

TOLL-LIKE RECEPTOR-4 DEPENDENT INTESTINAL GENE EXPRESSION DURING *ARCOBACTER BUTZLERI* INFECTION OF GNOTOBIOTIC IL-10 DEFICIENT MICE

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We have previously shown that *Arcobacter butzleri* infection induces Toll-like receptor (TLR) -4 dependent immune responses in perorally infected gnotobiotic IL-10^{-/-} mice. Here, we analyzed TLR-4-dependent expression of genes encoding inflammatory mediators and matrix-degrading gelatinases MMP-2 and -9 in the small and large intestines of gnotobiotic TLR-4-deficient IL-10^{-/-} mice that were perorally infected with *A. butzleri* strains CCUG 30485 or C1, of human and chicken origin, respectively. At day 6 following *A. butzleri* infection, colonic mucin-2 mRNA, as integral part of the intestinal mucus layer, was downregulated in the colon, but not ileum, of IL-10^{-/-} but not TLR-4^{-/-} IL-10^{-/-} mice. CCUG 30485 strain-infected TLR-4-deficient IL-10^{-/-} mice displayed less distinctly upregulated IFN- γ , IL-17A, and IL-1 β mRNA levels in ileum and colon, which was also true for colonic IL-22. These changes were accompanied by upregulated colonic MMP-2 and ileal MMP-9 mRNA exclusively in IL-10^{-/-} mice. In conclusion, TLR-4 is essentially involved in *A. butzleri* mediated modulation of gene expression in the intestines of gnotobiotic IL-10^{-/-} mice.

Keywords: *Arcobacter butzleri*, IL-23/Th17 axis, IL-22/IL-18 axis, TLR-4, lipooligosaccharide, lipopolysaccharide, pro-inflammatory immune responses, intestinal innate and adaptive immunity, gnotobiotic IL-10^{-/-} mouse infection model, gelatinases

Introduction

The gram-negative *Arcobacter* species belong to the family of *Campylobacteraceae* and can be isolated from a broad range of habitats including surface water. In animals, *Arcobacter* spp. form part of the commensal gastrointestinal microbiota [1]. In susceptible humans, however, distinct *Arcobacter* species acquired via the oral route may cause infections characterized by acute gastroenteritis or prolonged watery diarrhea. Depending on the pathogenic properties of the respective bacterial strain and/or host susceptibility, symptoms last for several weeks or months. Single cases as well as outbreaks of *Arcobacter* associated human diseases have been reported, but solid epidemiological data on the prevalence of *Arcobacter* infection are limited [2, 3]. This might be due to the fact that only insufficient methods for cultivation and species identification of *Arcobacter* spp. are applied in routine diagnostic

departments [2, 3]. Nevertheless, several authors reported in retrospective studies that *Arcobacter* spp. are among the fourth most common *Campylobacterales* species derived from diarrheal patients [4–6]. Until now, 21 *Arcobacter* spp. have been described [7]. Among those, the International Commission on Microbiological Specifications for Foods (ICMSF) rated *Arcobacter butzleri* and *Arcobacter cryaerophilus* as serious hazards for human health [8]. So far, however, only very limited information about the responsible virulence genes and underlying immunopathogenic mechanisms of infection is available. The presence of putative virulence genes such as *cadF*, *mviN*, *pIdA*, *tlyA*, *cj1349*, *hecB*, *irgA*, *hecA*, *ciaB*, and *iroE* have been described within the genomic sequence of *A. butzleri* strain RM 4018 [9], for instance, and shown to contribute to adhesion (CadF, HecA, Cj1349), invasion (CiaB), lysis of erythrocytes (HecB, TlyA, PIdA), iron acquisition, and maintaining of infection (IrgA, IroE) as well as peptido-

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glycan biosynthesis (MviN) in other bacteria [10–18]. It is, however, unclear whether these putative virulence factors exert similar functions in *Arcobacter*. Whereas *A. butzleri* has been shown to adhere to and invade into several cell lines *in vitro*, no correlation between virulence gene pattern of *A. butzleri* isolates and adhesive and invasive phenotypes, however, could be shown [19, 20]. Even though *in vitro* cytotoxic effects of distinct *A. butzleri* strains have been observed, the corresponding toxin has not been identified so far [5, 21–25]. A potential diarrhea-inducing mechanism of human *Arcobacter* infection might be a compromised intestinal epithelial barrier function observed upon *A. butzleri* infection of HT29/B6 cells [26]. Investigations of *Arcobacter*–host interactions, however, are hampered due to scarcity of appropriate *in vivo* models. So far, *A. butzleri* infection models revealed conflicting results that were depending on respective *A. butzleri* strains, the host species and the breed. *A. butzleri* did not induce disease in conventional chicken, but infected turkey strains displayed variable disease outcomes [27]. Whereas adult rats presented with watery diarrhea and imbalanced serum electrolytes depending on pathogenic loads, neonatal albino rats were suffering from self-limited diarrhea and small intestinal as well as hepatic necroses [28, 29]. Furthermore, *A. butzleri* exerted invasive properties in neonatal piglets as indicated by bacterial translocation to extra-intestinal tissue sites such as liver, kidney, and even the brain [30].

To date, however, reports on murine *Arcobacter* infection are scarce. One study revealed enhanced adherent properties of initially low-adherent *A. butzleri* strains upon serial intraperitoneal passages in mice [31]. Very recently, our group has performed murine *A. butzleri* studies applying the gnotobiotic (i.e., secondary abiotic) IL-10^{-/-} mouse model that had initially been established by us for investigating *Campylobacter jejuni*–host interactions [32]. Following peroral infection, *A. butzleri* was able to readily colonize the murine intestinal tract. Whereas mice did not display any overt clinical signs of infection-induced disease, the bacteria did not only induce small and large intestinal but also extra-intestinal including systemic immune responses depending on the respective strain and the time course of infection [33, 34]. These immune responses were dependent on Toll-like receptor (TLR) -4, the innate immune receptor for bacterial lipooligosaccharide (LOS), and lipopolysaccharide (LPS) derived from cell walls of gram-negative bacteria, given that gnotobiotic IL-10^{-/-} mice lacking TLR-4 presented with less pronounced intestinal and systemic immune responses [35, 36]. The exact mechanisms underlying arcobacteriosis, however, are virtually unknown. In a very recently published study (Heimesaat et al., 2016, this issue), we surveyed *A. butzleri* strain-dependent induced expression profiles of regulatory, epithelial barrier function preserving, pro-inflammatory, and matrix-degrading molecules in the small and large intestines that are presumably involved in mediating infection-induced sequelae. We were able to show, for instance, that mucin-2, a pivotal constituent of the intestinal

mucus providing first line defence against intestinal pathogens [37], was downregulated in the colon, but not ileum of *A. butzleri*-infected mice (Heimesaat et al., 2016, this issue). Alike *C. jejuni* infection [38], *A. butzleri* induced intestinal expression of cytokines such as IL-17A, IL-22, and IFN- γ that are produced by T helper (Th) 17 cells and share important functions in antimicrobial host immunity, as well as their master regulator IL-23 (Heimesaat et al., 2016, this issue). Our group recently showed that IL-22 was able to induce IL-18 upon bacterial and parasitic infection *in vitro* and, conversely, IL-18 amplified IL-22 release during Th1 mediated intestinal inflammation [39]. The observation that both intestinal IL-22 and IL-18 were upregulated during murine *A. butzleri* infection in a strain-dependent manner points towards that the IL-22/IL-18 axis might also be involved in mediating/orchestrating immune responses in arcobacteriosis (Heimesaat et al., 2016, this issue). Upon *A. butzleri* infection, gnotobiotic IL-10^{-/-} mice additionally displayed increased mRNA levels of pro-inflammatory cytokines including TNF and IL-1 β that were accompanied by increased intestinal upregulation of the tissue-degrading matrix metalloproteinases (MMP) -2 and -9 (also referred to as gelatinases A and B, respectively) [40]. Based upon our previous study published within this issue, we addressed here whether TLR-4 regulates the ileal and colonic immune responses during *A. butzleri* infection at the transcriptional level. Therefore, we analyzed respective gene expression profiles in the small and large intestines of *A. butzleri*-infected gnotobiotic IL-10^{-/-} mice lacking TLR-4.

Materials and methods

Gnotobiotic 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 deprived gastrointestinal microbiota were generated following broad-spectrum antibiotic treatment as described earlier [32, 41]. 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 infec-

tion experiment [42]. Three days before infection, the antibiotic cocktail was replaced by sterile tap water (*ad libitum*). Abiotic status of gnotobiotic mice was confirmed as described earlier [41, 43].

Arcobacter butzleri infection of mice

Age-matched female gnotobiotic TLR-4^{-/-} IL-10^{-/-} and IL-10^{-/-} mice were perorally infected with approximately 10⁹ 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) as described previously [35, 36]. Naive age-matched female gnotobiotic IL-10^{-/-} and TLR-4^{-/-} 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 [44], whereas the C1 strain was isolated from fresh chicken meat [19]. 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 [33, 34].

Sampling procedures

Mice were sacrificed by isofluran treatment (Abbott, Greifswald, Germany) on day 6 or 16 p.i. Tissue samples from ileum and colon were removed under sterile conditions.

Analysis of gene expression by real-time PCR

Total RNA was isolated from snap frozen colonic and ileal *ex vivo* biopsies, reverse transcribed, and analyzed as described previously [45]. Briefly, murine mRNAs coding for mucin-2, IFN- γ , TNF, IL-17A, IL-1 β , IL-23p19, IL-22, IL-18, MMP-2, MMP-9, TIMP-1, and TIMP-3 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-phosphoribosyl-transferase (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 *U* test (GraphPad Prism v6.05, 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 and discussion

Peroral A. butzleri infection of gnotobiotic IL-10^{-/-} mice lacking TLR-4

We have recently shown that TLR-4 is essentially involved in inducing large and small intestinal but also systemic immune responses upon murine *A. butzleri* infection [35, 36]. In the present study, we analyzed potential TLR-4-dependent changes in the expression of genes encoding key mediators of inflammation including Th17 cytokines and the gelatinases MMP-2 and MMP-9. To address this, we applied the gnotobiotic IL-10^{-/-} mouse infection model [32] and challenged IL-10^{-/-} mice lacking TLR-4 (TLR-4^{-/-} IL-10^{-/-}) perorally with 10⁹ CFU of two different *A. butzleri* strains, i.e., either strain CCUG 30485 (of human origin) or C1 (of chicken origin) perorally by gavage as described previously in more detail [33, 34]. Either *A. butzleri* strain readily colonized the intestinal tract of mice until day 16 p.i. Bacterial loads, however, were approximately 0.5 to 2.0 orders of magnitude lower in fecal samples derived from IL-10^{-/-} mice lacking TLR-4 as compared to IL-10^{-/-} controls [35, 36]. Even though mice of either genotype were clinically unaffected, distinct TLR-4-dependent pro-inflammatory immune responses could be observed in the small and large intestinal tract [33, 34]. For instance, *A. butzleri*-infected TLR-4^{-/-} IL-10^{-/-} mice displayed less distinct intestinal apoptosis and less influx of innate and adaptive immune cells into the intestinal mucosa and lamina propria as compared to IL-10^{-/-} mice [35, 36]. One could argue that the beneficial outcome in mice lacking TLR-4 might be due to differences in the colonization status, which we can indeed not exclude *per se*. However, during the early phase of infection, differences of intraluminal bacterial loads (i.e., less than one log) were rather too low for a biological relevant impact. This is further supported by the fact that the large intestinal *A. butzleri* loads (ranging between 10⁶ and 10⁸ CFU per gram feces) in the late course of infection were relatively high and the threshold to induce disease most likely far lower. Hence, it is rather questionable whether two orders of magnitude in intraluminal intestinal *A. butzleri* burden would have biological relevance. Furthermore, bacterial loads did not differ in the small intestines of mice with respective genotypes [35].

Colonic expression of the mucin-2 gene in *A. butzleri*-infected gnotobiotic IL-10^{-/-} mice lacking TLR-4

Given that mucin-2 comprises an important mucus layer constituent and pivotally contributes to intestinal epithelial barrier function [46, 47], we addressed whether *A. butzleri* infection affects mucin-2 expression in the colon of gnotobiotic IL-10^{-/-} mice in a TLR-4-dependent fashion. Whereas mucin-2 expression was downregulated in large intestines of IL-10^{-/-} control animals 6 days following infection with either strain and additionally at day 16 post C1 strain infection ($p < 0.05$ – 0.01 ; Fig. 1), mRNA levels did not differ between infected and uninfected TLR-4^{-/-} IL-10^{-/-} mice. At day 16 p.i., however, CCUG 30485 strain-infected TLR-4^{-/-} IL-10^{-/-} mice displayed lower large intestinal mucin-2 mRNA levels as compared to infected IL-10^{-/-} animals ($p < 0.05$; Fig. 1, left panel). Until day 16 following C1 strain infection, however, mucin-2 expression decreased in TLR-4^{-/-} IL-10^{-/-} mice as compared to day 6 p.i. but was still comparable to naive controls ($p < 0.05$; Fig. 1, right panel). Hence, colonic mucin-2 is not TLR-4 dependently expressed upon *A. butzleri* infection.

Colonic expression of genes encoding defined immune mediators in *A. butzleri*-infected gnotobiotic IL-10^{-/-} mice lacking TLR-4

We next addressed whether distinct cytokines belonging to the IL-23/Th17 and IL-22/IL-18 axis were expressed in a TLR-4-dependent fashion following *A. butzleri* infection. At day 16 postinfection with either strain, colonic IFN- γ , TNF, IL-17A, and IL-1 β mRNA levels were higher in TLR-4^{-/-} IL-10^{-/-} as compared to uninfected control mice ($p < 0.01$; Fig. 2). At day 6 p.i., *A. butzleri* CCUG 30485 strain induced increases in colonic IFN- γ , IL-17A, and IL-1 β mRNA in IL-10^{-/-} mice ($p < 0.01$ – 0.001 ; Fig. 2A, C, and D, left panels). In CCUG 30485 strain-infected

IL-10^{-/-} mice lacking TLR-4, however, large intestinal IFN- γ mRNA levels were lower as compared to infected IL-10^{-/-} mice ($p < 0.05$; Fig. 2A, left panel). Moreover, at day 16 following C1 strain infection, colonic IL-23p19 mRNA was downregulated ($p < 0.05$; Fig. 3A, right panel), whereas colonic IL-22 mRNA level was upregulated at day 16 post CCUG 30485 strain infection in TLR-4^{-/-} IL-10^{-/-} mice only ($p < 0.05$; Fig. 3B, left panel). Furthermore, colonic IL-22 mRNA level increased as early as 6 days following CCUG 30485 strain infection ($p < 0.001$; Fig. 3B, left panel). In large intestines of IL-10^{-/-}, but not TLR-4-deficient IL-10^{-/-} mice, IL-18 mRNA levels increased until day 16 following *A. butzleri* infection with either strain ($p < 0.05$ – 0.001 ; Fig. 3C), that was also the case at day 6 upon C1 strain infection ($p < 0.01$; Fig. 3C, right panel). Hence, depending on the respective bacterial strain and on the time course of infection, pro-inflammatory Th17 cytokines and mediators of the IL-22/IL-18 axis are TLR-4 dependently expressed in large intestines upon *A. butzleri* infection.

Colonic expression of genes encoding matrix-degrading gelatinases in *A. butzleri*-infected gnotobiotic IL-10^{-/-} mice lacking TLR-4

In the following, we focused on the matrix-degrading gelatinases MMP-2 and MMP-9 during *A. butzleri* infection. MMP-2 mRNA levels were lower in large intestines of IL-10^{-/-} mice lacking TLR-4 at day 16 following infection with either strain ($p < 0.05$; Fig. 4A), whereas MMP-9 mRNA was downregulated in IL-10^{-/-} mice, but irrespective of the *A. butzleri* strain only at day 6 p.i. ($p < 0.05$ – 0.01 ; Fig. 4B). The mRNA levels of genes encoding for the endogenous inhibitors of gelatinases TIMP-1 and TIMP-3 were both downregulated in the large intestines of TLR-4^{-/-} IL-10^{-/-} mice at day 6 following CCUG 30485 strain infection ($p < 0.05$; Fig. 5, left panels), whereas colonic TIMP-3 mRNA levels had

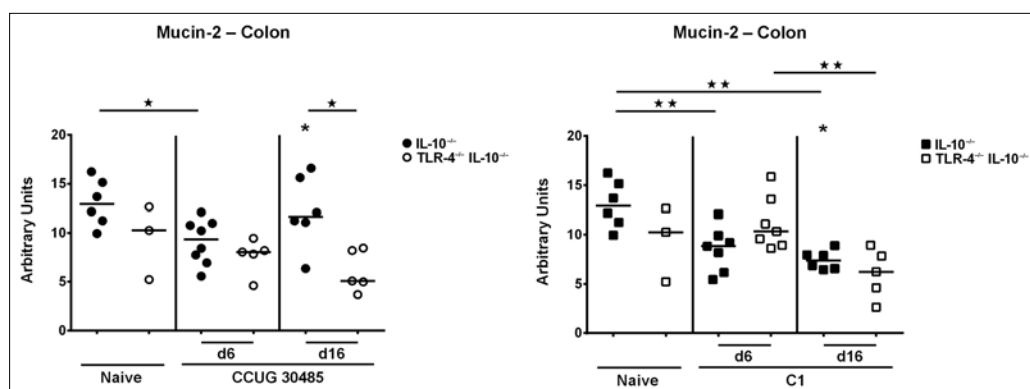


Fig. 1. Colonic mucin-2 mRNA levels in gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (open symbols) and IL-10^{-/-} mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (circles; left panel) or strain C1 (squares; right panel). Uninfected mice served as negative control (naive). Mucin-2 mRNA levels were determined in *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 (* $p < 0.05$; ** $p < 0.01$). Significant differences between *A. butzleri* strains are indicated by stars within the graph. Data shown were pooled from three independent experiments

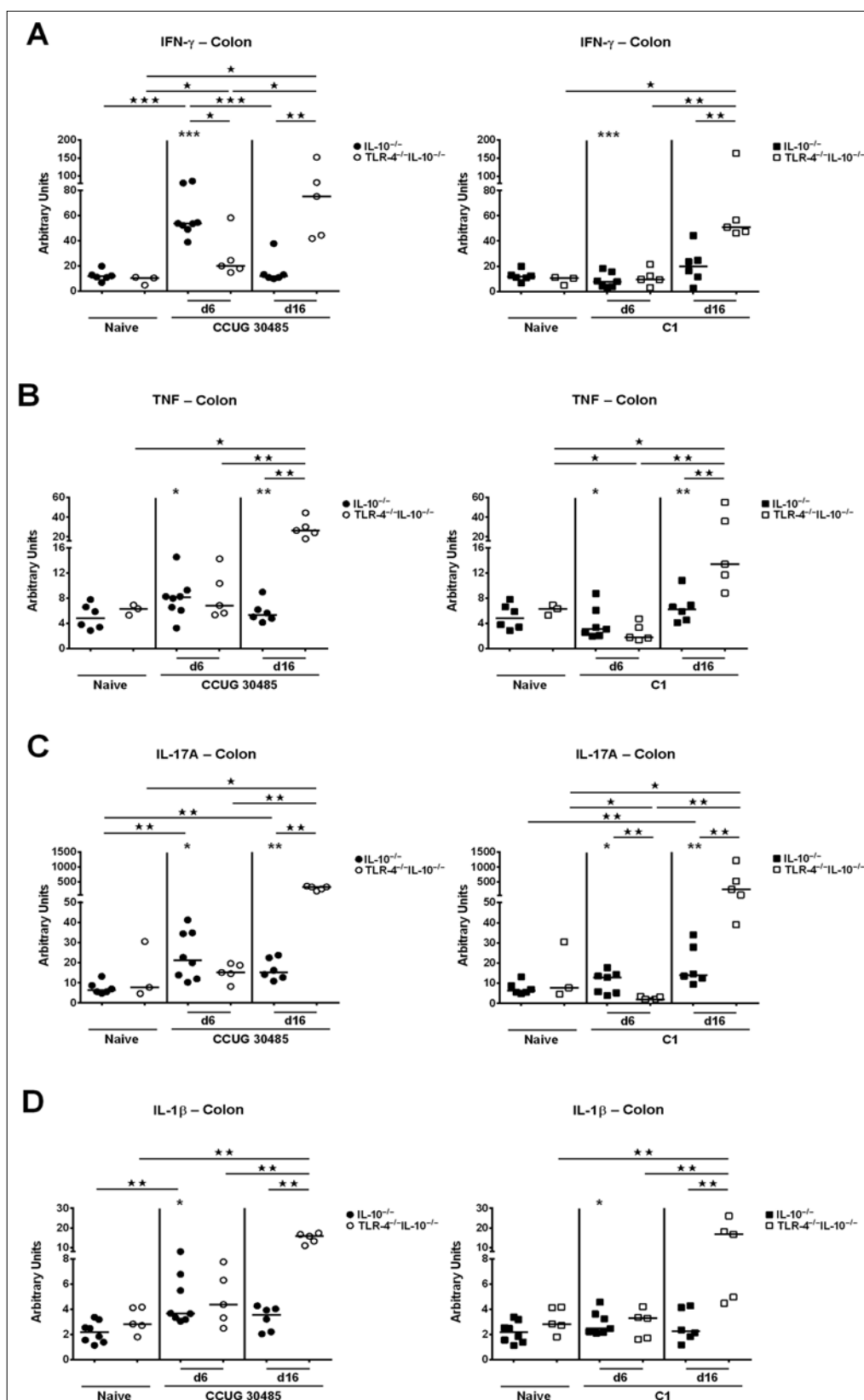


Fig. 2. Colonic expression of genes encoding pro-inflammatory cytokines in gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (open symbols) and IL-10^{-/-} mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (circles; left panel) or strain C1 (squares; right panel). Uninfected mice served as negative control (naive). A) IFN- γ , B) TNF, C) IL-17A, and D) IL-1 β mRNA levels were determined in *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 (**p* < 0.05; ***p* < 0.01; ****p* < 0.001). Significant differences between *A. butzleri* strains are indicated by stars within the graph. Data shown were pooled from three independent experiments

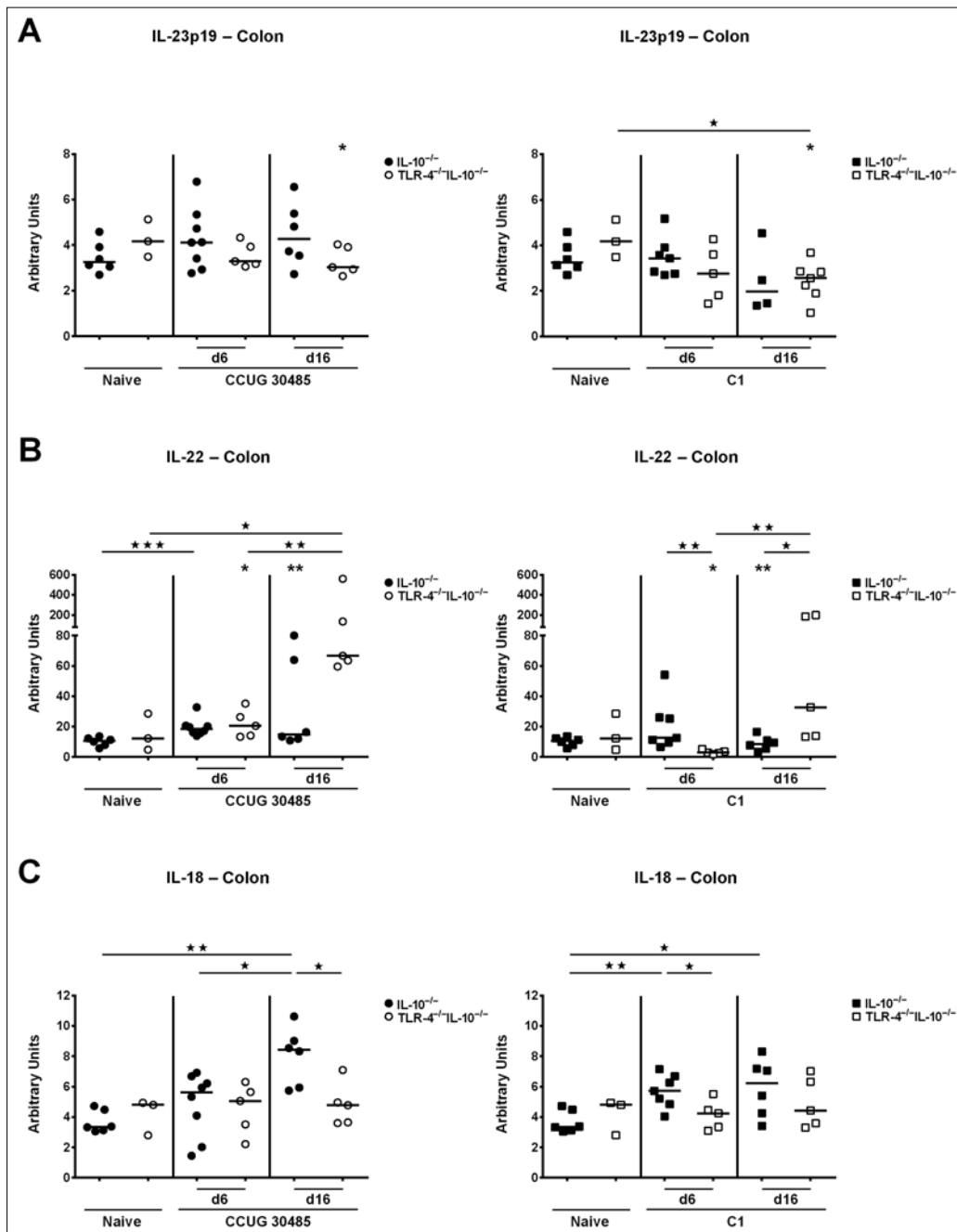


Fig. 3. Colonic expression of genes coding for IL-23p19, IL-22, and IL-18 in gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (open symbols) and IL-10^{-/-} mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (circles; left panel) or strain C1 (squares; right panel). Uninfected mice served as negative control (naive). A) IL-23p19, B) IL-22, and C) IL-18 mRNA levels were determined in *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 (**p* < 0.05; ***p* < 0.01; ****p* < 0.001). Significant differences between *A. butzleri* strains are indicated by stars within the graph. Data shown were pooled from three independent experiments

additionally decreased 16 days following infection with either strain (*p* < 0.01; Fig. 5B). Thus, following murine *A. butzleri* infection, colonic MMP-2 but not MMP-9 mRNA is regulated in a TLR-4-dependent manner. Taken together, distinct colonic pro-inflammatory and matrix-degrading mediators contributing to *A. butzleri* strain-dependent induced immune responses are expressed in a TLR-4-dependent fashion, whereas others are not.

Ileal mucin-2 mRNA levels in A. butzleri-infected gnotobiotic IL-10^{-/-} mice lacking TLR-4

We next addressed whether TLR-4-dependent expression of respective mediators observed in the large intestines of *A. butzleri*-infected mice was similar in the small intestinal tract. Ileal mucin-2 mRNA expression was enhanced following CCUG 30485 strain infection of TLR-4^{-/-} IL-10^{-/-} mice only (*p* < 0.05, Fig. 6).

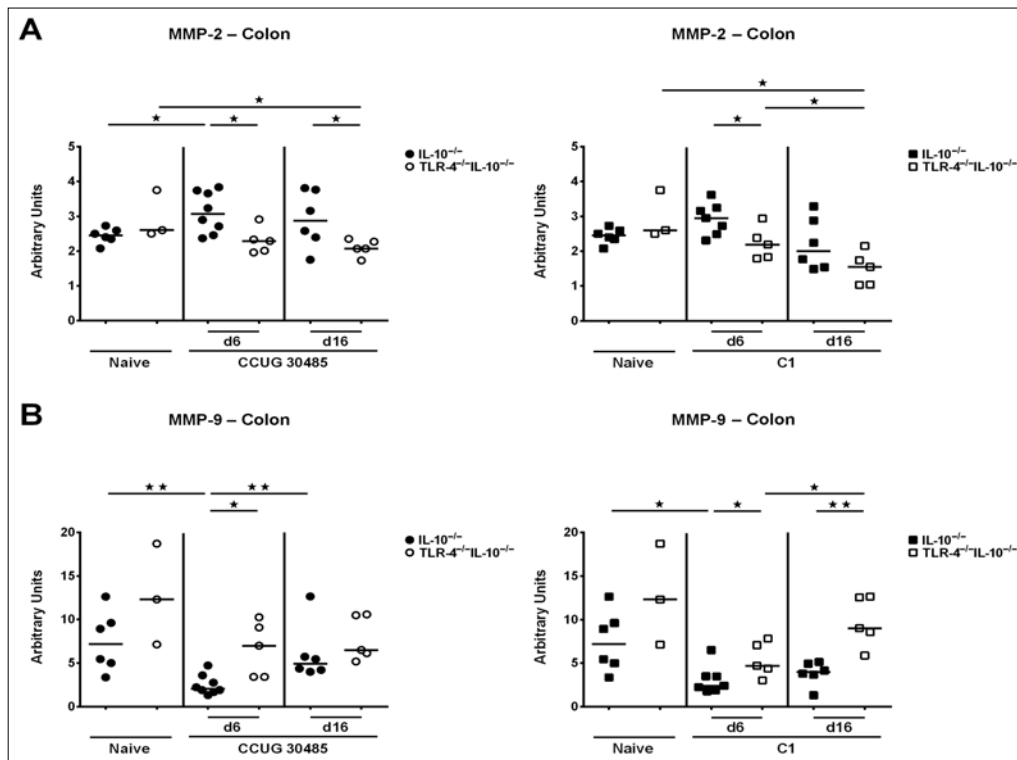


Fig. 4. Colonic MMP-2 and MMP-9 mRNA levels in gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (open symbols) and IL-10^{-/-} mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (circles; left panel) or strain C1 (squares; right panel). Uninfected mice served as negative control (naive). A) MMP-2 and B) MMP-9 mRNA levels were determined in *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 (**p* < 0.05; ***p* < 0.01). Data shown were pooled from three independent experiments

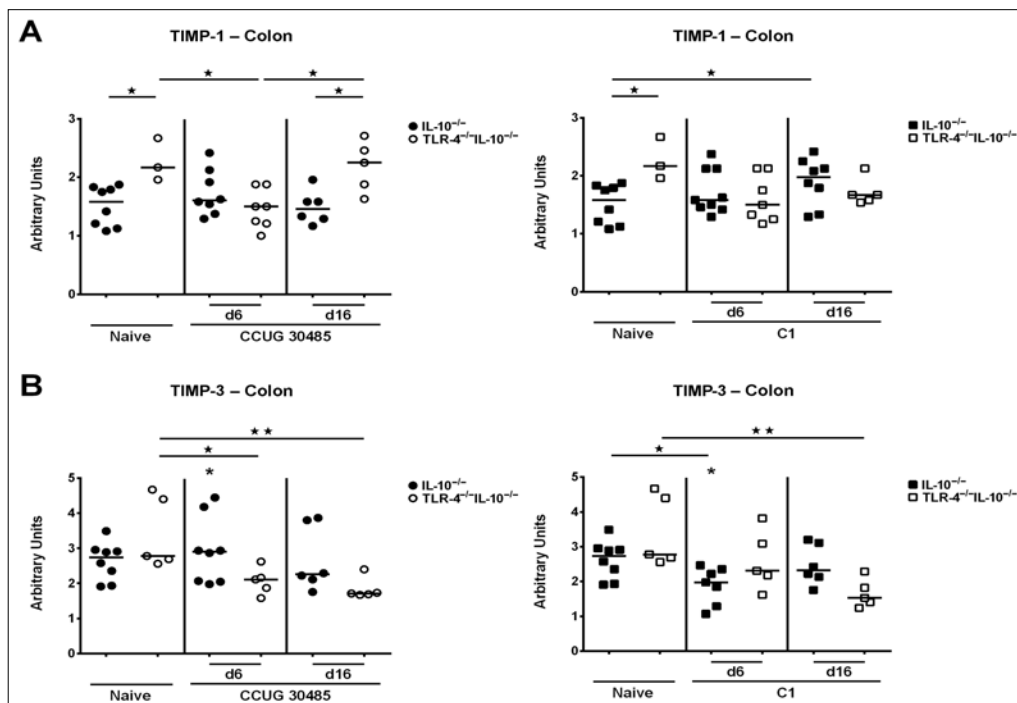


Fig. 5. Colonic expression of genes encoding TIMP-1 and TIMP-3 in gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (open symbols) and IL-10^{-/-} mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (circles; left panel) or strain C1 (squares; right panel). Uninfected mice served as negative control (naive). A) TIMP-1 and B) TIMP-3 mRNA levels were determined in *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 (**p* < 0.05; ***p* < 0.01). Data shown were pooled from three independent experiments

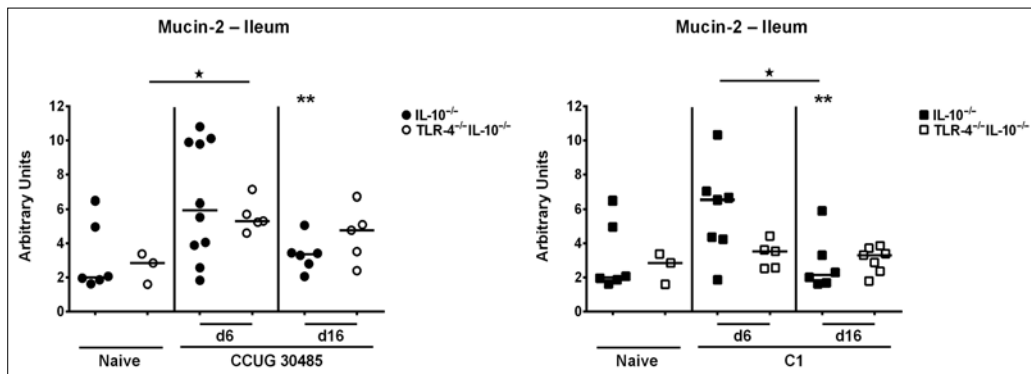


Fig. 6. Ileal expression of the mucin-2 gene in gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (open symbols) and IL-10^{-/-} mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (circles; left panel) or strain C1 (squares; right panel). Uninfected mice served as negative control (naive). Mucin-2 mRNA levels were determined in *ex vivo* ileal 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 (**p* < 0.05; ***p* < 0.01). Significant differences between *A. butzleri* strains are indicated by stars within the graph. Data shown were pooled from three independent experiments

Ileal expression of genes encoding defined immune mediators in *A. butzleri*-infected gnotobiotic IL-10^{-/-} mice lacking TLR-4

At day 6 p.i. with either strain, mRNA of both the pro-inflammatory cytokines IFN- γ and IL-1 β was upregulated in the ileum of IL-10^{-/-}, but not IL-10^{-/-} mice lacking TLR-4 (*p* < 0.05–0.001; Fig. 7A and D). This was also the case in ileal TNF expression at either time point upon CCUG 30485 strain infection and at day 16 following C1 strain infection (*p* < 0.01; Fig. 7B). Notably, basal TNF as well as IL-1 β mRNA levels were higher in naive TLR-4^{-/-} IL-10^{-/-} as compared to IL-10^{-/-} control mice (*p* < 0.05 and *p* < 0.01, respectively; Fig. 7B and D). Furthermore, ileal IL-17A mRNA expression levels increased at both days 6 and 16 post CCUG 30485 strain infection of mice irrespective of their genotype (*p* < 0.01–0.001; Fig. 7C, left panel). These increases, however, were less pronounced in TLR-4^{-/-} IL-10^{-/-} as compared to IL-10^{-/-} mice at day 6 p.i. (*p* < 0.01; Fig. 7C, left panel). Following C1 strain infection, ileal IL-17A mRNA levels did not differ from naive mice (Fig. 7C, right panel).

At either time point following CCUG 30485 strain infection, ileal IL-23p19 mRNA was upregulated in TLR-4^{-/-} IL-10^{-/-} mice (*p* < 0.01; Fig. 8A, left panel), whereas in IL-10^{-/-} controls, IL-23p19 mRNA levels had increased at day 6 following infection with either strain (*p* < 0.05–0.001; Fig. 8A). Upon *A. butzleri* CCUG 30485 strain infection at either time point, IL-22 mRNA levels were elevated in ilea of both IL-10^{-/-} and TLR-4^{-/-} IL-10^{-/-} mice, whereas expression levels were higher in double-deficient mice as compared to controls at day 16 p.i. (*p* < 0.01; Fig. 8B, left panel). At days 6 and 16 following C1 strain infection, ileal IL-22 mRNA levels were increased in TLR-4^{-/-} IL-10^{-/-} mice, but not in IL-10^{-/-} mice, at the earlier time point only (*p* < 0.05–0.01; Fig. 8B, right panel). Furthermore, IL-18 mRNA expression was upregulated 6 days following CCUG 30485 strain infection of TLR-4^{-/-} IL-10^{-/-} mice only (*p* < 0.05, Fig. 8C, left

panel). Notably, 6 and 16 days following *A. butzleri* infection with either strain, but also in the naive state, TLR-4^{-/-} IL-10^{-/-} mice displayed higher ileal IL-18 mRNA level as compared to respective IL-10^{-/-} control animals (*p* < 0.05–0.01; Fig. 8C). Hence, similar to the colon, distinct pro-inflammatory Th17 cytokines and mediators of the IL-22/IL-18 axis are TLR-4 dependently expressed in small intestines upon *A. butzleri* infection, depending on the respective strain and on the time course of infection.

Ileal expression of genes encoding matrix-degrading gelatinases in *A. butzleri*-infected gnotobiotic IL-10^{-/-} mice lacking TLR-4

Ileal MMP-2 mRNA levels were upregulated in mice of either genotype at day 6 following CCUG 30485 strain infection (*p* < 0.05–0.01; Fig. 9A, left panel). MMP-9 mRNA levels, however, increased in ilea of IL-10^{-/-} but not TLR-4^{-/-} IL-10^{-/-} mice until day 6 following infection with either strain (*p* < 0.01–0.001; Fig. 9B). Moreover, TIMP-1 mRNA was upregulated in ilea of TLR-4^{-/-} IL-10^{-/-} mice 6 days upon CCUG 30485 strain infection (*p* < 0.05; Fig. 10A, left panel). At both days 6 and 16 p.i., TLR-4^{-/-} IL-10^{-/-} mice infected with either strain exhibited higher TIMP-1 mRNA levels in the ileum as compared to respective IL-10^{-/-} control mice (*p* < 0.05–0.01; Fig. 10A). Small intestinal TIMP-3 gene expression, however, was virtually unaffected by *A. butzleri* infection (Fig. 10B). Thus, converse to the colon, ileal MMP-9, but not MMP-2 mRNA, is regulated in a TLR-4-dependent manner following murine *A. butzleri* infection.

In summary, these data indicate that in both the small as well as the large intestinal tract *A. butzleri* infection affects gene expression patterns of distinct regulatory and pro-inflammatory mediators via TLR-4. The TLR-4-dependent expression of respective molecules during murine *A. butzleri* infection is supported by our previous *C. jejuni* studies in different murine infection and inflammation models

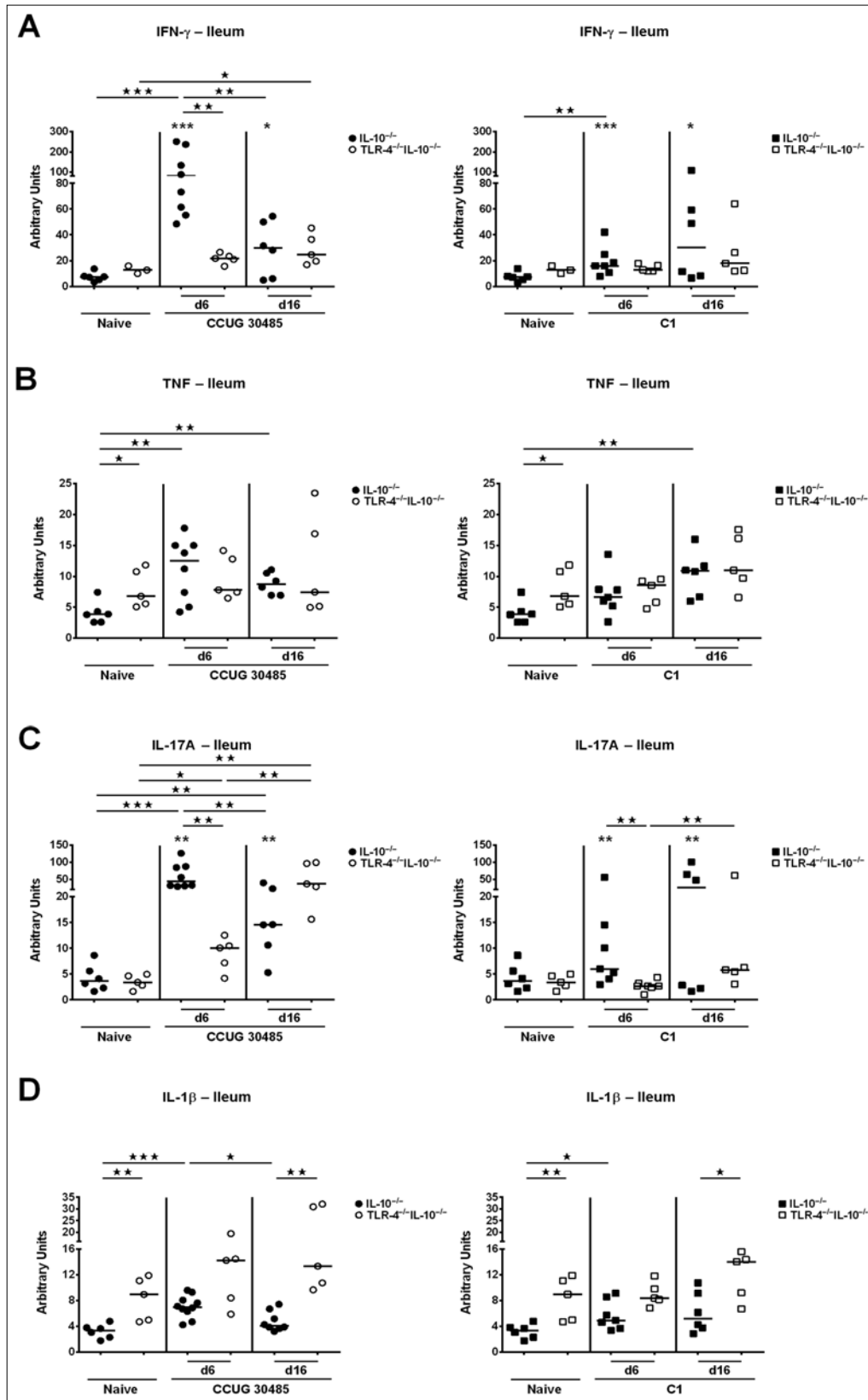


Fig. 7. Ileal expression of genes encoding pro-inflammatory cytokines in gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (open symbols) and IL-10^{-/-} mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (circles; left panel) or strain C1 (squares; right panel). Uninfected mice served as negative control (naive). A) IFN- γ , B) TNF, C) IL-17A, and D) IL-1 β mRNA levels were determined in *ex vivo* ileal 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 (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Significant differences between *A. butzleri* strains are indicated by stars within the graph. Data shown were pooled from three independent experiments

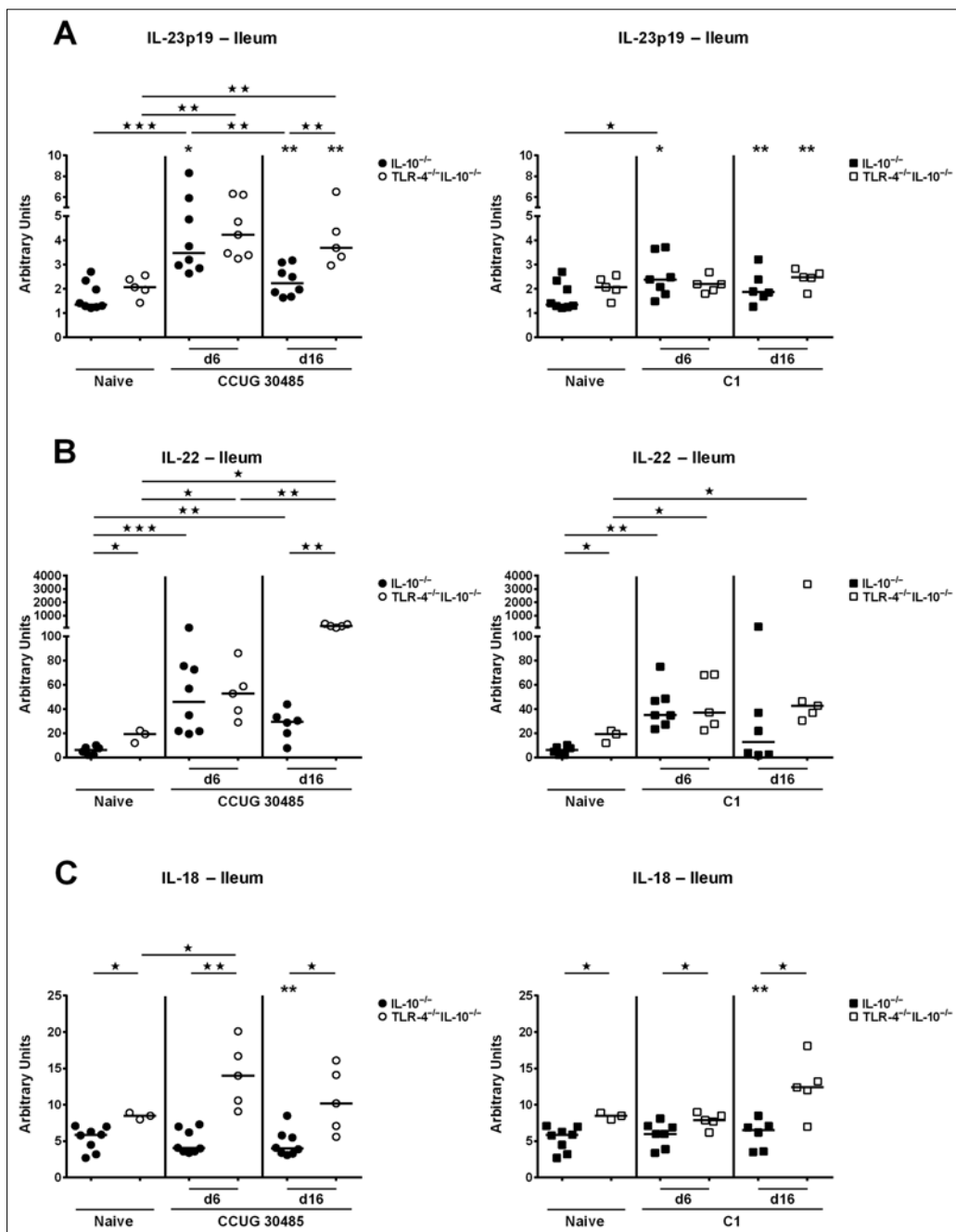


Fig. 8. Ileal expression of genes coding for IL-23p19, IL-22, and IL-18 in gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (open symbols) and IL-10^{-/-} mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (circles; left panel) or strain C1 (squares; right panel). Uninfected mice served as negative control (naive). A) IL-23p19, B) IL-22, and C) IL-18 mRNA levels were determined in *ex vivo* ileal 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 (**p* < 0.05; ***p* < 0.01; ****p* < 0.001). Significant differences between *A. butzleri* strains are indicated by stars within the graph. Data shown were pooled from three independent experiments

including conventionally colonized as well as gnotobiotic IL-10^{-/-} mice [32, 48, 49], gnotobiotic wildtype mice [43], conventional infant mice [50, 51], conventional mice fed viable *E. coli* or Western diet [49, 52], mice harboring a human microbiota [32], and conventional mice suffering from *Toxoplasma gondii*-induced acute ileitis [49, 53]. These studies all revealed that *C. jejuni*-induced intestinal and extra-intestinal sequelae are mediated by TLR-4 signalling of pathogenic LOS. However, neither *A. butzleri*

LPS nor LOS has been identified yet. Only in the halophilic bacterium *A. halophilus*, the structure of the carbohydrate backbone of LOS has been characterized [54].

Conclusion

In conclusion, TLR-4 is essentially involved in mediating pro-inflammatory immune responses in murine *Arcobac-*

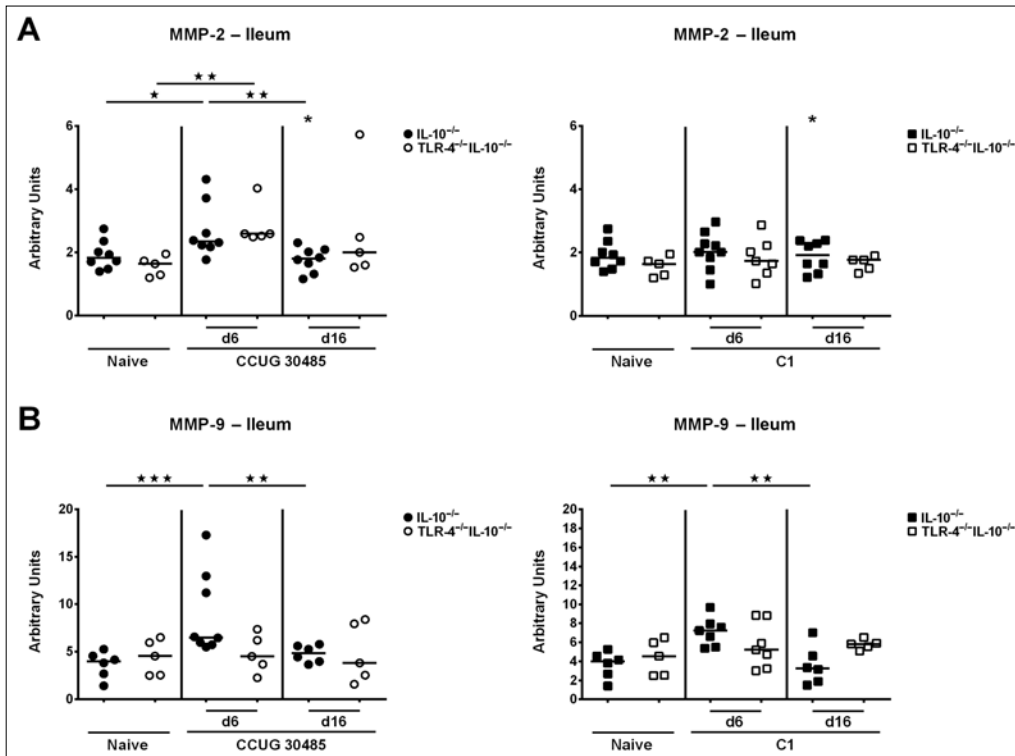


Fig. 9. Ileal expression of genes encoding MMP-2 and MMP-9 in gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (open symbols) and IL-10^{-/-} mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (circles; left panel) or strain C1 (squares; right panel). Uninfected mice served as negative control (naive). A) MMP-2 and B) MMP-9 mRNA levels were determined in *ex vivo* ileal 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 (**p* < 0.05; ***p* < 0.01; ****p* < 0.001). Significant differences between *A. butzleri* strains are indicated by stars within the graph. Data shown were pooled from three independent experiments

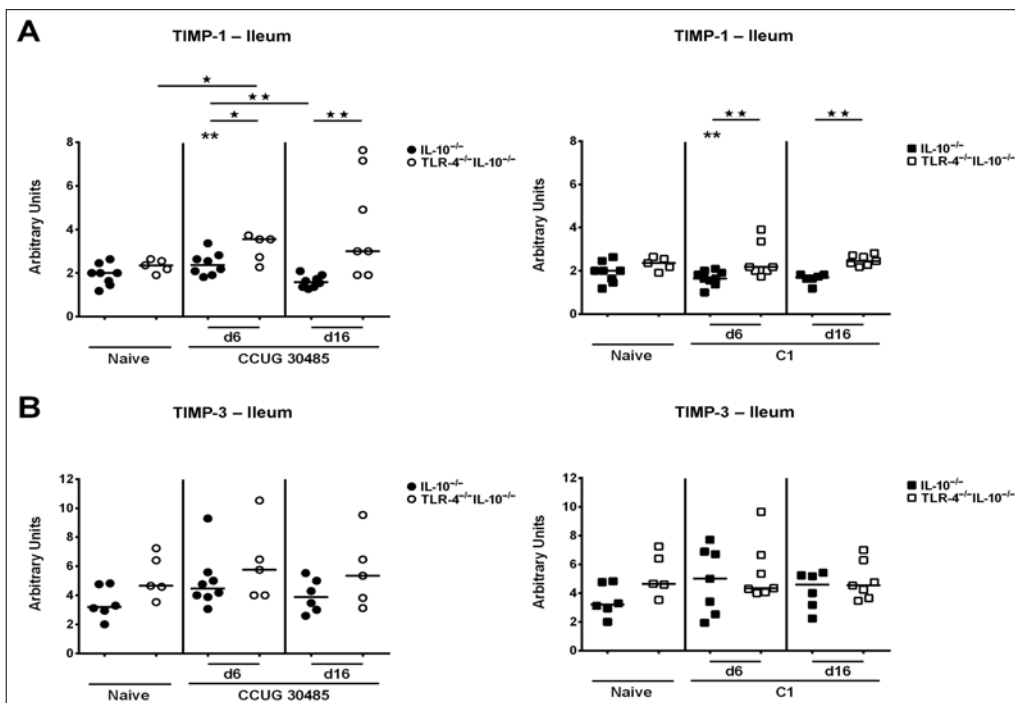


Fig. 10. Ileal TIMP-1 and TIMP-3 mRNA in gnotobiotic TLR-4^{-/-} IL-10^{-/-} mice following *A. butzleri* infection. Gnotobiotic TLR-4^{-/-} IL-10^{-/-} (open symbols) and IL-10^{-/-} mice (closed symbols) were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (circles; left panel) or strain C1 (squares; right panel). Uninfected mice served as negative control (naive). A) TIMP-1 and B) TIMP-3 mRNA levels were determined in *ex vivo* ileal 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 (**p* < 0.05; ***p* < 0.01). Significant differences between *A. butzleri* strains are indicated by stars within the graph. Data shown were pooled from three independent experiments

ter infection. Hence, bacterial LOS and/or LPS acts as a virulence factor in arcobacteriosis.

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Competing interests

The authors have declared that no competing interests exist.

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