Gut microbiota depletion is a pivotal prerequisite to warrant Campylobacter jejuni infection and induced inflammation in IL-10−/− mice used as acute campylobacteriosis model. We here assessed the impact of an 8-week antibiotic regimen of ampicillin, ciprofloxacin, imipenem, metronidazole, and vancomycin (ABx) as compared to ampicillin plus sulbactam (A/S) on gut microbiota depletion and immunopathological responses upon oral C. jejuni infection. Our obtained results revealed that both antibiotic regimens were comparably effective in depleting the murine gut microbiota facilitating similar pathogenic colonization alongside the gastrointestinal tract following oral infection. Irrespective of the preceding microbiota depletion regimen, mice were similarly compromised by acute C. jejuni induced enterocolitis as indicated by comparable clinical scores and macroscopic as well as microscopic sequelae such as colonic histopathology and apoptosis on day 6 post-infection. Furthermore, innate and adaptive immune cell responses in the large intestines were similar in both infected cohorts, which also held true for intestinal, extra-intestinal and even systemic secretion of pro-inflammatory cytokines such as TNF-α, IFN-γ, and IL-6. In conclusion, gut microbiota depletion in IL-10−/− mice by ampicillin plus sulbactam is sufficient to investigate both, C. jejuni infection and the immunopathological features of acute campylobacteriosis.

KEYWORDS
ampicillin plus sulbactam, broad-spectrum antibiotic treatment, differential gut microbiota depletion, enteropathogenic infection, Campylobacter jejuni, acute campylobacteriosis model, microbiota-depleted IL-10−/− mice, host-pathogen interaction

INTRODUCTION
Infections with the food-borne pathogen Campylobacter jejuni are progressively rising all around the globe and are among the most frequently reported causes of bacterial enteritis [1, 2]. After an incubation period of 2–5 days some infected individuals may complain about relatively mild intestinal symptoms whereas others suffer from acute campylobacteriosis characterized by fever, abdominal cramps, watery or even bloody diarrhea with mucous discharge [3, 4]. Microscopic analysis of human intestinal biopsies demonstrates that innate and adaptive immune cell populations such as neutrophils, macrophages, and monocytes as well as T lymphocytes and B cells, respectively, infiltrate the colonic mucosa and lamina propria. Histopathological observations include crypt abscesses, erosions and ulcerations as well as colonic epithelial cell apoptosis [5–8]. Diseased patients are usually treated symptomatically by rehydration and electrolyte substitution. However, antibiotic treatment with erythromycin and fluoroquinolones should be restricted to severely diseased individuals with immune-compromising comorbidities [3, 4]. In the majority of cases, the course of
campylobacteriosis is self-limiting. Nevertheless, on rare occasions post-infectious autoimmune disorders such as Guillain Barré syndrome, reactive arthritis or chronic inflammatory morbidities within the gastrointestinal tract might occur with a latency of weeks or even months post-infection (p.i.) [3, 4, 9]. Despite the clinical importance and serious socioeconomic burden of human campylobacteriosis, the molecular mechanisms underlying the interaction of the enteropathogens with the commensal gut microbiota and the immune system of the host are only incompletely understood. This has been mainly due to the fact that reliable in vivo including murine models of infection were scarce for a long time [10]. Conventional laboratory mice, for instance, exert a strong physiological colonization resistance determined by their specific commensal gut microbiota composition preventing the murine host from stable gastrointestinal colonization and infection with distinct enteropathogens including C. jejuni [11, 12]. Following microbiota depletion by broad-spectrum antibiotic pretreatment, however, mice can be readily infected following oral C. jejuni challenge [11, 13, 14]. One needs to take into consideration that when compared to humans, conventional wildtype mice are up to 10,000 times more resistant to Toll-like receptor-4 ligands such as lipopolysaccharide (LPS) and lipooligosaccharide (LOS) derived from the cell walls of Gram-negative bacteria [15]. Upon depletion of the anti-inflammatory cytokine gene interleukin-10 (il-10) both, LPS and LOS resistance can be overcome with the consequence that upon peroral C. jejuni infection, microbiota-depleted IL-10−/− mice can not only be colonized by the pathogen but also become infected and develop typical symptoms of acute campylobacteriosis such as wasting and bloody diarrhea within 6 days p.i. In frame of this immunopathological scenario, pronounced C. jejuni-LOS induced pro-inflammatory immune responses can be observed in intestinal as well as extra-intestinal including systemic compartments of infected microbiota-depleted IL-10−/− mice [8, 16].

So far, we successfully achieved gut microbiota depletion by orally treating mice with a quintuple antibiotic cocktail (ABx) consisting of ampicillin, ciprofloxacin, imipenem, vancomycin, and metronidazole for 8 weeks [13, 16]. When taking fecal samples from microbiota-depleted mice two days after withdrawal of the antibiotic cocktail and thus, immediately before C. jejuni infection, cultural analyses on solid and enriched media revealed negative results indicative for an absence of cultivable bacteria [13]. Strikingly, when applying culture-independent, molecular (i.e., 16S rRNA) based methods, commensal intestinal including eubacterial gene numbers detected in fecal samples did not differ from those measured in autoclaved food pellets proving successful gut microbiota depletion [17].

Nevertheless, one needs to take into consideration that a long-term broad-spectrum treatment with five different antibiotics might have adverse effects such as discomfort and diarrhea, for instance. Overall, from our experiences, challenged mice tolerated the treatment well and did not exert pronounced diarrheal symptoms. The first days after initiation of the antibiotic challenge, however, a slightly reduced daily drinking volume below average could be observed in some mice, most likely due to the well-known unpleasant metallic taste caused by metronidazole [18]. But the drinking volume normalized as soon as the mice got used to it within a few days. Most importantly, however, the increased risk of resistance development in members of the commensal gut bacterial ecosystem constitutes an adverse event of long-term antibiotic application [19].

This prompted us to apply a reductionistic approach for murine gut microbiota depletion by changing the antibiotic protocol from five different antibiotic compounds to ampicillin plus sulbactam (A/S) only. In our present study, we therefore tested if an 8-week course of oral A/S pretreatment could sufficiently i.) deplete the murine gut microbiota, and in turn, ii.) facilitate C. jejuni colonization and infection, and iii.) induce acute enterocolitis upon oral pathogen challenge of IL-10−/− mice.

**MATERIAL AND METHODS**

**Mice and antibiotic regimens for gut microbiota depletion**

IL-10−/− mice (C57BL/6 background) were bred and maintained in the Forschungsinstitute für Experimentelle Medizin, Charité – Universitätsmedizin Berlin, Germany. Mice were housed in cages including filter tops within an experimental semi-barrier under standard conditions (i.e., 22–24 °C room temperature, 55 ± 15% humidity, 12 h light/12 h dark cycle) and had free access to autoclaved water (ad libitum) and standard chow (food pellets: ssniff R/M-H, V1534-300, Sniff, Soest, Germany). To eradicate the commensal gut microbiota, 3-week-old female and male litter mate IL-10−/− mice were subjected to a quintuple antibiotic cocktail as described earlier [13, 20]. Briefly, mice were transferred to sterile cages (maximum of 3–4 animals per cage) and received an antibiotic cocktail for 8 weeks by adding ampicillin (1 g L−1; Dr. Friedrich Eberth Arzneimittel, Ursensollen, Germany), vancomycin (500 mg L−1; Hikma Pharmaceuticals, London, UK), ciprofloxacin (200 mg L−1; Fresenius Kabi, Bad Homburg, Germany), imipenem (250 mg L−1; Fresenius Kabi) and metronidazole (1 g L−1; B. Braun, Melsungen, Germany) to the drinking water (ad libitum). Another cohort of mice received ampicillin plus sulbactam only (2 g L−1; Dr. Friedrich Eberth Arzneimittel, Ursensollen, Germany). Two days before C. jejuni infection, the animals received autoclaved tap water to assure antibiotic washout.

**Gut microbiota analyses**

Cultural analyses of fecal samples were performed as described previously [13, 20]. For molecular quantification of intestinal microbiota including fastidious and uncultivable bacteria, total DNA extracted from fecal samples was subjected to quantitative real time (qRT)-PCR analysis of the
16S rRNA genes as described previously [20]. Briefly, DNA contents in fecal extracts were determined using Quant-iT PicoGreen reagent (Invitrogen, Paisley, UK) and adjusted to 1 ng per μL. Then, abundance of the main bacterial groups of murine intestinal microbiota was assessed by qRT-PCR with group-specific 16S rRNA gene primers (Tib MolBiol, Berlin, Germany) as described [21]. The number of 16S rRNA gene copies of individual gut bacterial groups per μg DNA was calculated for each sample and frequencies of respective bacterial groups were proportionally compared.

C. jejuni infection

C. jejuni strain 81-176 was thawed from frozen stocks and grown on Columbia agar (with 5% sheep blood) and selective karmali agar plates (both from Oxoid, Wesel, Germany). Age- and sex-matched microbiota-depleted IL-10-/- mice (4-month-old littermates) were infected perorally with 10^9 colony forming units (CFU) of the pathogen in 0.3 mL sterile phosphate-buffered saline (PBS, Thermo Fisher Scientific, Waltham, MA, USA) on days 0 and 1 by gavage.

Gastrointestinal C. jejuni loads

Six days following C. jejuni infection, the pathogen loads were determined in samples from the stomach, duodenum, ileum, and colon by culture as described previously [13, 22]. In brief, intraluminal gastrointestinal samples were homogenized in sterile PBS (Thermo Fisher Scientific, Waltham, MA, USA) with a sterile pestle and serial dilutions plated onto karmali agar (Oxoid, Wesel, Germany) and grown on Columbia agar (with 5% sheep blood) and selective karmali agar plates (both from Oxoid, Wesel, Germany). The detection limit of viable pathogens was 100 CFU per g.

Clinical conditions

The clinical outcome in infected mice was quantitatively surveyed by using a cumulative clinical score (maximum 12 points), addressing the abundance of blood in feces (0: no blood; 2: microscopic detection of blood by the Guajac method using Haemoccult, Beckman Coulter/PCD, Kreffield, Germany; 4: macroscopic blood visible), the stool consistency (0: formed feces; 2: pasty feces; 4: liquid feces) and the clinical aspect (i.e., wasting symptoms; 0: normal; 1: ruffled fur; 2: less locomotion; 3: isolation; 4: severely compromised locomotion, pre-final aspect) as described earlier [23].

Sampling procedures

On day 6 p.i., mice were sacrificed by CO2 asphyxiation. Ex vivo biopsies from mesenteric lymph nodes (MLN), the colon, liver, and kidneys, as well as luminal samples from stomach, duodenum, ileum, and colon were derived under aseptic conditions. From each mouse, colonic samples were collected in parallel for subsequent microbiological and immunohistopathological analyses.

Histopathology

Histopathological analyses were performed in colonic ex vivo biopsies that had been immediately fixed in 5% formalin and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (H&E), by light microscopy (100 × magnification), and histopathological changes in the large intestines quantitatively assessed with histopathological scores [24]: Score 1, minimal inflammatory cell infiltrates in the mucosa with intact epithelium. Score 2, mild inflammatory cell infiltrates in the mucosa and submucosa with mild hyperplasia and mild goblet cell loss. Score 3, moderate inflammatory cell infiltrates in the mucosa with moderate goblet cell loss. Score 4, marked inflammatory cell infiltration into the mucosa and submucosa with marked goblet cell loss, multiple crypt abscesses, and crypt loss.

In situ immunohistochemistry

Quantitative in situ immunohistochemical analyses were performed in colonic ex vivo biopsies following immediate fixation in 5% formalin and embedding in paraffin as reported previously [21, 25]. In brief, to detect apoptotic epithelial cells, macrophages and monocytes, neutrophils, T lymphocytes, regulatory T cells, and B lymphocytes, colonic paraffin sections (5 μm) were stained with primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, Beverly, MA, USA; 1:200), F4/80 (no. 14-4801, clone BM8, eBioscience, San Diego, CA, USA; 1:50), MPO7 (No. A0398, Dako, Glostrup, Denmark; 1:100), CD3 (no. N1580, Dako, Glostrup, Denmark; 1:10), FOXP3 (clone FJK-165, no. 14-5773, eBioscience, San Diego, CA, USA; 1:200) and B220 (no. 14-0452-81, eBioscience, San Diego, CA, USA; 1:100), respectively. Positively stained cells were quantitated by a blinded independent investigator applying light microscopy. The average number of respective positively stained cells in each sample was determined within at least six high power fields (HPF, 0.287 mm²; 400 × magnification).

Pro-inflammatory mediator secretion

Intestinal ex vivo biopsies collected from MLN (3 nodes) and from the colon (longitudinally cut strips of approximately 1 cm²), washed in PBS (Thermo Fisher Scientific, Waltham, MA, USA) were transferred to 24-flat-bottom well culture plates (Thermo Fisher Scientific, Waltham, MA, USA) containing 500 mL serum-free RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with penicillin (100 μg mL⁻¹); Biochrom, Berlin, Germany) and streptomycin (100 μg mL⁻¹; Biochrom, Berlin, Germany). After an 18-h incubation period at 37 °C, respective culture supernatants and serum samples were tested for tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and interleukin-6 (IL-6) by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences, Heidelberg, Germany) on a BD FACSCanto II flow cytometer (BD Biosciences, Heidelberg, Germany). Nitric oxide concentrations were determined by the Griess reaction as stated earlier [20].
Statistical analyses

Medians and significance levels were calculated using GraphPad Prism (version 8; San Diego, CA, USA). Normalization of data was assessed by the Anderson-Darling test. The Student’s t-test and Mann-Whitney test were used for pairwise comparisons of normally and not normally distributed data, respectively. For multiple comparisons, the one-sided ANOVA with Tukey post-correction (for normally distributed data) and the Kruskal-Wallis test with Dunn’s post-correction (for not normally distributed data) were applied. Two-sided probability (p) values ≤0.05 were considered significant. Data were pooled from two independent experiments.

Ethics statement

All animal experiments were carried out according to the European animal welfare guidelines (2010/63/EU) following approval by the commission for animal experiments (“Landesamt für Gesundheit und Soziales”, LaGeSo, Berlin; registration numbers G0173/07 and G0104/19). The clinical conditions of mice were monitored daily.

Fig. 1. Quantification of microbial communities in conventional mice, differentially microbiota-depleted mice and autoclaved food pellets. Conventionally colonized IL-10−/− mice (SPF; diamonds) were subjected to a quintuple antibiotic cocktail (ABx; squares) or to ampicillin plus sulbactam only (A/S: circles) via the drinking water (ad libitum) for eight weeks in order to deplete the murine commensal gut microbiota. Two days after replacement of the antibiotic compounds by autoclaved tap water, gut microbial communities were assessed by qRT-PCR amplifying variable regions of the bacterial 16S rRNA genes as described (expressed as copy numbers per ng DNA) and compared to those detected in autoclaved food pellets (triangles). Total sample sizes (in parentheses), medians (black bars) and significance levels (p values) determined by the Kruskal-Wallis test and Dunn’s post-correction are indicated. Data were pooled from two independent experiments.
RESULTS AND DISCUSSION

Detection of gut microbiobial communities in conventionally colonized and differentially microbiota-depleted mice as compared to autoclaved food pellets

We first surveyed to what extent the commensal gut microbiota could be depleted in conventional IL-10−/− mice following an 8-week course of treatment with a broad-spectrum antibiotic cocktail consisting of five different antibiotic compounds (ABx) as compared to ampicillin plus sulbactam (A/S) only. Therefore, fecal samples were collected two days after withdrawal of respective antibiotic regimen and replacement by autoclaved tap water, hence, constituting the day of infection when applying our C. jejuni induced campylobacteriosis model. In addition, fecal samples from conventionally colonized (specific pathogen free, SPF) IL-10−/− mice and autoclaved food pellets were used as positive and negative control samples, respectively. We then performed a comprehensive culture-independent gut microbial survey by applying qRT-PCR based on amplification of 16S rRNA gene fragments specific for individual gut bacterial groups. Remarkably, the obtained results revealed that the quintuple ABx regimen and the A/S mono-treatment were comparably effective and depleted the commensal gut microbiota (p < 0.05–0.001 versus SPF; Fig. 1) as indicated by comparable gene numbers of enterobacteria, enterococci, lactobacilli, bifidobacteria, Bacteroides/Prevotella species, Clostridium cocoides group, Clostridium leptum group, and Mouse Intestinal Bacteroides (not significant (n.s.); Fig. 1). Strikingly, respective gene copies did not differ from those detected in autoclaved food pellets indicative for a virtually complete gut microbiota depletion. Of note, all autoclaved food pellets and fecal samples derived from both antibiotic cohorts were culture-negative (data not shown). Hence, the A/S mono-treatment and the quintuple ABx regimens were comparably effective in depleting the commensal murine gut microbiota completely.

Gastrointestinal pathogen loads following C. jejuni infection of differentially microbiota-depleted IL-10−/− mice

Two days after cessation of the antibiotic regimens, microbiota-depleted IL-10−/− mice were perorally infected with 10⁹ viable C. jejuni cells (strain 81–176) by gavage on days 0 and 1. Upon necropsy on day 6 p.i., the pathogenic burdens were quantitatively assessed in distinct parts of the gastrointestinal tract. Our cultural analyses revealed comparable C. jejuni counts in the stomach, duodenum, ileum, and colon of ABx as compared to A/S pretreated mice (n.s.; Fig. 2), with increasing luminal numbers from proximal to distal intestinal compartments. Hence, following oral enteropathogenic infection both antibiotic microbiota depletion regimens facilitated C. jejuni colonization alongside the gastrointestinal tract to a comparable extent.

Clinical Outcomes (d6 p.i.)

Fig. 2. Gastrointestinal pathogen loads following C. jejuni infection of differentially microbiota-depleted IL-10−/− mice. Conventionally colonized IL-10−/− mice were subjected to a quintuple antibiotic cocktail (ABx; squares) or to ampicillin plus sulbactam only (A/S; circles) via the drinking water (ad libitum) for eight weeks, in order to deplete the murine commensal gut microbiota and facilitate C. jejuni infection. Two days after replacement of the antibiotic compounds by autoclaved tap water, microbiota-depleted mice were perorally challenged with C. jejuni strain 81-176 on days 0 and 1 (grey symbols). On day 6 post-infection, the C. jejuni loads were assessed in luminal samples taken from distinct gastrointestinal parts (as indicated) by culture and expressed as colony forming units per gram (CFU g⁻¹). Medians (black bars) and the number of total numbers of analyzed animals (in parentheses) are given. Data were pooled from two independent experiments.
Clinical outcome following C. jejuni infection of differentially microbiota-depleted IL-10-/- mice

We further surveyed the clinical outcome of C. jejuni infection in mice from each respective microbiota depletion cohort. On day 6 p.i., mice from the ABx and A/S groups were both severely suffering from acute enterocolitis as indicated by similarly high scores for abundance of fecal blood, diarrhea, wasting symptoms, and the overall clinical conditions (n.s.; Fig. 3). Hence, irrespective of the preceding

Fig. 4. Macroscopic and microscopic inflammatory changes following C. jejuni infection of differentially microbiota-depleted IL-10-/- mice. Conventional IL-10-/- mice were subjected to a quintuple antibiotic cocktail (ABx, squares) or to ampicillin plus sulbactam only (A/S; circles) via the drinking water (ad libitum) for eight weeks in order to deplete the murine commensal gut microbiota. Two days after replacement of the antibiotic compounds by autoclaved tap water, differentially microbiota-depleted mice were perorally infected with C. jejuni strain 81-176 on days 0 and 1 (grey symbols). On day 6 post-infection, (A) the colonic lengths were measured with a ruler (in cm) and the (B) histopathological changes were quantified in hematoxylin and eosin (H&E) stained colonic paraffin sections by using defined histopathological scores (see methods). In addition, (C) the average numbers of apoptotic colonic epithelial cells were assessed microscopically from six high power fields (HPF, 400 × magnification) per animal in paraffin sections positive for cleaved caspase-3 (Casp3*). Naïve mice (open symbols) served as uninfected control animals. Medians (black bars), significance levels (p values) as determined by the Mann-Whitney U test and the total number of analyzed mice (in parentheses) are given. Data were pooled from two independent experiments.

Fig. 5. Large intestinal innate and adaptive immune cell responses following C. jejuni infection of differentially microbiota-depleted IL-10-/- mice. Conventional IL-10-/- mice were subjected to a quintuple antibiotic cocktail (ABx, squares) or to ampicillin plus sulbactam only (A/S; circles) via the drinking water (ad libitum) for eight weeks in order to deplete the murine commensal gut microbiota. Two days after replacement of the antibiotic compounds by autoclaved tap water, differentially microbiota-depleted mice were perorally infected with C. jejuni strain 81-176 on days 0 and 1 (grey symbols). On day 6 post-infection, the average numbers of (A) macrophages and monocytes (F4/80*), (B) neutrophils (MPO7*), (C) T lymphocytes (CD3*), (D) regulatory T cells (FOXP3*) and (E) B lymphocytes (B220*) per animal of were determined in immunohistochemically stained colonic paraffin sections from six high power fields (HPF, 400 × magnification). Naïve mice (open symbols) served as uninfected control animals. Medians (black bars), significance levels (p values) as determined by the Mann-Whitney U test and the total number of analyzed mice (in parentheses) are given. Data were pooled from two independent experiments.
gut microbiota depletion regimen, IL-10−/− mice were similarly compromised by acute enterocolitis upon oral C. jejuni infection.

**Macroscopic and microscopic inflammatory changes following C. jejuni infection of differentially microbiota-depleted IL-10−/− mice**

Since intestinal inflammation is accompanied by shrinkage of the affected intestinal compartment [20, 23], we measured the colonic lengths upon necropsy. In fact, C. jejuni infection was associated with shorter colonic lengths (p < 0.001 versus naive; Fig. 4A), irrespective of the preceding microbiota depletion regimen. We then quantitatively surveyed microscopic sequelae of infection and assessed to a comparable extent markedly increased histopathological scores in paraffin sections of colonic ex vivo biopsies derived from mice of the ABx and A/S groups on day 6 p.i. (p < 0.001 versus naive; Fig. 4B). This was also the case when counting colonic epithelial cells that were positive for cleaved caspase3 as assessed in immunohistochemically stained colonic paraffin sections (p < 0.001 versus naive; Fig. 4C) indicative for pronounced cell apoptosis. Hence, the preceding gut microbiota depletion regimen did neither affect macroscopic nor microscopic sequelae in C. jejuni infected IL-10−/− mice.

**Large intestinal innate and adaptive immune cell responses following C. jejuni infection of differentially microbiota-depleted IL-10−/− mice**

We then tested for innate and adaptive immune responses in the large intestines following C. jejuni challenge and
performed immunohistochemical stainings of colonic paraffin section with antibodies directed against distinct immune cell subsets. On day 6 following infection of mice from the ABx and A/S groups, numbers of innate immune cells such as macrophages, monocytes and neutrophils were elevated \((p < 0.001 \text{ versus naive; Fig. 5A,B})\), whereas macrophages and monocytes were slightly less abundant in the colonic mucosa and lamina propria of A/S as compared to ABx mice on day 6 p.i. \((p < 0.05; \text{ Fig. 5A})\). When quantifying defined adaptive immune cell populations, *C. jejuni* induced increases in large intestinal T lymphocytes, regulatory T cells, and B lymphocytes were comparable in mice from both microbiota depletion cohorts \((p < 0.001 \text{ versus naive; Fig. 5C,D,E})\). Hence, large intestinal immune cell responses were similar following *C. jejuni* infection of microbiota-depleted IL-10\(^{-/-}\) mice from the ABx and A/S groups.

**Intestinal pro-inflammatory mediator secretion following *C. jejuni* infection of differentially microbiota-depleted IL-10\(^{-/-}\) mice**

Next, we measured pro-inflammatory mediators in distinct intestinal compartments. *C. jejuni* infection was accompanied by elevated colonic TNF-\(\alpha\), IFN-\(\gamma\), IL-6, and nitric oxide concentrations \((p < 0.05–0.001; \text{ Fig. 6})\). Except for higher nitric oxide concentrations in the colons of A/S as compared to ABx mice \((p < 0.05; \text{ Fig. 6D})\), increases in the remaining pro-inflammatory cytokines were comparable in both microbiota depleted cohorts on day 6 p.i. (n.s.; Fig. 6A,B,C) with no differences between both cohorts. When assessing respective pro-inflammatory mediators in MLN draining the infected and inflamed intestines (Fig. 7), similarly increased IFN-\(\gamma\) and IL-6 concentrations were measured on day 6 p.i., \((p < 0.001 \text{ and } p < 0.05 \text{ versus naive, Fig. 7})\).
respectively; Fig. 7B,C), whereas TNF-α and nitric oxide concentrations were even higher in MLN derived from A/S versus ABx mice \((p < 0.01; \text{Fig. 7A,D})\). Hence, \textit{C. jejuni} infection of mice from both microbiota depletion regimens resulted in enhanced pro-inflammatory mediator secretion in distinct intestinal compartments.

**Pro-inflammatory cytokine secretion in distinct extra-intestinal and systemic compartments following \textit{C. jejuni} infection of differentially microbiota-depleted IL-10⁻/⁻ mice**

We further tested potential effects of preceding microbiota-depleting regimens on \textit{C. jejuni} induced pro-inflammatory cytokine secretion in extra-intestinal including systemic compartments. On day 6 following infection, increased TNF-α and IFN-γ concentrations could be assessed in the livers of mice from both ABx and A/S cohorts \((p < 0.05–0.001 \text{ versus naive; Fig. 8A,B})\), with higher hepatic TNF-α levels in the latter versus the former \((p < 0.05; \text{Fig. 8A})\).

Upon \textit{C. jejuni} infection of mice from both microbiota depletion groups, comparably increased IFN-γ \((p < 0.001 \text{ versus naive; Fig. 8A,B})\) as opposed to TNF-α concentrations \((\text{n.s. versus naive; Fig. 8C})\) could be measured in the kidneys. In addition, pro-inflammatory cytokine secretion upon \textit{C. jejuni} challenge was similarly enhanced systemically as indicated by comparably increased TNF-α, IFN-γ, and IL-6 concentrations measured in serum samples from mice of ABx and A/S mice on day 6 p.i. \((\text{n.s.}; p < 0.01–0.001 \text{ versus naive; Fig. 9})\), with higher IL-6 levels in the latter versus the

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**Fig. 8.** Pro-inflammatory cytokine secretion in distinct extra-intestinal compartments following \textit{C. jejuni} infection of differentially microbiota-depleted IL-10⁻/⁻ mice. Conventional IL-10⁻/⁻ mice were subjected to a quintuple antibiotic cocktail (ABx, squares) or to ampicillin plus sulbactam only (A/S; circles) via the drinking water \((\text{ad libitum})\) for eight weeks in order to deplete the murine commensal gut microbiota. Two days after replacement of the antibiotic compounds by autoclaved tap water, differentially microbiota-depleted mice were perorally infected with \textit{C. jejuni} strain 81-176 on days 0 and 1 (grey symbols). On day 6 post-infection, pro-inflammatory cytokines such as (A,C) TNF-α and (B,D) IFN-γ were measured \textit{ex vivo} biopsies derived from the liver (A,B) and the kidneys (C,D). Naive mice (open symbols) served as uninfected control animals. Medians (black bars), significance levels \((p \text{ values})\) as determined by the Mann-Whitney \(U\) test and the total number of analyzed mice \((\text{in parentheses})\) are given. Data were pooled from two independent experiments.
former \((p < 0.001; \text{Fig. 9C})\). Hence, irrespective of the preceding microbiota depleting regimens \textit{C. jejuni} infection of IL-10-/- mice resulted in enhanced extra-intestinal including systemic pro-inflammatory cytokine secretion.

**SUMMARY AND CONCLUSION**

Results indicate that the 8-week course of pretreatment of IL-10-/- mice with A/S alone and a quintuple antibiotic regimen consisting of ampicillin, ciprofloxacin, imipenem, metronidazole, and vancomycin (ABx) were comparably effective in gut microbiota depletion enabling \textit{C. jejuni} colonization. Remarkably, both antibiotic regimens resulted in the absence of not only cultivable, but also of fastidious and even uncultivable bacteria as indicated by the fact that bacterial 16S rRNA gene numbers in fecal samples of treated mice were as low as in autoclaved food pellets. Furthermore, irrespective of the antibiotic pretreatment regimen, \textit{C. jejuni} could stably establish within the gastrointestinal tract upon oral infection of microbiota-depleted IL-10-/- mice.

On day 6 p.i., mice from both antibiotic pretreatment cohort were similarly compromised and suffered from acute \textit{C. jejuni} induced enterocolitis as indicated by comparable clinical scores, and macroscopic as well as microscopic sequelae such as large intestinal histopathology and colonic epithelial cell apoptosis. Most importantly, \textit{C. jejuni} induced accumulation of innate and adaptive immune cells such as macrophages, monocytes, neutrophils, T lymphocytes, regulatory T cells, and B lymphocytes in the large intestinal mucosa and lamina propria was similar in both infected cohorts.

This also held true for intestinal, extra-intestinal and even systemic secretion of pro-inflammatory cytokines such as TNF-\(\alpha\), IFN-\(\gamma\), and IL-6.

Importantly, mice tolerated the A/S drinking solution even better than the ABx cocktail given that some of the mice from the latter group disliked the solution due to the unpleasant metallic taste caused my metronidazole.

From the economical point of view, application of A/S instead of the ABx cocktail has the advantage of lower costs and the A/S regimen more resembles the clinical situation in human medicine than the quintuple ABx regimen.

Finally, replacing ABx by A/S lowers the risk of resistance development against multiple antibiotics in the bacterial gut microbiota and reduces the environmental contamination.

In conclusion, our obtained data support the advantage of our reductionistic approach in replacing ABx by A/S pretreatment in generating microbiota-depleted mice with more pros than cons. Hence, sometimes less is even more.

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\textbf{Authors’ contributions:} MMH: Designed and performed experiments, analyzed data, wrote the paper.

SM: Performed experiments, analyzed data, co-wrote the paper.

RB: Performed experiments, analyzed data, edited the paper.

SB: Provided advice in experimental design, critically discussed results, co-wrote the paper.
Conflict of interests: SB and MMH are Editorial Board members.

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