 following infection with either strain ($p < 0.005$; *Fig. 2A and B*). However, TLR-4$^{-/-}$ IL-10$^{-/-}$ mice exhibited lower apoptotic cell numbers than IL-10$^{-/-}$ control animals at day 16 following *A. butzleri* strain CCUG 30485 infection ($p < 0.005$; *Fig. 2A*; *Fig. S1A*). Furthermore, apoptotic cell numbers were lower in the ileal mucosa of TLR-4$^{-/-}$ IL-10$^{-/-}$ as compared to IL-10$^{-/-}$ mice at days 6 and 16 following C1 strain infection ($p < 0.05$; *Fig. 2B*), and relative infection-induced increases were less pronounced in mice of the former genotype *(Fig. S1B)*. Taken together, despite lack of clinical and histopathological changes, TLR-4 gene deficiency was associated with lower abundances of ileal apoptotic cells during *A. butzleri* infection with either strain.
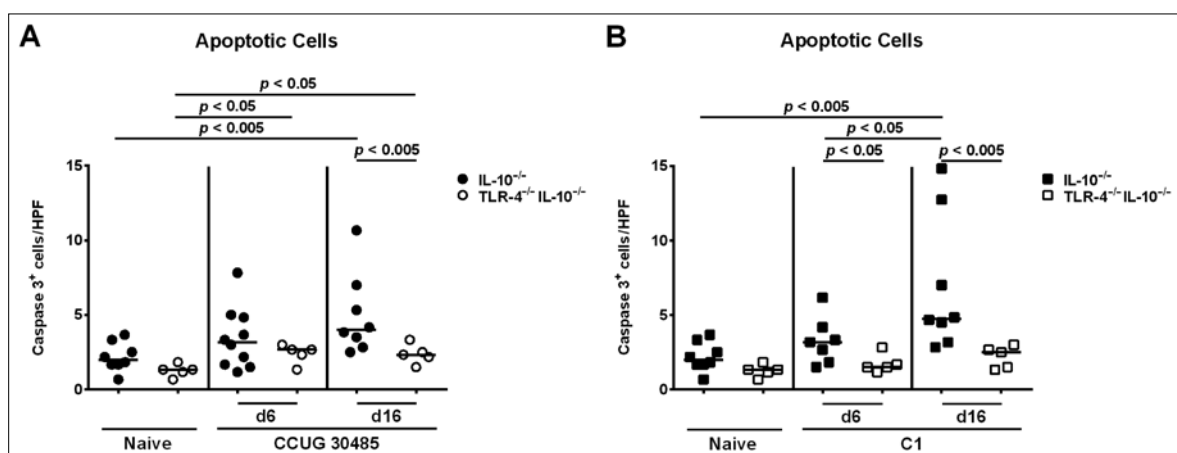

Fig. 2. Kinetics of apoptotic cells in ileal tissue of gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ mice following *A. butzleri* infection. Gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ (open symbols) and IL-10$^{-/-}$ mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *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 ileal sections at days 6 or 16 p.i. Medians (black bars) and levels of significance (*p*-value) determined by Mann–Whitney *U* test are indicated. Data shown were pooled from three independent experiments
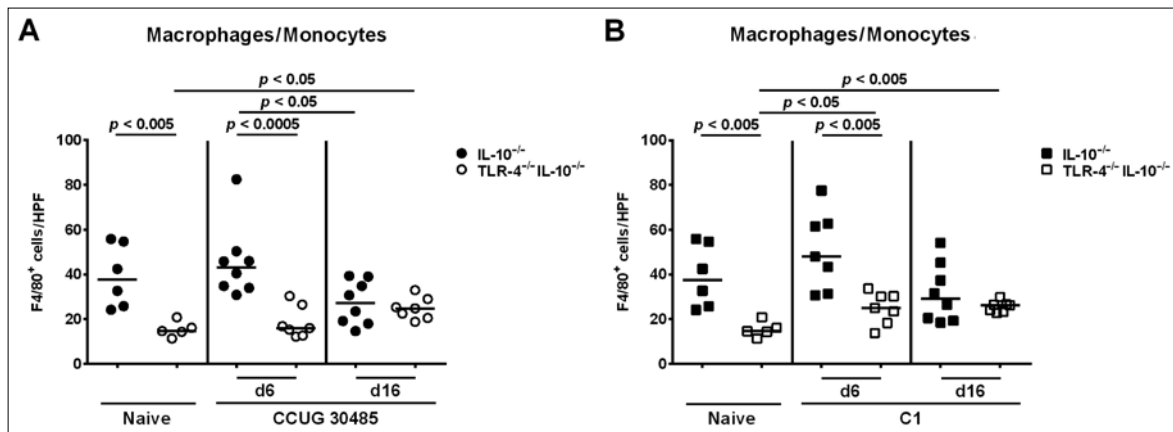
Fig. 3. Kinetics of innate immune cell influx into ileal tissue of gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ mice following *A. butzleri* infection. Gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ (open symbols) and IL-10$^{-/-}$ mice (closed symbols) were generated by antibiotic treatment and perorally infected 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 ileal sections at days 6 or 16 p.i. Medians (black bars) and levels of significance (*p*-value) determined by Mann–Whitney *U* test are indicated. Data shown were pooled from three independent experiments

Role of TLR-4 in small intestinal inflammation induced by A. butzleri *in gnotobiotic IL-10$^{-/-}$ mice*

Given that 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, we next quantitatively determined distinct immune cell populations by *in situ* immunohistochemical staining of small intestinal paraffin sections. Interestingly, already in the naive state, TLR-4$^{-/-}$ IL-10$^{-/-}$ mice exhibited lower numbers of F4/80+ macrophages and monocytes as compared to IL-10$^{-/-}$ mice ($p < 0.005$; *Fig. 3*), which also held true at day 6 following infection with either strain ($p < 0.0005$ and $p < 0.005$, respectively; *Fig. 3*). Six and 16 days following *A. butzleri* strain C1 infection, F4/80+ cell numbers increased in the ileal mucosa of TLR-4$^{-/-}$ IL-10$^{-/-}$ mice ($p < 0.05$ and $p < 0.005$, respectively; *Fig. 3B*), whereas ileal macrophages and monocytes increased as early as 6 days following *A. butzleri* CCUG 30485 strain infection in TLR-4$^{-/-}$ IL-10$^{-/-}$ mice only ($p < 0.05$; *Fig. 3A*). As compared to uninfected mice, numbers of CD3+ T lymphocytes were higher in small intestines of IL-10$^{-/-}$ than TLR-4$^{-/-}$ IL-10$^{-/-}$ mice at days 6 and 16 p.i. with either strain ($p < 0.05$–0.0005; *Fig. 4A*). Furthermore, *A. butzleri* induced relative increases in T lymphocytes were less pronounced in TLR-4$^{-/-}$ IL-10$^{-/-}$ as compared to IL-10$^{-/-}$ mice (*Fig. S1C*). Ileal Treg numbers increased in IL-10$^{-/-}$ but not TLR-4$^{-/-}$ IL-10$^{-/-}$ mice until days 6 and 16 following *A. butzleri* CCUG 30485 strain infection ($p < 0.05$; *Fig. 4B*, left panel; *Fig. S1D*). At day 16, but not day 6 following *A. butzleri* C1 strain infection, only IL-10$^{-/-}$ mice displayed higher Tregs in their small intestines as compared to uninfected control animals ($p < 0.05$; *Fig. 4B*, right panel; *Fig. S1D*). Furthermore, Treg numbers were higher in small intestines of IL-10$^{-/-}$ mice at day 6 following *A. butzleri* CCUG 30485 strain as compared

to C1 strain infection ($p < 0.05$; *Fig. 4B*). As for the innate F4/80+ cells, naive as well as infected TLR-4$^{-/-}$ IL-10$^{-/-}$ mice exhibited lower ileal B220+ B lymphocytes as compared to respective IL-10$^{-/-}$ control mice ($p < 0.05$–0.0001; *Fig. 4C*). Only 16 days following *A. butzleri* strain C1 infection, elevated B220+ cells could be determined in the ilea of IL-10$^{-/-}$ mice ($p < 0.005$; *Fig. 4C*, right panel; *Fig. S1E*) that were significantly higher as compared to those observed in *A. butzleri* CCUG 30485 strain infected mice at day 6 ($p < 0.01$; *Fig. 4C*). Taken together, TLR-4$^{-/-}$ IL-10$^{-/-}$ mice exhibited lower numbers of ileal innate and adaptive immune cell populations following *A. butzleri* infection with either strain.

Small intestinal and systemic pro-inflammatory cytokine levels in A. butzleri-*infected gnotobiotic IL-10$^{-/-}$ mice lacking TLR-4*

We next measured concentrations of the pro-inflammatory cytokine IFN-γ in supernatants of *ex vivo* biopsies derived from ileum, MLN, and spleen. Irrespective of the applied strain, ileal IFN-γ concentration significantly increased as early as 6 days following *A. butzleri* infection of IL-10$^{-/-}$, but not TLR-4$^{-/-}$ IL-10$^{-/-}$ mice ($p < 0.005$ and $p < 0.05$, respectively; *Fig. 5, Fig. S2A*). At day 6 p.i., IFN-γ concentrations were higher in small intestines of *A. butzleri* CCUG 30485 strain as compared to C1 strain infected IL-10$^{-/-}$ mice ($p < 0.05$; *Fig. 5*). Six days following *A. butzleri* CCUG 30485 strain infection, IFN-γ levels increased in MLNs of mice of either genotype, but less distinctly in IL-10$^{-/-}$ mice lacking TLR-4 ($p < 0.005$ and $p < 0.05$; *Fig. 6A, Fig. S2B*). There was, however, a trend towards lower IFN-γ concentrations in MLN of IL-10$^{-/-}$ mice lacking TLR-4 as compared to IL-10$^{-/-}$ control animals (n.s.; *Fig. 6A*). Interestingly, no signifi-

cant increases of the pro-inflammatory cytokine could be observed in MLN at either time point following *A. butzleri* C1 strain infection, irrespective of the genotype of mice *(Fig. 6B)*. Remarkably, pro-inflammatory cytokine responses were not restricted to the intestinal tract, given that IFN-γ concentrations significantly increased
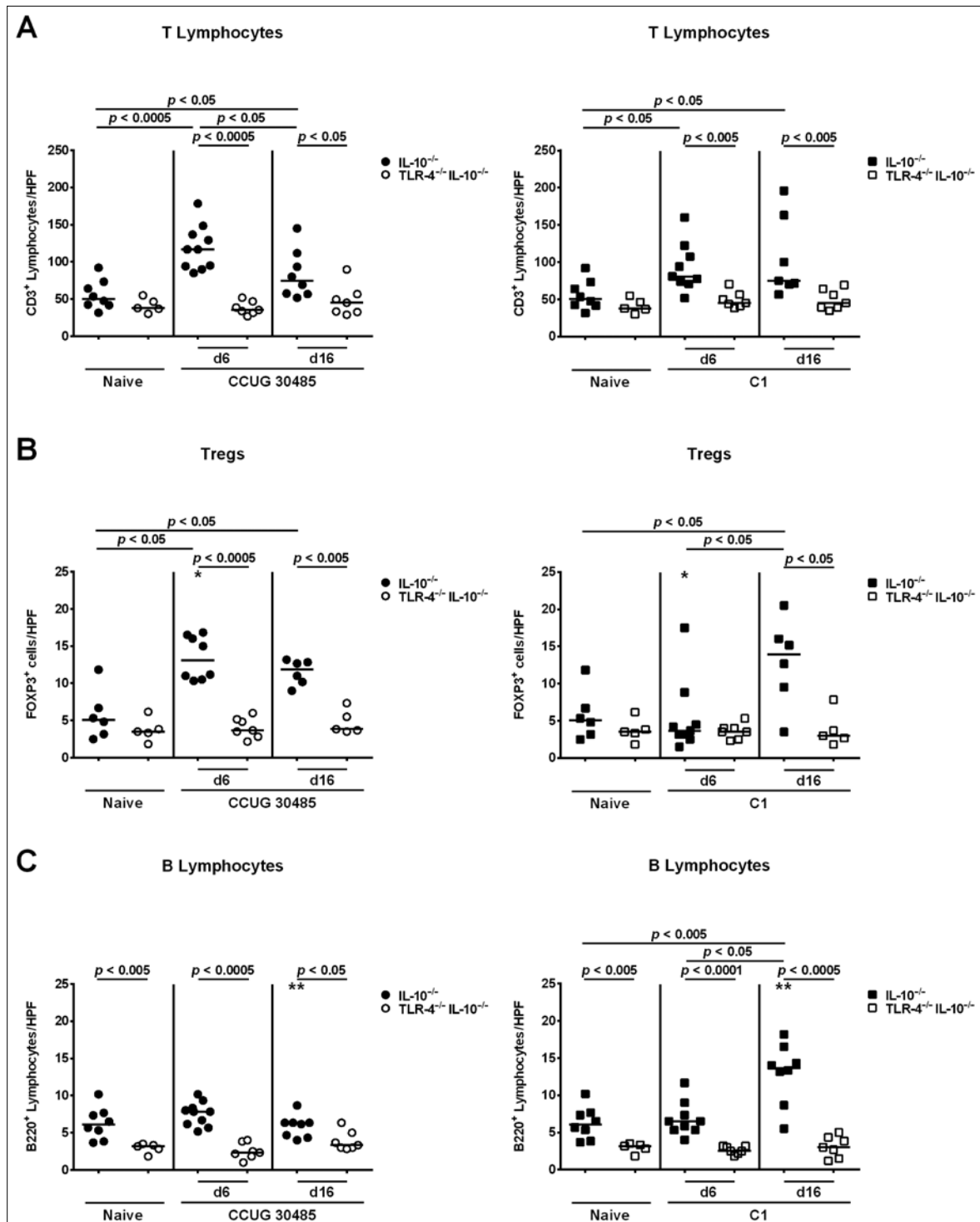

Fig. 4. Kinetics of adaptive immune cell influx into ileal tissue of gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ mice following *A. butzleri* infection. Gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ (open symbols) and IL-10$^{-/-}$ mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (circles; left panels) or strain C1 (squares; right panels). Uninfected mice served as negative controls (Naive). The average number of cells positive for (A) CD3 (T lymphocytes), (B) FOXP3 (regulatory T cells, Tregs), and (C) B220 (B lymphocytes) per mouse was determined microscopically in immunohistochemically stained ileal sections at days 6 or 16 p.i. Medians (black bars) and levels of significance (*p*-value) determined by 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
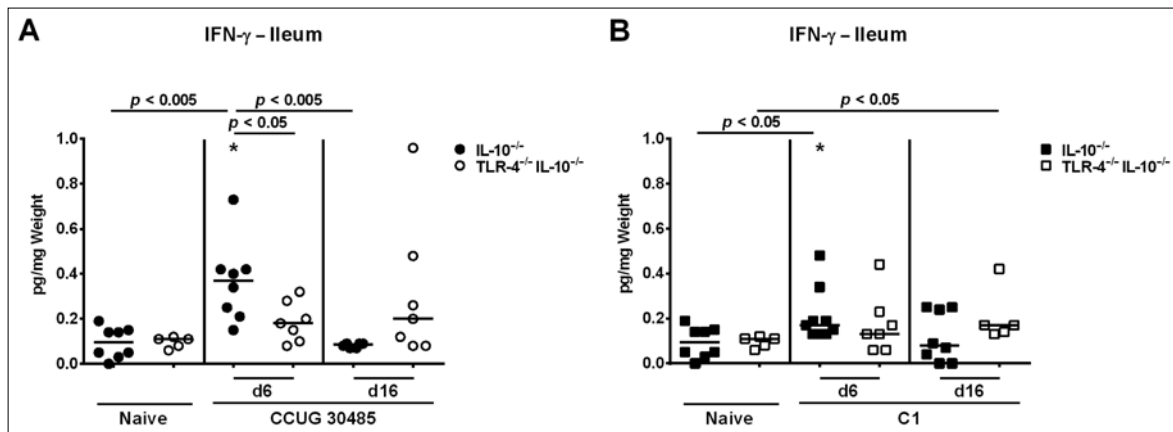
Fig. 5. Kinetics of pro-inflammatory cytokine responses in the ileum of gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ mice following *A. butzleri* infection. Gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ (open symbols) and IL-10$^{-/-}$ mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* (A) strain CCUG 30485 (circles) or (B) strain C1 (squares). Uninfected mice served as negative controls (Naive). IFN-γ concentrations (pg per mg ileal tissue) were determined in supernatants of *ex vivo* ileal biopsies taken at days 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 ($^*p < 0.05$). Data shown were pooled from three independent experiments
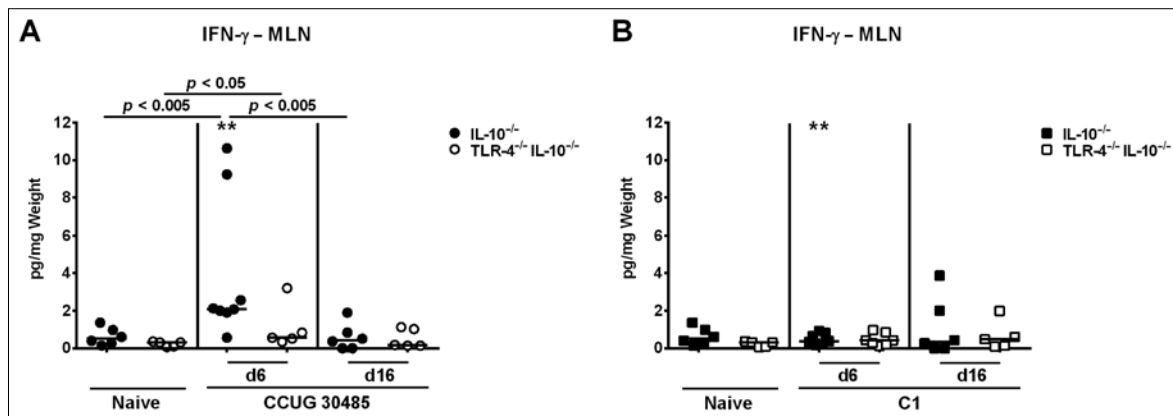

Fig. 6. Kinetics of pro-inflammatory cytokine responses in mesenetric lymphnodes of gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ mice following *A. butzleri* infection. Gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ (open symbols) and IL-10$^{-/-}$ mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* (A) strain CCUG 30485 (circles) or (B) strain C1 (squares). Uninfected mice served as negative controls (Naive). IFN-γ concentrations (pg per mg tissue) were determined in supernatants of *ex vivo* biopsies taken from mesenterial lymphnodes (MLN) at days 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

in spleens of IL-10$^{-/-}$ versus TLR-4$^{-/-}$ IL-10$^{-/-}$ mice as early as 6 days following CCUG 30485 strain infection ($p < 0.005$; *Fig. 7A, Fig. S2C*), but declined back to naive levels until day 16 p.i. *(Fig. 7A)*. At day 6 p.i., splenic IFN-γ concentrations were lower in C1 strain as compared to CCUG 30485 strain infected IL-10$^{-/-}$ mice ($p < 0.05$; *Fig. 7*). Interestingly, IFN-γ concentrations were higher in spleens of TLR-4$^{-/-}$ IL-10$^{-/-}$ as compared to IL-10$^{-/-}$ mice at day 16 following *A. butzleri* infection with either strain ($p < 0.005$; *Fig. 7, Fig. S2C*). In TLR-4$^{-/-}$ IL-10$^{-/-}$ mice, however, splenic IFN-γ levels measured at day 16 p.i. did not differ from uninfected, naive mice *(Fig. 7)*. Taken together, *A. butzleri*-induced IFN-γ production was strain dependent, but less pronounced in the small intestinal compartment of IL-10$^{-/-}$ mice lacking TLR-4$^{-/-}$.

Discussion

The immunopathological impact of *A. butzleri* infection *in vivo* is under current debate. Data regarding the molecular mechanisms underlying potential host interactions with this emerging pathogen are lacking due to the scarcity of appropriate *in vivo* models [20]. In the present study, we have applied our gnotobiotic (i.e., secondary abiotic) IL-10$^{-/-}$ mouse infection model in order to unravel the role of TLR-4, the major innate immune receptor for sensing of LPS and LOS derived from the cell wall of Gram-negative bacteria, in murine *Arcobacter* infection. Both gnotobiotic IL-10$^{-/-}$ mice lacking TLR-4 and gnotobiotic IL-10$^{-/-}$ control animals harbored comparable *A. butzleri* loads in their proximal (i.e., duodenum) and distal (i.e.,
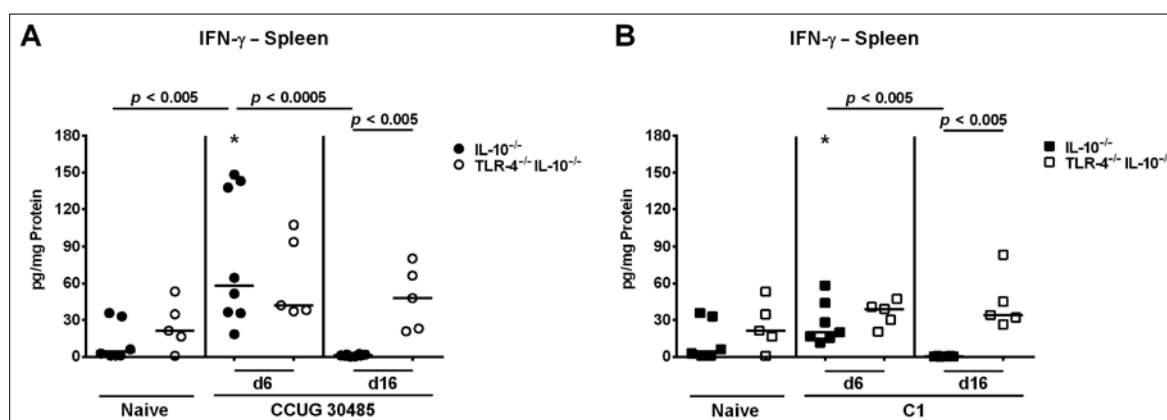
Fig. 7. Kinetics of pro-inflammatory cytokine responses in spleens of gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ mice following *A. butzleri* infection. Gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ (open symbols) and IL-10$^{-/-}$ mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* (A) strain CCUG 30485 (circles) or (B) strain C1 (squares). Uninfected mice served as negative controls (Naive). IFN-γ concentrations (pg per mg total protein) were determined in supernatants of *ex vivo* splenic biopsies taken at days 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 ($^*p < 0.05$). Data shown were pooled from three independent experiments

ileum) small intestines following peroral infection. The small intestinal colonization potential of *A. butzleri* was strain-independent, given that either strain colonized the proximal as well as distal small intestines of gnotobiotic mice at comparably high concentrations. Interestingly, *A. butzleri* strain-dependent differences in infection-induced immune cell responses could be observed in IL-10$^{-/-}$ control, but not IL-10$^{-/-}$ mice lacking TLR-4. For instance, small intestinal Treg numbers were lower at day 6 following C1 versus CCUG 30485 strain infection, whereas at day 16 p.i., B cell counts were higher in ilea of C1 as compared to CCUG 30485 strain-infected IL-10$^{-/-}$ mice. Remarkably, TLR-4 was essential for *A. butzleri*-induced ileal apoptosis and inflammatory immune cell responses as indicated by lower numbers of ileal T lymphocytes, Tregs and B cells as well as less distinct small intestinal pro-inflammatory IFN-γ secretion in infected IL-10$^{-/-}$ mice lacking TLR-4. Surprisingly, splenic IFN-γ concentrations were even higher in TLR-4$^{-/-}$ IL-10$^{-/-}$ as compared to IL-10$^{-/-}$ control mice 16 but not 6 days following *A. butzleri* infection with either strain. Notably, viable bacteria could not be detected in extra-intestinal and systemic compartments including the spleen by direct plating in infected mice of either genotype. It is tempting to speculate that, in the course of the systemic immune response upon infection, more immune cells such as dendritic cells and lymphocytes might have been imprinted and activated in the spleen of IL-10$^{-/-}$ mice lacking TLR-4 as compared to control mice. The TLR-4 dependence of local (i.e., small intestinal) pro-inflammatory immune responses in *A. butzleri* infected mice is well in line with results from our *C. jejuni in vivo* studies demonstrating that *C. jejuni*-induced colonic and extra-intestinal disease is mediated by TLR-4 signalling of pathogenic LOS [19, 26, 31]. The structure of the carbohydrate backbone of LOS has been successfully characterized in the halophilic bac-

terium *A. halophilus* [32]. To date, however, molecular structures of neither LPS nor LOS have been identified in *A. butzleri*. It is highly likely, however, that, besides TLR-4, other so far unknown pattern recognition factors including TLR and NOD-like receptors are involved in mediating *Arcobacter* infection. For murine *C. jejuni* infection, for instance, we were able to demonstrate that, in addition to TLR-4-dependent signalling of *C. jejuni* LOS, sensing of pathogenic lipoproteins and CpG-DNA by TLR-2 and -9, respectively, contributed to infection-induced pro-inflammatory sequelae [19, 26].

Taken together, we here show that TLR-4 is essential for pro-inflammatory immune responses induced by *A. butzleri* in the small intestinal tract of infected mice.

In conclusion, further *in vivo* studies should further elucidate the molecular mechanisms underlying pathogen–host interactions in arcobacteriosis.

Acknowledgements

Financial disclosure and grant support

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the article.

Competing interests

The authors have declared that no competing interests exist.

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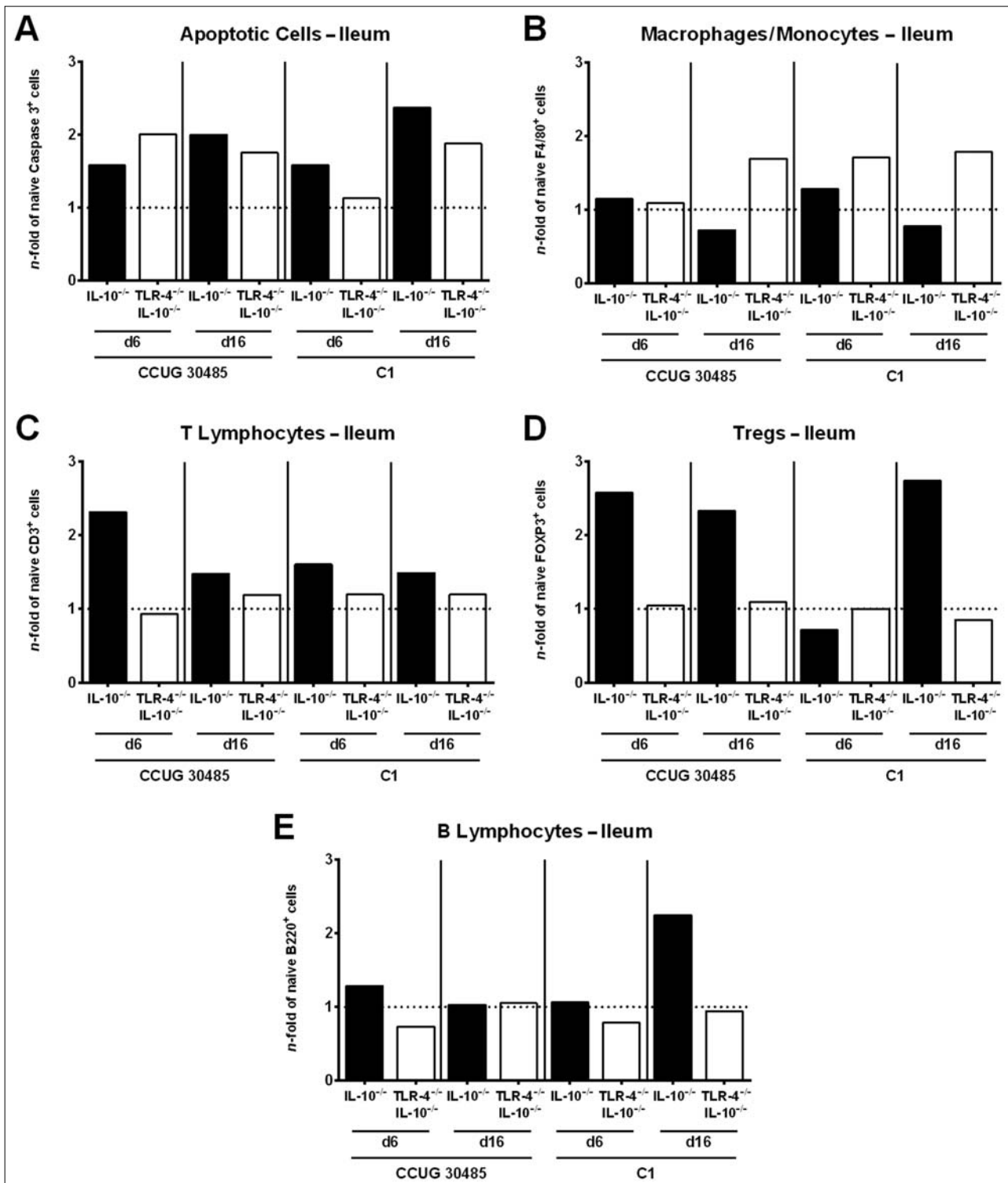
Fig. S1. Relative increases in adaptive and innate immune cell populations and apoptotic cells in ileal tissue of gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ mice following *A. butzleri* infection. Gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ (open bars) and IL-10$^{-/-}$ mice (closed bars) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 or strain C1 as indicated. Uninfected mice served as controls. The average number of (A) apoptotic cells (positive for caspase-3, Casp3+), (B) macrophages and monocytes (F4/80+), (C) T lymphocytes (CD3+), (D) regulatory T cells (Tregs, FOXP3+), and (E) B lymphocytes (B220+) per animal was determined microscopically in immunohistochemically stained ileal section at days 6 or 16 p.i. Respective bars indicate fold increases of respective cell abundance upon infection. Data shown were pooled from three independent experiments
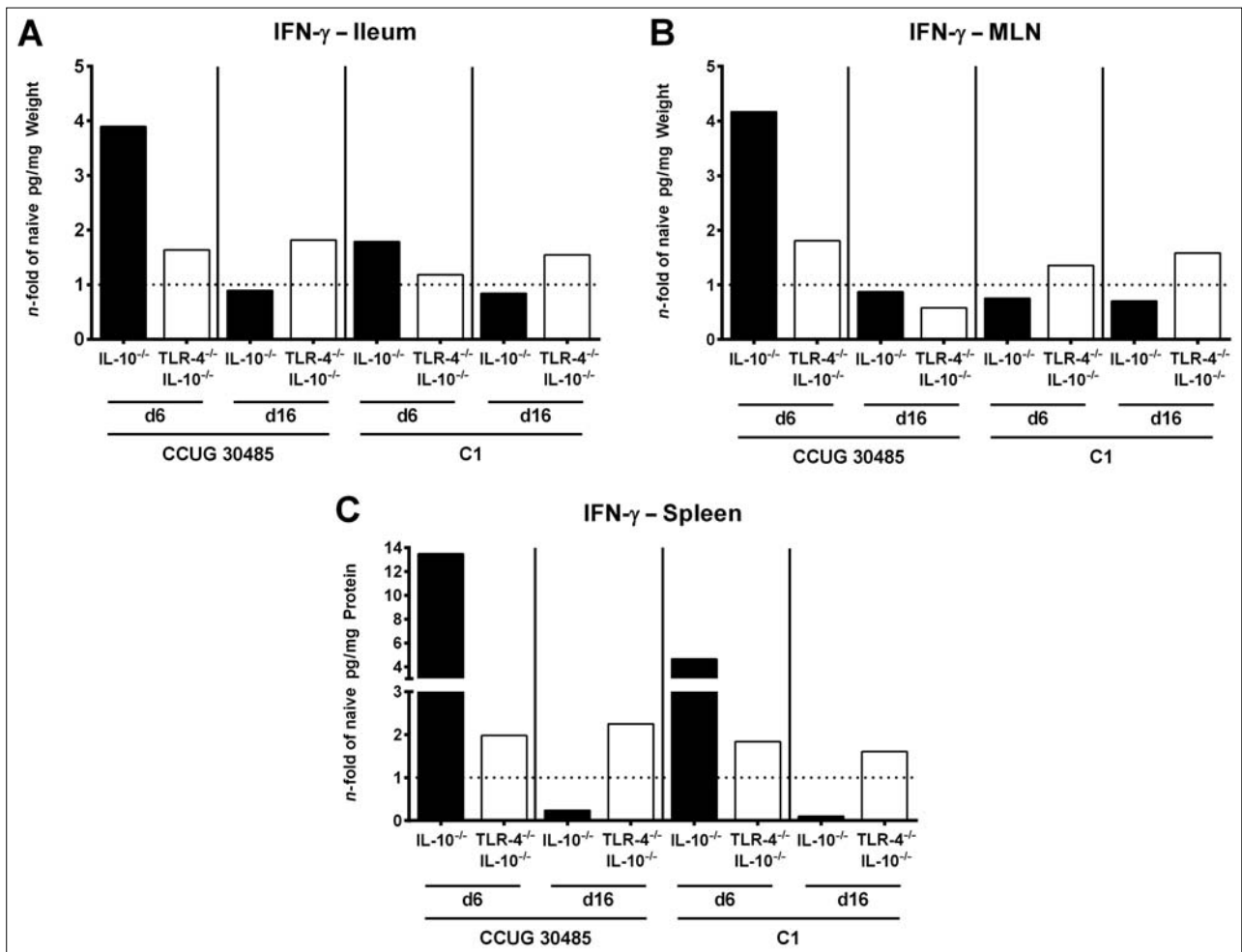
Fig. S2. Relative increases in intestinal and systemic IFN-γ concentrations in gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ mice following *A. butzleri* infection. Gnotobiotic TLR-4$^{-/-}$ IL-10$^{-/-}$ (open bars) and IL-10$^{-/-}$ mice (closed bars) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 or strain C1 as indicated. Uninfected mice served as controls. IFN-γ concentrations were determined in supernatants of *ex vivo* biopsies taken from (A) ilea, (B) mesenteric lymphnodes (MLN), and (C) spleen at days 6 or 16 p.i. Bars indicate fold increases of IFN-γ levels in respective compartments upon infection. Data shown were pooled from three independent experiments