INTESTINAL EXPRESSION OF GENES CODING INFLAMMATORY MEDIATORS AND GELATINASES DURING ARCOBACTER BUTZLEI INFECTION OF GNOTOBIOTIC IL-10 DEFICIENT MICE

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We have previously shown that Arcobacter butzleri induces intestinal, extra-intestinal, and systemic immune responses in perorally infected gnotobiotic IL-10−/− mice in a strain-dependent fashion. Here, we present a comprehensive survey of small and large intestinal expression profiles of inflammatory and regulatory mediators as well as of the matrix-degrading gelatinases MMP-2 and MMP-9 following murine A. butzleri infection. Gnotobiotic IL-10−/− mice were infected with A. butzleri strains CCUG 30485 or C1 of human and chicken origin, respectively. At day 6 following A. butzleri infection, mucin-2 mRNA, an integral part of the intestinal mucus layer, was downregulated in the colon, whereas TNF and IL-23p19 mRNA, an integral part of the intestinal mucus layer, was downregulated in the colon, whereas TNF and IL-23p19 mRNA were upregulated in the ileum. Furthermore, IFN-γ, IL-17A, IL-1β, and IL-22 mRNA were upregulated in both colonic and ileal ex vivo biopsies at day 6 post strain CCUG 30485 infection. These changes were accompanied by downregulated colonic MMP-9 levels, whereas both MMP-2 and MMP-9 mRNA were upregulated in the ileum. In conclusion, these data indicate that A. butzleri infection induces changes in the expression of genes involved in pro-inflammatory and regulatory immune responses as well as in tissue degradation.

Keywords: Arcobacter butzleri, Campylobacterales, IL-23/Th17 axis, IL-22/IL-18 axis, pro-inflammatory immune responses, intestinal tract, gnotobiotic IL-10−/− mouse infection model, gelatinases, host defence, mucosal immunology

Introduction

Motile, spiral-shaped gram-negative bacteria of the genus Arcobacter represent a taxonomical branch of the Campylobacteraceae family of the clades Campylobacterales. In the intestinal tract of animals, Arcobacter species are considered commensals [1], whereas, in humans, Arcobacter butzleri and Arcobacter cryaerophilus have been both classified as potential hazards by the International Commission on Microbiological Specifications for Food [2]. Given that routine diagnostic measures usually fail to reliably detect and identify Arcobacter, robust epidemiological data in humans are scarce [1]. In retrospective studies, however, Arcobacter spp. have been graded as the fourth most common species within the Campylobacterales clade isolated from diarrheal subjects. Both sporadic cases and outbreaks have been reported in humans [3, 4]. Patients acquire Arcobacter spp. predominantly via the peroral route upon ingestion of contaminated food or water and present with acute, self-limiting, gastroenteritis or prolonged watery diarrhea that can even persist for several weeks [3, 4]. So far, however, information regarding Arcobacter–host interaction has been limited due to scarcity of appropriate animal infection models. In order to explore mechanisms underlying A. butzleri infection in vivo, we have recently applied gnotobiotic (i.e., secondary abiotic) IL-10−/− mice generated by broad-spectrum antibiotic treatment. Initially, this infection model had been established for investigating Campylobacter jejuni infection in vivo [5]. Given the taxonomic relationship between A. butzleri with the Campylobacteraceae family, we infected gnotobiotic IL-10−/− mice with A. butzleri strains CCUG 30485 or C1, that had initially been isolated from a diseased patient and fresh chicken meat, respectively [6, 7]. Within 1 week, infected mice displayed small and large intestinal as well as extra-intestinal and systemic immune responses but did not exhibit clinical or macroscopic disease [8, 9].

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In the present study, we dissected the contribution of defined molecules to the observed local (i.e., intestinal) immune responses in more detail. We therefore surveyed the expression of genes encoding inflammatory and regulatory mediators as well as matrix-degrading metalloproteinases (MMP) such as gelatinases and mucin-2 within the colon and ileum during A. butzleri infection.

### 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. To overcome physiological colonization resistance and assure stable colonization, gnotobiotic (i.e., secondary abiotic) IL-10−/− mice with a deprived gastrointestinal microbiota were generated following broad-spectrum antibiotic treatment as described earlier [5, 10]. 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 [12]. Three days prior infection, the antibiotic cocktail was replaced by sterile tap water (ad libitum). Abiotic status of gnotobiotic mice was confirmed as described earlier [5, 10].

#### Arcobacter butzleri infection of mice

Four-month-old female gnotobiotic IL-10−/− mice were perorally infected with approximately 10⁷ 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) as described previously [13, 14]. Naive age-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 [6], whereas the C1 strain was isolated from fresh chicken meat [7]. 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 [5, 8, 9].

#### Sampling procedures

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

#### 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 of ≤0.05 were considered significant. Experiments were reproduced twice.

**Fig. 1.** Changes in colonic mucin-2 mRNA levels in gnotobiotic IL-10−/− mice following A. butzleri infection. Gnotobiotic IL-10−/− mice were generated by antibiotic treatment and perorally infected either with A. butzleri strain CCUG 30485 (circles) or strain C1 (squares). Uninfected mice served as negative control (naive; closed diamonds). Mucin-2 mRNA levels were determined in ex vivo colonic biopsies taken at days (d) 6 (closed symbols) or 16 (open symbols) postinfection. Medians (black bars), numbers of analyzed animals, 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.
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

Peroral A. butzleri infection of gnotobiotic IL-10−/− mice

In the present study, we performed for the first time a comprehensive survey of intestinal expression profiles of inflammatory mediators including T helper cell (Th) 17 cytokines important for host antimicrobial immunity and matrix-degrading gelatinases during A. butzleri infection. To address this, we infected gnotobiotic IL-10−/− mice [11] with the A. butzleri strains CCUG 30485 or C1 (10^9 CFU each) perorally by gavage [8, 9]. Mice were stably colonized by either strain with median intestinal loads of 10^8 CFU per gram feces until the end of the experiment at day 16 p.i. [8, 9]. Interestingly, even though mice did not display any overt clinical signs of enteric disease including wasting, diarrhea, or occurrence of blood in fecal samples at day 6 or day 16 p.i., distinct pro-inflammatory immune responses could be observed in small and large intestinal as well as extra-intestinal and systemic compartments [8, 9].

![Fig. 2. Colonic expression of genes coding for pro-inflammatory cytokines in gnotobiotic IL-10−/− mice following A. butzleri infection. Gnotobiotic IL-10−/− mice were generated by antibiotic treatment and perorally infected either with A. butzleri strain CCUG 30485 (circles) or strain C1 (squares). Uninfected mice served as negative control (naive; closed diamonds). A) IFN-γ, B) TNF, C) IL-17A, and D) IL-1β mRNA levels were determined in ex vivo colonic biopsies taken at days (d) 6 (closed symbols) or 16 (open symbols) postinfection. Medians (black bars), numbers of analyzed animals, and significance levels as determined by the Mann–Whitney U test are indicated (*p < 0.05; **p < 0.01; ***p < 0.001). Data shown were pooled from three independent experiments](image-url)
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Large intestinal gene expression of mucin-2, pro-inflammatory mediators, and gelatinases in \textit{A. butzleri}-infected gnotobiotic IL-10$^{-/-}$ mice

Within the mucus layer of the small and large intestinal tract, mucin-2 is expressed and contributes significantly to combating bacterial infection and, subsequently, maintenance of intestinal epithelial barrier function [16, 17]. We therefore investigated whether \textit{A. butzleri} infection affects mucin-2 expression in the colon of gnotobiotic IL-10$^{-/-}$ mice. At either time point of C1 strain infection and at day 6 following CCUG 30485 strain challenge, colonic mucin-2 expression was downregulated ($p < 0.05$; Fig. 1). Next, we determined expression of pro-inflammatory mediators in large intestines of \textit{A. butzleri}-infected mice. Whereas colonic TNF mRNA levels were rather comparable between infected and naive mice (Fig. 2B), IFN-$\gamma$, IL-17A, and IL-1$\beta$ mRNA expression were upregulated as early as 6 days p.i. ($p < 0.01$–0.001; Fig. 2A, C, and D) and remained elevated until day 16 p.i. in case of IL-17A ($p < 0.05$; Fig. 2C), but not of the remaining ones after infection with \textit{A. butzleri} strain CCUG 30485 (Fig. 2A and D). In addition, colonic IL-17A mRNA levels increased at day 16 following C1 strain infection ($p < 0.05$; Fig. 2C). Moreover, large intestinal expression of IL-23p19, a master regulator of mucosal immunity during intestinal infection and inflammation [18], did not change upon \textit{A. butzleri} infection (Fig. 3A), whereas colonic IL-22 was upregulated at day 6 following CCUG 30485 strain infection ($p < 0.001$; Fig. 3B), as observed for IFN-$\gamma$, IL-17A, and IL-1$\beta$. Colonic IL-18 mRNA levels, however, were increased at day 16, but not day 6, follow-
In the CCUG 30485 strain infection, and upregulated in C1 strain-infected mice at either time point ($p < 0.05$ – $0.01$; Fig. 3C). Given that matrix-degrading gelatinases are upregulated during intestinal inflammation upon infection with *Toxoplasma gondii* [15] and *C. jejuni* [19–21], we analyzed expression of the genes encoding MMP-2 and -9 following *A. butzleri* infection. Moreover, we determined large intestinal expression of the tissue inhibitors of metalloproteinases (TIMP) -1 and -3, that counteract gelatinase expression and function [22, 23]. Whereas colonic MMP-2 expression was virtually unchanged in *A. butzleri*-infected mice (Fig. 4A), MMP-9 was downregulated at day 6 following CCUG 30485 and C1 strain infection ($p < 0.01$ and $p < 0.05$, respectively; Fig. 4B). Of the analyzed TIMPs, only TIMP-1 mRNA was upregulated in colonic *ex vivo* biopsies at day 16 following *A. butzleri* C1 strain infection ($p < 0.05$; Fig. 5). Hence, large intestinal immune responses upon *A. butzleri* infection shown previously [8, 13] are
Fig. 6. Changes in ileal mucin-2 mRNA levels in gnotobiotic IL-10\(^{-}\)/− mice following A. butzleri infection. Gnotobiotic IL-10\(^{-}\)/− mice were generated by antibiotic treatment and perorally infected either with A. butzleri strain CCUG 30485 (circles) or strain C1 (squares). Uninfected mice served as negative control (naive; closed diamonds). Mucin-2 mRNA levels were determined in ex vivo ileal biopsies taken at days (d) 6 (closed symbols) or 16 (open symbols) postinfection. Medians (black bars), numbers of analyzed animals, and significance levels as determined by the Mann–Whitney \(U\) test are indicated (\(*\ p < 0.05\)). Data shown were pooled from three independent experiments.

Fig. 7. Ileal expression of genes coding for pro-inflammatory cytokines in gnotobiotic IL-10\(^{-}\)/− mice following A. butzleri infection. Gnotobiotic IL-10\(^{-}\)/− mice were generated by antibiotic treatment and perorally infected either with A. butzleri strain CCUG 30485 (circles) or strain C1 (squares). Uninfected mice served as negative control (naive; closed diamonds). A) IFN-\(\gamma\), B) TNF, C) IL-17A, and D) IL-1\(\beta\) mRNA levels were determined in ex vivo ileal biopsies taken at days (d) 6 (closed symbols) or 16 (open symbols) postinfection. Medians (black bars), numbers of analyzed animals, and significance levels as determined by the Mann–Whitney \(U\) test are indicated (\(*\ p < 0.05; \^{*}\ p < 0.01; \^{\star\star}\ p < 0.001\)). Data shown were pooled from three independent experiments.
associated with a strain-dependent expression pattern of mucus constituents, pro-inflammatory mediators including Th17 cytokines and their regulatory molecules as well as of matrix-degrading gelatinases.

Small intestinal gene expression of mucin-2, pro-inflammatory mediators, and gelatinases in A. butzleri-infected gnotobiotic IL-10−/− mice

We next surveyed respective mRNA levels within a different compartment of the gastrointestinal tract, namely, the distal small intestines of A. butzleri-infected gnotobiotic IL-10−/− mice. Ileal mucin-2 expression was rather unaffected by A. butzleri infection. At day 16 following C1 strain infection, however, mice displayed lower mucin-2 levels than at day 6 p.i. (p < 0.05; Fig. 6). At day 6 following infection with either A. butzleri strain, ileal IFN-γ mRNA expression was upregulated (p < 0.01–0.001; Fig. 7A), whereas TNF mRNA levels were higher at either time point of CCUG 30485 strain infection and additionally at day 16 following C1 strain infection (p < 0.05–0.01; Fig. 7B). Ileal IL-17A and IL-1β were both upregulated at day 6 and the former additionally at day 16 following CCUG 30485 strain infection (p < 0.01–0.001; Fig. 7C and D). Increased IL-23p19 and IL-22 mRNA levels could be detected in small intestines of A. butzleri CCUG 30485 strain-infected mice at day 6 and day 16 p.i. (p < 0.05–0.001; Fig. 8A and B) and the latter additionally at day 6 following C1 strain infection (p < 0.01; Fig. 8B), whereas ileal IL-18 mRNA expression remained virtually unchanged during infection with either strain (Fig. 8C).
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A. *butzleri* CCUG 30485 strain-induced upregulation of pro-inflammatory cytokines at day 6 p.i. was accompanied by elevated ileal MMP-2 and MMP-9 mRNA expression (*p* < 0.05–0.01; Fig. 9). As opposed to the large intestines, MMP-9 was upregulated in the distal small intestinal tract at day 6 following C1 strain infection but returned to basal levels thereafter (*p* < 0.01 and *p* < 0.05, respectively; Fig. 9B). Interestingly, ileal expression levels of TIMP-1 and TIMP-3 were rather comparable between infected and naive mice (Fig. 10). Only TIMP-1 mRNA levels of CCUG 30485 strain infected mice were higher at day 6 versus day 16 p.i. (*p* < 0.01; Fig. 10A). These data clearly indicate not only strain- and kinetic- but also tissue-dependent differences in expression patterns of mucus constituents, pro-inflammatory mediators including Th17 cytokines and their regulatory molecules as well as of matrix-degrading gelatinases.

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**Fig. 9.** Ileal expression of genes coding for MMP-2 and MMP-9 in gnotobiotic IL-10−/− mice following *A. butzleri* infection. Gnotobiotic IL-10−/− mice were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (circles) or strain C1 (squares). Uninfected mice served as negative control (naive; closed diamonds). A) MMP-2 and B) MMP-9 mRNA levels were determined in *ex vivo* ileal biopsies taken at days (d) 6 (closed symbols) or 16 (open symbols) postinfection. Medians (black bars), numbers of analyzed animals, 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. 10.** Ileal expression of genes coding for TIMP-1 and TIMP-3 in gnotobiotic IL-10−/− mice following *A. butzleri* infection. Gnotobiotic IL-10−/− mice were generated by antibiotic treatment and perorally infected either with *A. butzleri* strain CCUG 30485 (circles) or strain C1 (squares). Uninfected mice served as negative control (naive; closed diamonds). A) TIMP-1 and B) TIMP-3 mRNA levels were determined in *ex vivo* ileal biopsies taken at days (d) 6 (closed symbols) or 16 (open symbols) postinfection. Medians (black bars), numbers of analyzed animals, and significance levels as determined by the Mann–Whitney *U* test are indicated (**p** < 0.01). Data shown were pooled from three independent experiments.
Discussion

In the present study, we performed a survey of small and large intestinal gene expression levels of molecules that are involved in mediating host resistance against bacterial infections during murine A. butzleri infection. Upon peroral infection, pathogens are confronted with the mucus layer protecting the underlying mucosal epithelial tissue from damage and preserving epithelial barrier function [24]. Mucins including mucin-2 are secreted complex glycoproteins that give mucus its viscous consistency and act as first line defence against pathogens [24]. To date, however, no information regarding Arcobacter–mucin interactions is available. In our study, colonic mucin-2 mRNA was downregulated in mice infected with either strain at day 6 p.i. and additionally at day 16 following A. butzleri C1 strain infection. Mucins have further been shown to act as major chemoattractants for bacterial pathogens such as C. jejuni [25]. Upon binding, C. jejuni–mucin interaction results in reduced pathogenic growth and, besides other transcriptomic changes, in enhanced transcription of mucin-degrading enzymes in C. jejuni [26]. We could recently show that C. jejuni infection of conventionally colonized IL-10−/− mice was accompanied by a decrease in intestinal mucin-2 mRNA expression [27], which was also true for A. butzleri-infected gnotobiotic IL-10−/− animals as shown here. Notably, isolator raised germfree mice exhibited an extremely thin colonic mucus layer that upon exposure with bacterial products such as peptidoglycan or lipopolysaccharide could be quickly restored to levels observed in conventionally colonized mice [28]. Nevertheless, our data presented here need to be interpreted with caution given that we analyzed mRNA expression of mucin-2 but neither the translated protein nor the complex mucus layer.

Following stable intestinal colonization, A. butzleri-infected mice did not exhibit macroscopic disease but distinct pro-inflammatory sequelae that were not restricted to the small and large intestines, given that pronounced immune responses could be observed in extra-intestinal including systemic compartments [8, 9, 13, 14]. Both innate and adaptive immune responses upon C. jejuni infection were characterized by elevated expression of genes encoding Th17 cytokines in human intestinal ex vivo biopsies [29]. Accordingly, our results indicate that IFN-γ, IL-22, and IL-17A were upregulated in both murine colon and ileum after A. butzleri infection. This is well in line with our previous studies revealing colonic increases in respective mediators upon murine C. jejuni infection [19]. In C. jejuni-induced colitis, both T lymphocytes and innate lymphoid cells (ILCs) were sources for IFN-γ, IL-22, and IL-17A secretion in a time- and organ-specific fashion [30], whereas IFN-γ played a critical role particularly during the early phase of acute C. jejuni infection [29]. The triad IFN-γ, IL-22, and IL-17A exert potential bactericidal properties including enhanced β-defensin production, which has been shown an effective antimicrobial mechanism directed against C. jejuni [31] and presumably also against A. butzleri. Elevated expression of the gene for IL-17A is further associated with early neutrophil recruitment to sites of infection [32], and enhanced IL-17A and IFN-γ secretion with tissue damage upon C. jejuni infection [30]. Given that IL-23 is a known master regulator of mucosal immune responses upon intestinal infection and inflammation [18], we assessed intestinal IL-23p19 expression in A. butzleri-infected mice. Following A. butzleri CCUG 30485, but not C1 infection, ileal, but not colonic IL-23p19 mRNA levels, were upregulated. Hence, it is tempting to speculate that IL-23 might also act as a central regulator of immune responses during Arcobacter infections in a strain- and organ-specific fashion. The exact underlying mechanisms, however, remain to be elucidated.

In the intestinal tract, IL-22, as member of the IL-10 family, exerts a dichotomous mode of action in a tissue-specific manner. Whereas pro-inflammatory properties of IL-22 have been shown in the small intestines [15], IL-22 induced anti-inflammatory responses and tissue repair in the colon [33, 34]. Recently, we showed that colonic apoptosis and pro-inflammatory immune responses were accompanied by increased IL-22 but also IL-18 mRNA levels in the large intestines of C. jejuni-infected mice [19, 21]. These data support our results obtained from A. butzleri-infected mice presented here. In the colon, but not ileum, IL-18 was upregulated upon A. butzleri infection with either strain during both the early and late phase of infection, whereas colonic IL-22 mRNA increased until day 6 following CCUG 30485 strain infection. We could recently show that IL-22 induced the expression of IL-18 mRNA in intestinal epithelial cells following Citrobacter rodentium and T. gondii infection [35]. Conversely, IL-18 was able to amplify IL-22 production from ILCs and Th1 mediated intestinal inflammation [35]. Future studies need to unravel whether such a mutual regulation of IL-22 and IL-18 were also true for Arcobacter infection. Given that matrix-degrading enzymes such as gelatinases are upregulated upon intestinal inflammation [15, 36, 37], we determined intestinal expression levels of MMP-2 and MMP-9 and their endogenous inhibitors including TIMP-1 and TIMP-3 during murine Arcobacter infection. As early as 6 days following A. butzleri strain CCUG 30485 infection, both ileal MMP-2 and MMP-9 mRNA were upregulated, whereas, unexpectedly, MMP-9 was even downregulated in the large intestines at day 6 following infection with either strain. In our previous C. jejuni infection studies, MMP-2, but not MMP-9 mRNA, was upregulated in large intestines of infected infant mice, whereas mRNA levels of genes for both TIMP-1 and TIMP-3 were virtually unchanged similar to A. butzleri infection investigated here.

Conclusion

In conclusion, our data indicate that murine A. butzleri infection induces changes in the intestinal expression levels of genes encoding distinct pro-inflammatory, regulatory,
and matrix-degrading molecules essential for immunity and tissue turnover, respectively.

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**Competing interests**

The authors have declared that no competing interests exist.

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