THE ROLE OF GELATINASES IN *CAMPYLOBACTER JEJUNI* INFECTION OF GNOTOBIOTIC MICE

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Matrix metalloproteinases (MMP)-2 and -9 (also referred to gelatinases-A and -B, respectively) are upregulated in the inflamed gut of mice and men. We recently demonstrated that synthetic gelatinase blockage reduced large intestinal pro-inflammatory immune responses and apoptosis following murine *Campylobacter (C.) jejuni* infection. In order to address which gelatinase mediates *C. jejuni*-induced immune responses, gnotobiotic MMP-2$^{-/-}$, MMP-9$^{-/-}$, and wildtype (WT) mice were generated by broadspectrum antibiotic treatment and perorally infected with *C. jejuni* strain 81-176. The pathogen stably colonized the murine intestinal tract irrespective of the genotype but did not translocate to extra-intestinal compartments. At days 8 and 14 postinfection (p.i.), less pronounced colonic histopathological changes were observed in infected MMP-2$^{-/-}$ mice, less distinct epithelial apoptosis, but more epithelial proliferation in both MMP-2$^{-/-}$ and MMP-9$^{-/-}$ mice, as compared to WT controls. Reduced immune responses in gelatinase-deficient mice were characterized by lower numbers of effector as well as innate and adaptive immune cells within the colonic mucosa and lamina propria. The expression of IL-22, IL-18, IL-17A, and IL-1β mRNA was higher in the colon of MMP-2$^{-/-}$ as compared to WT mice. In conclusion, both MMP-2 and MMP-9 are differentially involved in mediating *C. jejuni*-induced intestinal immunopathology.

Keywords: *Campylobacter jejuni, in vivo* infection model, gnotobiotic mice, matrix metalloproteinases, gelatinases, pro-inflammatory immune responses, translocation, IL-22, IL-18, apoptosis

Introduction

Human cases of bacterial gastroenteritis caused by the gram-negative bacterium *Campylobacter (C.) jejuni* are worldwide on the rise [1, 2]. Being part of the commensal gut microbiota in many wild and domestic animal species, zoonotic transmission to humans takes place from livestock animals via consumption of contaminated water or meat products [3–5]. Infected patients present symptoms of considerable variability ranging from rather mild, non-inflammatory, watery diarrhea to severe, inflammatory, bloody diarrhea, and abdominal pain lasting for up to a few weeks [6]. In most patients, the disease is self-limiting. In some cases, however, infected patients develop postinfectious sequelae such as reactive arthritis and peripheral neuropathies including the Guillain–Barré and Miller–Fisher syndromes later on [6, 7]. In intestinal tissues of infected patients histological changes such as apoptosis, crypt abscesses, ulcerations, and pronounced influx of pro-inflammatory immune cell populations including lymphocytes and neutrophils into the intestinal mucosa and lamina propria are observed [8, 9]. Although human campylobacteriosis is of global importance, our understanding of the molecular mechanisms underlying *C. jejuni* infection has been hampered for a long time due to the scarcity of appropriate vertebrate models. Newborn piglets, weanling ferrets, chicken, gnotobiotic canine pups, and primates have been more or less successfully used for studying *C. jejuni*–host interactions [6, 10]. Our group has recently shown that gnotobiotic mice lacking the commensal intestinal microbiota following broad-spectrum antibiotic treatment can be readily colonized by *C. jejuni* [11]. Subsequently, mice display *C. jejuni* induced pro-inflammatory immune responses and epithelial apoptosis in the large intestinal mucosa, hence, mimicking key feature of human campylobacteriosis [11]. Thus, gnotobiotic mice are very well suited as *C. jejuni* infection model to study host–pathogen interactions *in vivo*.

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Matrix metalloproteinases (MMPs) comprise a heterogenous family of zinc- and calcium-dependent matrix-degrading endopeptidases under tight control of endogenous inhibitors, namely, tissue inhibitors of matrix metalloproteinases (TIMPs) [12–14]. With respect to their substrate specificity, MMPs can be categorized into collagenases (MMP-1, -8, -13, -18), gelatinases (MMP-2, -9), stromelysins (MMP-3, -7, -10, -11), elastase (MMP-12), and membrane-type matrix metalloproteinases (MT-MMP-1 through -5) [15]. MMPs are crucial for embryonic development, differentiation, proliferation, and regeneration of tissues [13, 14]. A dysbalance between MMP activating and inhibiting factors, however, results in a plethora of diseases including intestinal inflammation, arthritis, atherosclerosis, or cancer [16, 17]. Importantly, expression levels of the gelatinases-A and -B (MMP-2 and MMP-9, respectively) are upregulated in intestinal inflammation models [18–20] and in patients suffering from human inflammatory bowel diseases such as Crohn's disease or ulcerative colitis [21–23]. To date, however, data regarding the impact of MMPs, particularly the gelatinases, in intestinal infection caused by bacteria including *C. jejuni* are limited. Distinct MMPs have been shown to be upregulated during infection with gram-negative bacteria [24]. Whereas increased MMP-3 transcripts could be detected in Peyer's patches following *Salmonella* and *Yersinia* infection [25], MMP-2 and MMP-9 were upregulated in *Helicobacter pylori* infected mice [26]. Furthermore, human and murine *Helicobacter*-induced gastritis were associated with increased MMP-9 levels derived from macrophages [27]. Interestingly, distinct serine proteases derived from *E. coli* have been shown to act as specific pro-MMP-2 activators [28].

Recently, we could show that selective gelatinase blockage by the synthetic compound RO28-2653 (blocking both MMP-2 and MMP-9) reduced large intestinal apoptosis and pro-inflammatory immune cell responses in *C. jejuni*-infected gnotobiotic IL-10$^{-/-}$ mice [29]. This prompted us to investigate in more detail which gelatinase is involved in *C. jejuni*-induced disease. To address this, we infected gnotobiotic MMP-2$^{-/-}$, MMP-9$^{-/-}$, and corresponding wildtype (WT) control mice perorally with *C. jejuni* strain 81-176. We then investigated 1) the gastrointestinal colonization and translocation properties of *C. jejuni,* 2) the clinical course of infection, 3) the histopathological colonic changes including apoptosis *in situ,* 4) the abundances of immune cell populations in the colonic mucosa and lamina propria *in situ,* and, furthermore, 5) the large intestinal expression of pro-inflammatory cytokines belonging to the IL-22/IL-18/IL-17 axis at two distinct time points postinfection (p.i.).

Methods

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 G0135/10). Animal welfare was monitored twice daily by assessment of clinical conditions.

Mice and C. jejuni *infection*

Female MMP-2$^{-/-}$ and MMP-9$^{-/-}$ mice (all in C57BL/6j background [20]) as well as age- and sex-matched C57BL/6j WT control mice were bred and maintained within the same specific pathogen free (SPF) unit in the Forschungseinrichtungen für Experimentelle Medizin (FEM, Charité – University Medicine Berlin). In order to confirm absence of MMP-2 or MMP-9 gene expression, genomic DNA was isolated, and disruption of either gene was confirmed by polymerase chain reaction (PCR) [20]. Gnotobiotic mice (with a virtually depleted gastrointestinal microbiota) were generated by broad-spectrum antibiotic treatment as described earlier [30]. Briefly, 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, Holzkirchen, Germany), imipenem (250 mg/L; Fresenius Kabi, Graz, Austria), and metronidazole (1 g/L; Braun, Melsungen, Germany) to the drinking water *ad libitum* starting at 8 weeks of age and continued for approximately 10 weeks before the infection experiment [11]. Three days prior infection, the antibiotic cocktail was replaced by sterile tap water *(ad libitum)*. Then, mice were perorally infected with 10^9 colony forming units (CFU) of viable *C. jejuni* strain 81-176 in a volume of 0.3 mL phosphate buffered saline (PBS) on two consecutive days (day 0 and day 1) by gavage as described earlier [11].

Clinical score

To assess clinical signs of *C. jejuni* induced infection on a daily basis, a standardized cumulative clinical score (maximum 12 points), addressing the occurrence of blood in feces (0: no blood; 2: microscopic detection of blood by the Guajac method using Haemoccult, Beckman Coulter/PCD, Krefeld, Germany; 4: macroscopic blood visible), diarrhea (0: formed feces; 2: pasty feces; 4: liquid feces), and the clinical aspect (0: normal; 2: ruffled fur, less locomotion; 4: isolation, severely compromized locomotion, pre-final aspect) was used [31].

Sampling procedures

Mice were sacrificed at day 8 or day 14 p.i. by isofluran treatment (Abbott, Greifswald, Germany). Cardiac blood and tissue samples from colon, ileum, mesenteric lymphnodes (MLNs), spleen, liver, and kidney were asserved

under sterile conditions. Intestinal *ex vivo* biopsies were collected in parallel for immunohistochemical, microbiological, and immunological analyses. Immunohistopathological changes were assessed in colonic samples that were immediately fixed in 5% formalin and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (H&E) or respective antibodies for *in situ* immunohistochemistry as described earlier [32].

Histopathological grading of large intestinal lesions

Histopathological changes were quantitatively assessed in H&E stained large intestinal paraffin sections applying a histopathological scoring system by two independent double-blinded investigators as described previously [33]. In brief:

Colonic histopathology (max. 4 points; according to [34]): 0: no inflammation; 1: single isolated cell infiltrates within the mucosa; no epithelial hyperplasia; 2: mild scattered to diffuse cell infiltrates within the mucosa and submucosa; mild epithelial hyperplasia; starting loss of goblet cells; 3: cell infiltrates within mucosa, submucosa, and sometimes transmural; epithelial hyperplasia; loss of goblet cells; 4: cell infiltrates within mucosa, submucosa, and transmural; severe inflammation; loss of goblet cells, loss of crypts; ulcerations; severe epithelial hyperplasia.

Immunohistochemistry

In situ immunohistochemical analysis of colonic paraffin sections was performed as described previously [35–37]. Primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, Beverly, MA, USA, 1:200), Ki67 (TEC3, Dako, Denmark, 1:100), myeloperoxidase (MPO-7, no. A0398, Dako, 1:500), F4/80 (no. 14-4801, clone BM8, eBioscience, San Diego, CA, USA, 1:50), CD3 (no. N1580, Dako, 1:10), FOXP3 (FJK-16s, eBioscience, 1:100), and B220 (eBioscience, 1:200) were used. For each animal, the average number of positively stained cells within at least six high power fields (HPF, 0.287 mm^2, 400× magnification) were determined microscopically by a double-blinded investigator.

Quantitative analysis of C. jejuni *colonization and translocation*

Viable *C. jejuni* were detected in feces over time p.i. or at time of necropsy (day 8 or day 14 p.i.) in luminal samples taken from the the terminal ileum and colon, dissolved in sterile PBS and serial dilutions cultured on Karmali- and Columbia-Agar supplemented with 5% sheep blood (Oxoid) for 2 days at 37 °C under microaerobic conditions using CampyGen gas packs (Oxoid). To quantify bacterial translocation, *ex vivo* biopsies derived from mesenteric lymph nodes (MLNs), spleen, liver, and kidney were homogenized in 1 mL sterile PBS, whereas cardiac blood (≈100 μL) was directly streaked onto Karmali-Agar and Columbia-Agar supplemented with 5% sheep blood and cultivated accordingly. The respective weights of fecal or tissue samples were determined by the difference of the sample weights before and after asservation. The detection limit of viable pathogens was ≈100 CFU per gram.

Cytokine detection in supernatants of colonic ex vivo biopsies

Ileal and colonic *ex vivo* biopsies were cut longitudinally and washed in PBS. Strips of approximately 1 cm^2 intestinal tissue were placed in 24-flat-bottom well culture plates (Nunc, Wiesbaden, Germany) containing 500 μL serum-free RPMI 1640 medium (Gibco, Life Technologies, Paisley, UK) supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL; PAA Laboratories). After 18 h at 37 °C, culture supernatants or serum samples were tested for TNF, IFN-γ, MCP-1, IL-6, and IL-10 by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences) on a BD FACSCanto II flow cytometer (BD Biosciences).

Real-time PCR

RNA was isolated from snap frozen colonic *ex vivo* biopsies, reverse transcribed, and analyzed as described previously [19]. Murine IL-23p19, IL-22, IL-18, IL-17A, and IL-1β mRNA expressions were detected and analyzed using Light Cycler Data Analysis Software (Roche). Expression levels were calculated relative to the HPRT expression and indicated as "Arbitrary Units" (fold expression).

Statistical analysis

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

Results

Colonization and translocation properties of C. jejuni *in gnotobiotic mice lacking MMP-2 or MMP-9*

In the present study, we dissected the role of the gelatinases-A and -B (also referred to MMP-2 and MMP-9, respectively) in murine campylobacteriosis. To address this, gnotobiotic MMP-2$^{-/-}$, MMP-9$^{-/-}$, and wildtype (WT) mice were generated in which the intestinal microbiota was virtually depleted by quintuple antibiotic treatment for 10 weeks [11, 29–33, 38]. Following peroral infection with 10^9 CFU of *C. jejuni* strain 81-176 on two consecu-
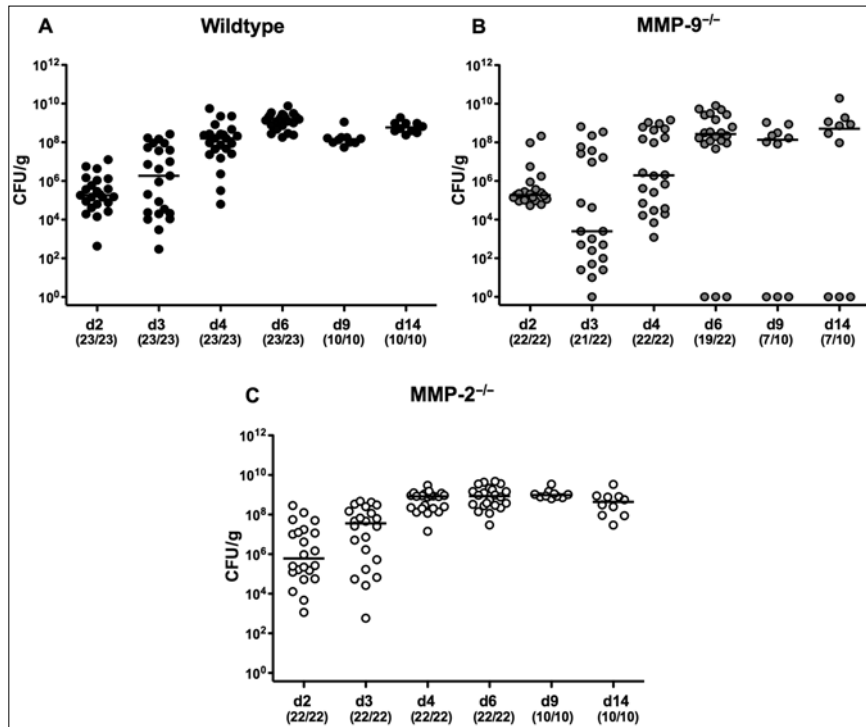
Fig. 1. Kinetics of intestinal *C. jejuni* colonization in perorally infected gnotobiotic mice lacking MMP-2 or MMP-9. Gnotobiotic (A) wildtype (black circles), (B) MMP-9$^{-/-}$ (grey circles), and (C) MMP-2$^{-/-}$ (white circles) mice were generated following broad-spectrum antibiotic treatment and perorally infected with *C. jejuni* strain 81-176 by gavage at day 0 and day 1. Pathogenic loads were determined in fecal samples (CFU, colony forming units per gram) at distinct time points (d, day) postinfection (p.i.) as indicated by culture. Numbers of mice harboring *C. jejuni* strain 81-176 out of the total number of analyzed animals are given in parentheses. Data were pooled from three independent experiments
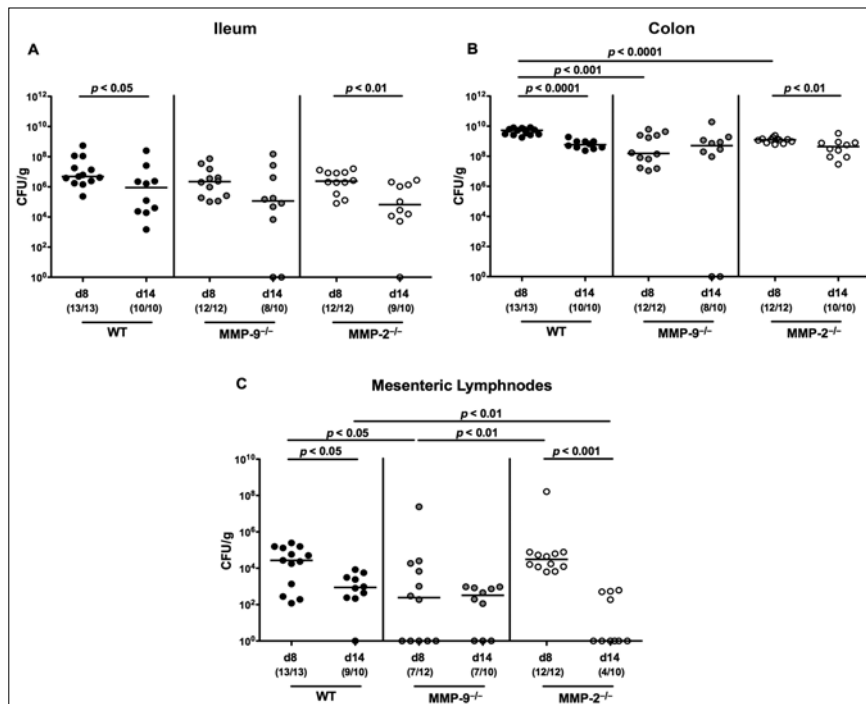

Fig. 2. Intestinal *C. jejuni* loads in perorally infected gnotobiotic mice lacking MMP-2 or MMP-9. Gnotobiotic wildtype (WT; black circles), MMP-9$^{-/-}$ (grey circles), and MMP-2$^{-/-}$ (white circles) mice were perorally infected with *C. jejuni* strain 81-176 at days 0 and 1. Pathogenic loads (CFU, colony forming units per gram) were determined in luminal samples taken from the (A) terminal ileum or (B) colon or in homogenates of (C) mesenteric lymphnodes at day (d) 8 or 14 postinfection (p.i.) as indicated by culture. Numbers of mice harboring *C. jejuni* strain 81-176 out of the total number of analyzed animals are given in parentheses. Medians (black bars) and level of significance (*p*-value) determined by Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments

tive days (day 0 and day 1), mice of either genotype harbored the pathogen with loads of 10^9 per gram fecal sample *(Fig. 1)*. In single cases, however, MMP-9$^{-/-}$ mice had expelled the pathogen as early as 6 days p.i. *(Fig. 1B)*. We next surveyed the effect of MMP-2 and MMP-9 in murine campylobacteriosis at two distinct time points, namely, as early as 8 days and 2 weeks following *C. jejuni* infection. In small and large intestines of WT and MMP-2$^{-/-}$ mice, *C. jejuni* loads slightly decreased by approximately 1 to 2 orders of magnitude between days 8 and 14 p.i. *(Fig. 2A,B)*. In the colon, MMP-2$^{-/-}$ and MMP-9$^{-/-}$ mice harbored approximately 1.5 orders of magnitude lower *C. jejuni* loads as compared to wildtype mice at day 8 p.i. *(Fig. 2B)*. Overall, irrespective of the genotype, gnotobiotic mice could be stably infected with *C. jejuni* strain 81-176 with relatively high loads over time. We next assessed whether viable *C. jejuni* were able to translocate from the intestinal tract to extra-intestinal compartments. As early as 8 days p.i., *C. jejuni* could be detected in the MLNs of all WT and MMP-2$^{-/-}$ mice, but only in 58.3% of MMP-9$^{-/-}$ mice *(Fig. 2C)*. Pathogenic loads were lower in MLNs of MMP-9$^{-/-}$ mice at day 8 p.i. and of MMP-2$^{-/-}$ at day 14 p.i. when compared to WT controls ($p < 0.05$ and $p < 0.01$, respectively; *Fig. 2C*). Between days 8 and 14 p.i., *C. jejuni* loads decreased in MLNs of WT and MMP-2$^{-/-}$ mice ($p < 0.05$ and $p < 0.001$, respectively; *Fig. 2C*). At day 14 p.i., *C. jejuni* detection rates in MLNs of WT, MMP-9$^{-/-}$, and MMP-2$^{-/-}$ mice were 90%, 70%, and 40%, respectively *(Fig. 2C)*. Remarkably, viable *C. jejuni* did not translocate beyond the intestinal tract since neither in liver, kidney, spleen, and blood viable pathogens could be detected by direct plating (not shown).

Macroscopic and microscopic sequelae of C. jejuni *infection in gnotobiotic mice lacking MMP-2 or MMP-9*

We next assessed potential clinical symptoms of *C. jejuni* infection (such as bloody diarrhea and wasting) in gelatinase-deficient mice. At day 8 p.i., occurrence of

blood could be detected microscopically in 8.7%, 18.2%, and 9.1% of fecal samples derived from WT, MMP-9$^{-/-}$, and MMP-2$^{-/-}$ mice, respectively, whereas 14 days p.i., no bloody diarrhea could be observed at all *(Fig. 3A)*. Hence, despite pathogenic colonization, infected gnotobiotic mice of either genotype were clinically virtually unaffected.

We next surveyed histopathological changes in the colonic mucosa in the course of *C. jejuni* infection. Until day 8 p.i., significant but rather mild histopathological changes such as single isolated cell infiltrates within the mucosa, but no epithelial hyperplasia could be observed in the colonic mucosa of mice irrespective of the genotype *(Fig. 3)*. At day 14 p.i., histopathological changes were even more severe in WT and MMP-9$^{-/-}$, but not MMP-2$^{-/-}$ mice as compared to those observed at day 8 p.i. ($p < 0.001$ and $p < 0.05$, respectively; *Fig. 3B*). Moreover, at day 14 p.i., histopathological changes were less distinct in the large intestines of MMP-2$^{-/-}$ as compared to WT and MMP-9$^{-/-}$ mice *(Fig. 3B)*. Notably, the variability of histopathological changes in the colon of MMP-9$^{-/-}$ mice at day 14 p.i. was relatively high as indicated by high standard deviations of histopathological scores.

Given that apoptosis is a commonly used diagnostic marker in the histopathological evaluation and grading of intestinal disease including campylobacteriosis [11], we quantitatively assessed numbers of caspase-3+ cells within the colonic epithelium of infected mice. Whereas following infection abundances of apoptotic cells increased in colons of mice of either genotype, this increase was less pronounced in MMP-2$^{-/-}$ as compared to WT mice, both at day 8 and day 14 p.i. ($p < 0.001$ and $p < 0.01$, respectively; *Fig. 4A*). Furthermore, MMP-9$^{-/-}$ mice displayed lower colonic apoptotic cell numbers at day 8, but not day 14 p.i., as compared to WT controls ($p < 0.05$; *Fig. 4A*). Given that Ki67 comprises a nuclear protein associated with and necessary for cellular proliferation [39], we stained colonic paraffin sections against Ki67 to determine proliferative measures of the colonic epithelium counter-
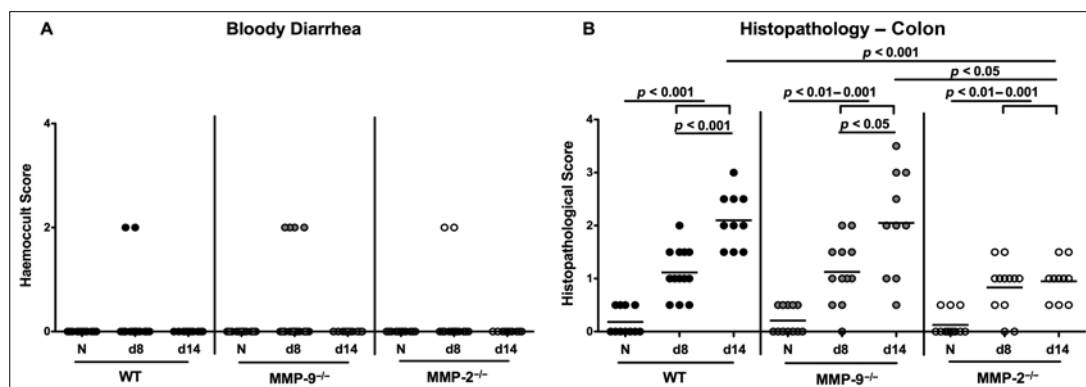

Fig. 3. Clinical symptoms and colonic histopathological changes in *C. jejuni* infected gnotobiotic mice lacking MMP-2 or MMP-9. Gnotobiotic wildtype (WT; black circles), MMP-9$^{-/-}$ (grey circles), and MMP-2$^{-/-}$ (white circles) mice were perorally infected with *C. jejuni* strain 81-176 at days 0 and 1. At day (d) 8 and day 14 postinfection (p.i.), (A) occurrence of bloody diarrhea and (B) histopathological changes in H&E stained colonic paraffin sections were assessed applying a standarized haemoccult and histopathological scoring system, respectively. Naive (N) mice served as uninfected controls. Medians (black bars) and level of significance (*p*-value) determined by Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments
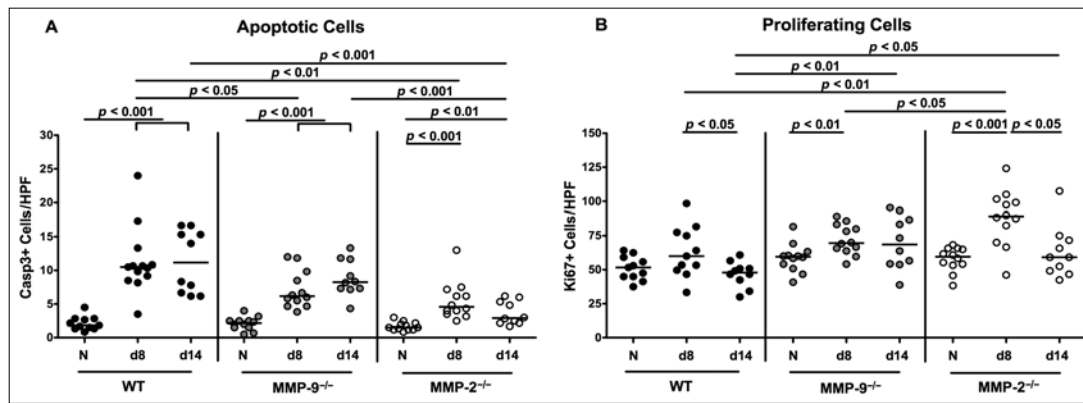
Fig. 4. Apoptosis and proliferation in the colonic epithelium of *C. jejuni* infected gnotobiotic mice lacking MMP-2 or MMP-9. Gnotobiotic wildtype (WT; black circles), MMP-9$^{-/-}$ (grey circles), and MMP-2$^{-/-}$ (white circles) mice were perorally infected with *C. jejuni* strain 81-176 at days 0 and 1. The average number of colonic (A) apoptotic cells (positive for caspase-3, Casp3) and (B) proliferating cells (positive for Ki67) from at least six high power fields (HPF, 400x magnification) per animal was determined microscopically in immunohistochemically stained colonic paraffin sections at day (d) 8 and day 14 postinfection (p.i.). Naive (N) mice served as uninfected controls. Medians (black bars) and level of significance (*p*-value) determined by Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments

acting apoptosis following *C. jejuni* infection. Proliferating cells increased in the colonic epithelium of MMP-9$^{-/-}$ and MMP-2$^{-/-}$, but not WT mice until day 8 p.i. ($p < 0.01$ and $p < 0.001$, respectively; *Fig. 4B*). In the colonic mucosa of MMP-2$^{-/-}$ mice, higher Ki67+ cell numbers could be observed at both day 8 and day 14 p.i. as compared to WT controls ($p < 0.01$ and $p < 0.05$, respectively; *Fig. 4B*), whereas MMP-9$^{-/-}$ mice displayed more proliferating cells in the colon at day 14 p.i. only, when compared to WT animals ($p < 0.01$; *Fig. 4B*). Hence, MMP-2$^{-/-}$ mice exhibited less microscopic disease following *C. jejuni* infection, which was accompanied by more regenerative epithelial measures counteracting the inflammatory sequelae.

Immune cell responses in C. jejuni *strain 81-176 infected gnotobiotic mice lacking MMP-2 or MMP-9*

Given that recruitment of pro-inflammatory immune cells to sites of inflammation is a hallmark of intestinal patho-

genic infection including campylobacteriosis [11], we quantitatively assessed the influx of effector cells as well as of innate and adaptive immune cells into the large intestinal mucosa and lamina propria by *in situ* immunohistochemical staining of colonic paraffin sections. Following *C. jejuni* infection, numbers of colonic MPO7+ neutrophils, F4/80+ macrophages and monocytes, CD3+ T lymphocytes, FOXP3+ regulatory T cells (Tregs), and B220+ B lymphocytes increased in mice of either genotype and were higher at both day 8 and day 14 p.i. as compared to uninfected control mice *(Fig. 5)*. Increases in neutrophils, Tregs, and B cells were less pronounced in large intestines of MMP-2$^{-/-}$ mice as compared to WT mice at both time points *(Fig. 5A, Fig. 6B,C)*. Moreover, MMP-2$^{-/-}$ mice exhibited lower numbers of macrophages and monocytes as well as of T lymphocytes at day 14 p.i. as compared to WT controls *(Fig. 5B, Fig. 6A)*. For MMP-9$^{-/-}$ mice, lower numbers of colonic neutrophils, macrophages and monocytes, T lymphocytes, and Tregs could be determined at
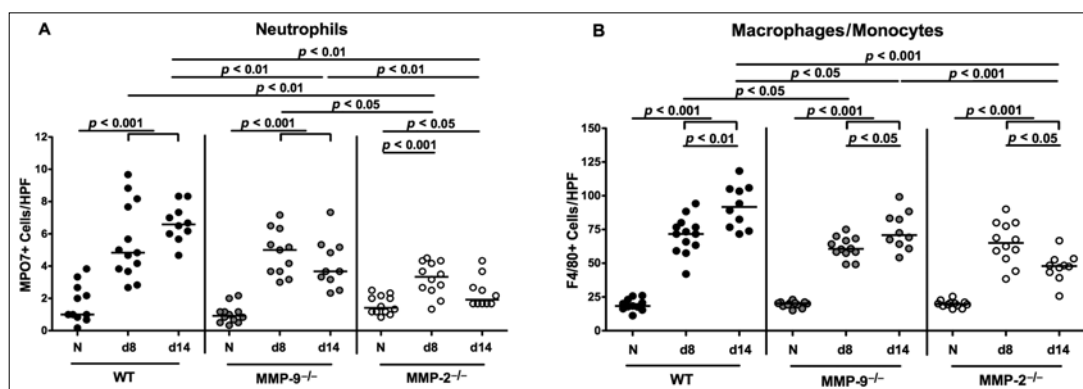

Fig. 5. Innate immune cells in the colon of *C. jejuni*-infected gnotobiotic mice lacking MMP-2 or MMP-9. Gnotobiotic wildtype (WT; black circles), MMP-9$^{-/-}$ (grey circles), and MMP-2$^{-/-}$ (white circles) mice were perorally infected with *C. jejuni* strain 81-176 at days 0 and day 1. The average number of cells positive for (A) MPO7 (neutrophils) and (B) F4/80 (macrophages and monocytes) from at least six high power fields (HPF, 400× magnification) per animal were determined microscopically in immunohistochemically stained colonic paraffin sections derived from mice at day (d) 8 and day 14 postinfection (p.i.). Naive (N) mice served as uninfected controls. Medians (black bars) and significance levels as determined by the Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments
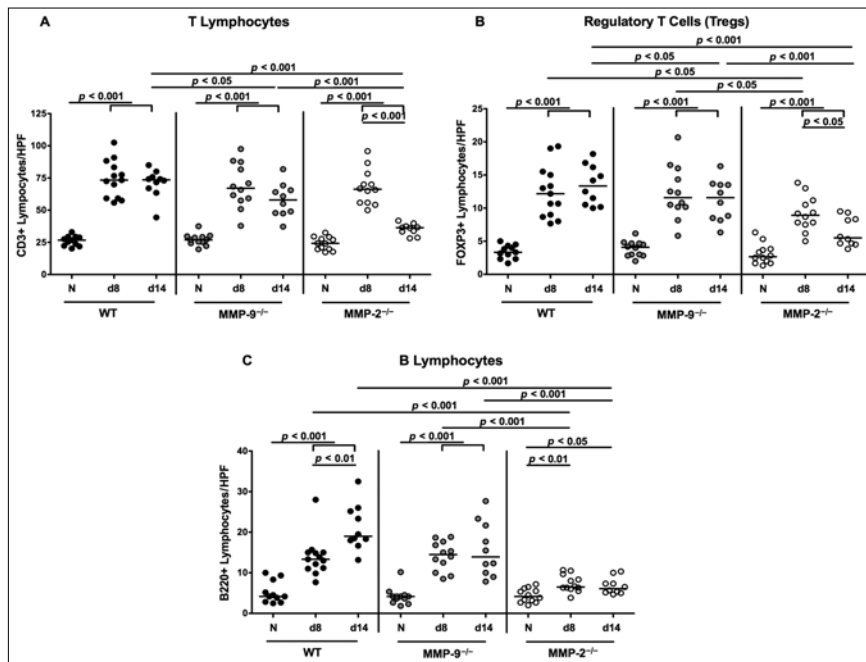
Fig. 6. Adaptive immune cells in the colon of *C. jejuni* infected gnotobiotic mice lacking MMP-2 or MMP-9. Gnotobiotic wildtype (WT; black circles), MMP-9$^{-/-}$ (grey circles), and MMP-2$^{-/-}$ (white circles) mice were perorally infected with *C. jejuni* strain 81-176 at days 0 and day 1. The average number of cells positive for (A) CD3 (T Lymphocytes), (B) FOXP3 (Regulatory T Cells, Tregs), and (C) B220 (B Lymphocytes) from at least six high power fields (HPF, 400× magnification) per animal were determined microscopically in immunohistochemically stained colonic paraffin sections derived from mice at day (d) 8 and day 14 postinfection (p.i.). Naive (N) mice served as uninfected controls. Medians (black bars) and significance levels as determined by the Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments

day 14 p.i. when compared to WT mice *(Fig. 5, Fig. 6A,B)*, whereas also at day 8 p.i. lower macrophages and monocytes could be determined in colons of MMP-9$^{-/-}$ versus WT mice *(Fig. 5B)*. Hence, influx of immune cells was overall less pronounced MMP-9$^{-/-}$ mice at day 14 p.i. and in MMP-2$^{-/-}$ at both time points.

Cytokine secretion in the intestines of C. jejuni *strain 81-176 infected gnotobiotic mice lacking MMP-2 or MMP-9*

We next determined pro- and anti-inflammatory cytokine secretion in *ex vivo* biopsies derived from small as well as large intestines of *C. jejuni* strain 81-176 infected gnotobiotic MMP-2$^{-/-}$ and MMP-9$^{-/-}$ mice. Until day 14 p.i., small intestinal TNF levels increased in mice of either genotype *(Fig. 7A)*, whereas higher ileal IFN-γ concentrations could be determined in WT and MMP-9$^{-/-}$ but not MMP-2$^{-/-}$ mice at day 8 p.i. *(Fig. 7B)*. The anti-inflammatory cytokine IL-10 increased as early as 8 days following *C. jejuni* infection in ilea of mice, irrespective of their genotype and was still elevated in small intestines of WT mice at day 14 p.i. *(Fig. 7D)*. In the colon, elevated TNF concentrations could be observed at either time point in WT and MMP-9$^{-/-}$ mice and at day 14 p.i. in MMP-2$^{-/-}$ animals as compared to naive controls *(Fig. 8A)*, whereas colonic IFN-γ levels increased as early as 8 days p.i. in WT and MMP-9$^{-/-}$ but not MMP-2$^{-/-}$ mice *(Fig. 8B)*.

C. jejuni infection was further accompanied by higher colonic MCP-1 levels at either time point, irrespective of the genotype of mice *(Fig. 8C)*, whereas large intestinal IL-10 was elevated in mice of either genotype at day 8 p.i. only *(Fig. 8D)*. Taken together, pro- and anti-inflammatory cytokines increased in the intestinal tract upon *C. jejuni* infection, but without significant differences between mice of the respective genotypes.

Colonic mRNA expression of pro- and anti-inflammatory cytokines in C. jejuni *strain 81-176 infected gnotobiotic mice lacking MMP-2 or MMP-9*

We next determined colonic mRNA expression levels of pro- and anti-inflammatory mediators of the IL-22/IL-18/IL-17 axis during *C. jejuni* infection of gelatinase-deficient mice. In WT and MMP-2$^{-/-}$ mice, colonic IL-23p19 mRNA levels were elevated at either time point and in the large intestines of MMP-9$^{-/-}$ mice at day 14 p.i. *(Fig. 9A)*. In MMP-9$^{-/-}$ mice, however, colonic IL-23p19 mRNA concentrations were lower as compared to WT mice at day 8 and at day 14 p.i. ($p < 0.001$ and $p < 0.01$, respectively; *Fig. 9A*). Following *C. jejuni* infection, IL-22 mRNA increased in colons of either gelatinase deficient mice as early as day 8 p.i. and until day 14 p.i. in WT mice *(Fig. 9B)*. Interestingly, large intestinal IL-22 mRNA levels were higher in MMP-2$^{-/-}$ mice at day 8 p.i. ($p < 0.001$), but lower in MMP-9$^{-/-}$ mice at day 14 p.i. ($p < 0.05$), when com-
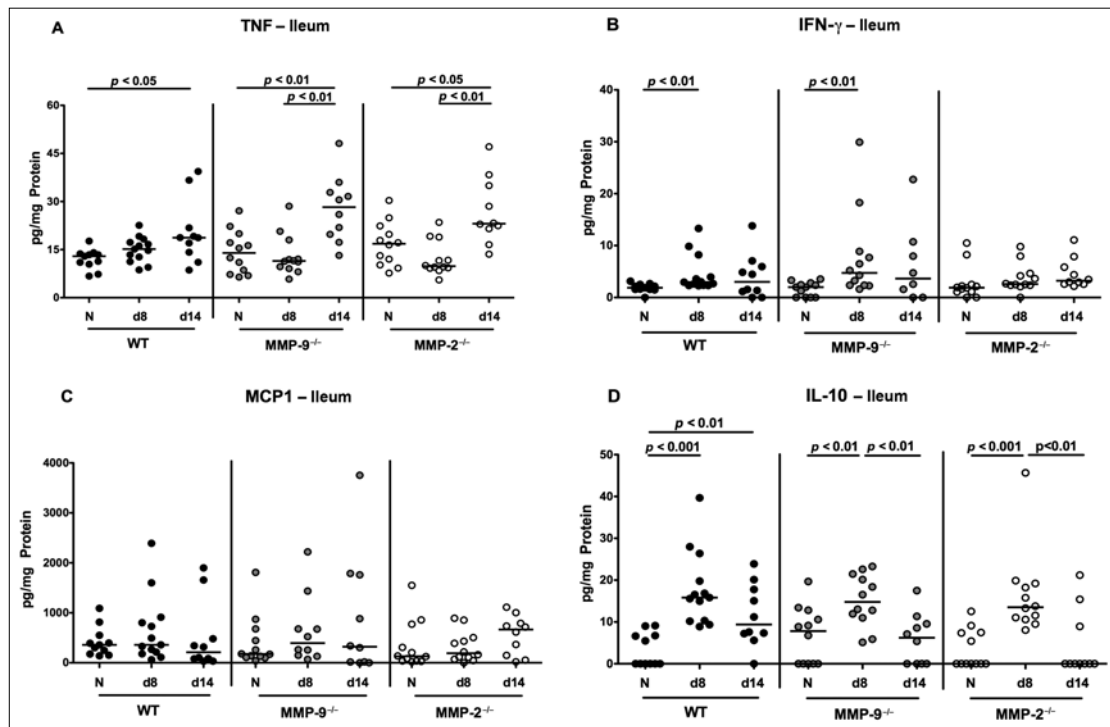
Fig. 7. Cytokine concentrations in the ileum of *C. jejuni*-infected gnotobiotic mice lacking MMP-2 or MMP-9. Gnotobiotic wildtype (WT; black circles), MMP-9$^{-/-}$ (grey circles), and MMP-2$^{-/-}$ (white circles) mice were perorally infected with *C. jejuni* strain 81-176 at days 0 and 1. (A) TNF, (B) IFN-γ, (C) MCP1, and (D) IL-10 concentrations were determined in supernatants of ileal *ex vivo* biopsies derived at day (d) 8 and day 14 postinfection (p.i.). Naive (N) mice served as uninfected controls. Medians (black bars) and significance levels as determined by the Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments
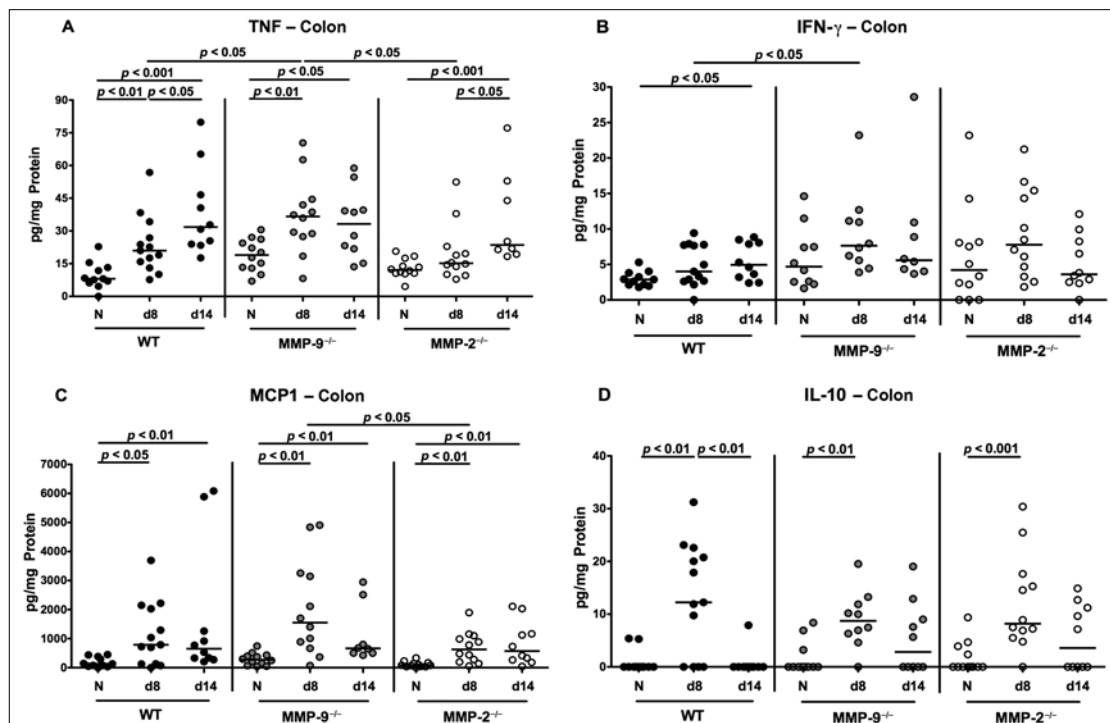

Fig. 8. Cytokine concentrations in the colon of *C. jejuni*-infected gnotobiotic mice lacking lacking MMP-2 or MMP-9. Gnotobiotic wildtype (WT; black circles), MMP-9$^{-/-}$ (grey circles), and MMP-2$^{-/-}$ (white circles) mice were perorally infected with *C. jejuni* strain 81-176 at days 0 and 1. (A) TNF, (B) IFN-γ, (C) MCP1, and (D) IL-10 concentrations were determined in supernatants of colonic *ex vivo* biopsies derived at day (d) 8 and day 14 postinfection (p.i.). Naive (N) mice served as uninfected controls. Medians (black bars) and significance levels as determined by the Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments
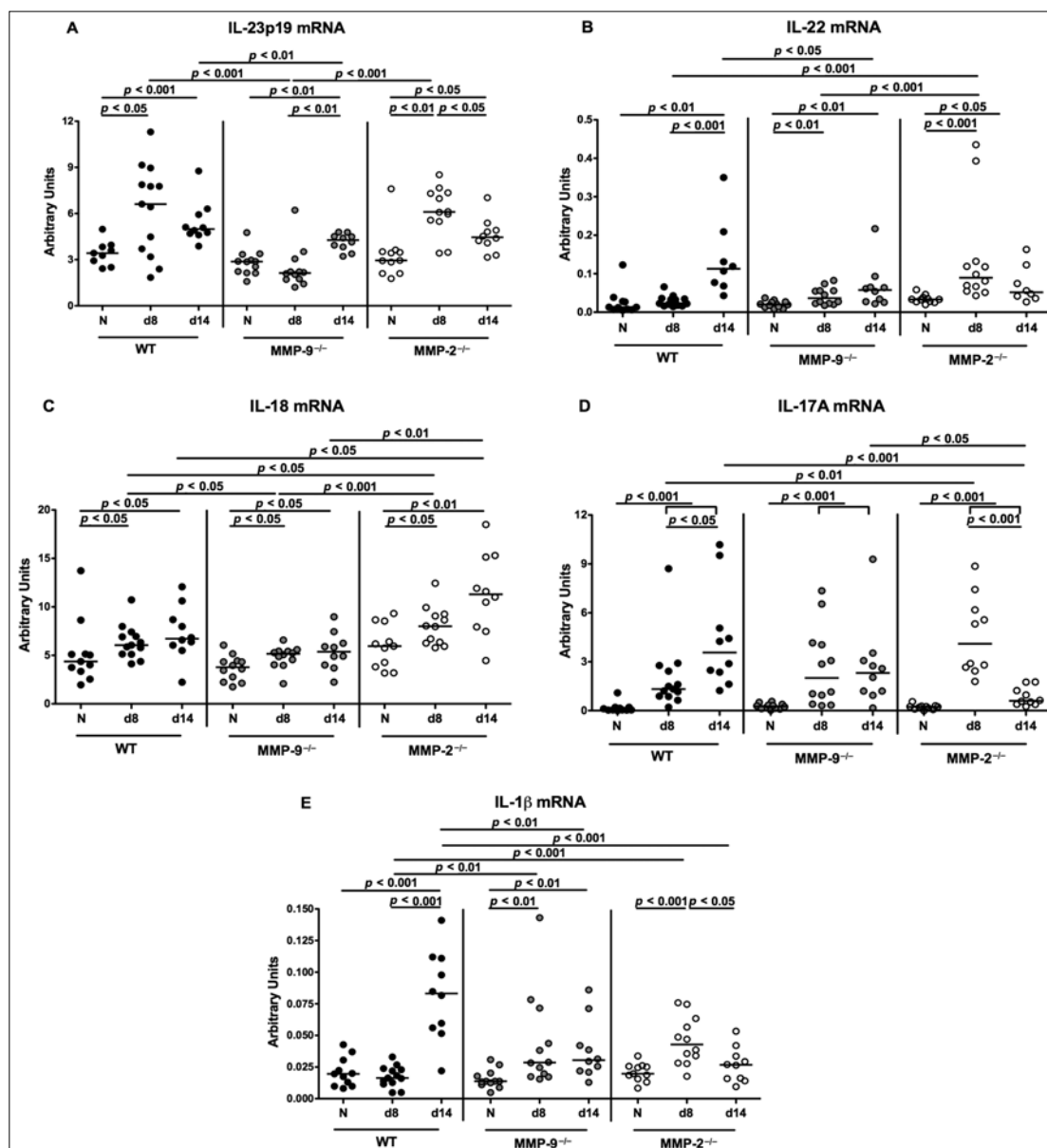
Fig. 9. Expression of pro- and anti-inflammatory mediators in the colon of *C. jejuni*-infected gnotobiotic mice lacking MMP-2 or MMP-9. Gnotobiotic wildtype (WT; black circles), MMP-9$^{-/-}$ (grey circles), and MMP-2$^{-/-}$ (white circles) mice were perorally infected with *C. jejuni* strain 81-176 at days 0 and 1. (A) IL-23p19, (B) IL-22, (C) IL-18, (D) IL-17A, and (E) IL-1β mRNA expression levels were determined in colonic *ex vivo* biopsies derived at day (d) 8 and day 14 postinfection (p.i.) by real-time PCR and expressed in arbitrary units (fold expression). Naive (N) mice served as uninfected controls. Medians (black bars) and significance levels as determined by the Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments

pared to WT control animals at the respective time points *(Fig. 9B)*. *C. jejuni*-infection induced increases in colonic IL-18 mRNA concentrations were less distinct in MMP-9$^{-/-}$ mice at day 8 p.i. ($p < 0.05$), but even more pronounced in MMP-2$^{-/-}$ mice at either time point ($p < 0.05$) as compared to WT controls *(Fig. 9C)*. Irrespective of the genotype, colonic IL-17A was upregulated upon *C. jejuni* infection. In MMP-2$^{-/-}$ mice, however, IL-17A mRNA expression levels were higher at day 8 p.i. ($p < 0.01$), but lower at day 14 p.i. ($p < 0.001$), when compared to WT animals *(Fig. 9D)*. Notably, this held also true for colonic IL-1β mRNA expression levels in both MMP-9$^{-/-}$ and MMP-2$^{-/-}$ as compared to WT mice *(Fig. 9E)*. Moreover, upon *C. jejuni* infection, colonic

IL-1β expression was upregulated at day 14 p.i. in WT mice, at day 8 p.i. in MMP-2$^{-/-}$ mice and at both time points in MMP-9$^{-/-}$ mice *(Fig. 9E)*. Hence, mRNA expression of pro- and anti-inflammatory cytokines belonging to the IL-22/IL-18/IL-17 axis are differentially regulated in *C. jejuni*-infected gnotobiotic MMP-2$^{-/-}$ and MMP-9$^{-/-}$ mice.

Discussion

The gelatinases MMP-2 and MMP-9 are pivotally involved in modulating intestinal inflammation of mice and men [18–23, 29, 40]. To date, however, only limited data

regarding the role of gelatinases in host interactions with enteric pathogens including *C. jejuni* exist. Results from our recent experiments revealed that selective gelatinase blockage by the synthetic compound RO28-2653 ameliorated colonic pro-inflammatory immune responses and apoptosis in *C. jejuni* 81-176 strain infected gnotobiotic IL-10$^{-/-}$ mice [29]. This provided strong evidence that gelatinases are involved in the pathogenesis of campylobacteriosis. To address the question which gelatinase in particular modulates the course of murine *C. jejuni* infection, we investigated campylobacteriosis in gnotobiotic MMP-2- or MMP-9-deficient mice at distinct time points p.i. Results revealed that *C. jejuni* stably colonized the intestinal tract of gnotobiotic MMP-2$^{-/-}$, MMP-9$^{-/-}$, and WT mice at high loads. Viable bacteria were further isolated from MLNs, but did not translocate to extra-intestinal compartments such as liver, kidney, spleen, or blood. This is well supported by our previous campylobacteriosis studies in different murine models including gnotobiotic, with human microbiota reconstituted, conventional WT infant and gnotobiotic IL-10$^{-/-}$ mice [11, 31–33, 37, 38, 41, 42]. Upon *C. jejuni* infection, gnotobiotic MMP-2$^{-/-}$ mice developed less colonic histopathology, whereas both infected MMP-2$^{-/-}$ and MMP-9$^{-/-}$ mice displayed less apoptotic, but more proliferating cells in the colonic epithelium as compared to WT mice. These data are partly supported by our very recent study obtained from different *C. jejuni* infection models. Following peroral infection with the *C. jejuni* B2 strain, conventionally colonized infant MMP-2$^{-/-}$, but not MMP-9$^{-/-}$ mice exhibited less pronounced colonic histopathological and apoptotic changes as compared to WT controls [43]. Interestingly, in both studies, less influx of effector as well as of innate and adaptive immune cells including neutrophils, T lymphocytes, and Tregs into the colonic mucosa and lamina propria of either gelatinase deficient mice could be observed. In contrast to infected infant MMP-2$^{-/-}$ and MMP-9$^{-/-}$ mice, which expelled the pathogen rather early in the course of infection [43], gnotobiotic mice of either genotype harbored the pathogen at high loads throughout the experiment.

Results presented here support the important role of MMP-2 in intestinal inflammation and are well in line with results from our earlier studies showing that MMP-2, but not MMP-9, is essential for the pathogenesis of acute ileitis [19] and colitis [20] in mice.

Less severe intestinal inflammation observed in *C. jejuni*-infected gnotobiotic MMP-2$^{-/-}$ mice was accompanied by increased IL-22 mRNA levels in the colon. IL-22 belongs to the IL-10 cytokine family and is well known for its tissue-protective and wound-healing responses from epithelial cells following infection and inflammation [44, 45]. In the intestinal tract, IL-22 exerts its dichotomous actions in a tissue-depending manner. Whereas IL-22 has been shown to act as an anti-inflammatory mediator in the large intestine, we have previously reported its pro-inflammatory properties in the small intestine. In acute *Toxoplasma gondii*-induced ileitis, IL-23 caused small intestinal immunopathology via the upregulation of MMP-2 and,

surprisingly, via the induction of IL-22 [19, 46]. Besides IL-22, higher IL-17A, IL-1β, and IL-18 mRNA expression levels were detected in large intestines of MMP-2$^{-/-}$, but not MMP-9$^{-/-}$ mice at day 8 p.i. as compared to infected WT mice in the present study. These results are supported by a study demonstrating that IL-22, IL-17A, and IL-1β secretion was increased in MLNs and colons of IL-10$^{-/-}$ mice as early as 4 days following oral *C. jejuni* infection, whereas both T cells and innate lymphoid cells (ILCs) induced upregulation of IL-17A and IL-22 in an organ- and time-specific manner [47]. Moreover, *C. jejuni* infection resulted in an upregulation of IL-22, IL-17A, and IL-1β in *ex vivo* colonic biopsies derived from humans [48]. Results further revealed that *C. jejuni* induced an innate IL-22/IL-17 as well as an adaptive Th17/Th1 host response [48]. Taken together, these data indicate that cytokines of the IL-22 and IL-17 families are essential mediators of host immunity in the acute and effector phase of *C. jejuni* infection *in vivo*. Upregulated IL-22 mRNA expression levels in the large intestines of *C. jejuni*-infected MMP-2$^{-/-}$ mice were accompanied by increased IL-18 concentrations. Data on the role of IL-18 in *C. jejuni*–host interaction are lacking so far, but we could recently show that IL-22 induced IL-18 expression in gut epithelial cells after oral *Toxoplasma gondii* or *Citrobacter rodentium* infection [49]. Furthermore, IL-18 amplified Th1-mediated intestinal inflammation and IL-22 production from ILCs during pathogenic infection [49]. It is therefore tempting to speculate that IL-22 and IL-18 might also exert a mutual regulation during intestinal *C. jejuni* infection.

Conclusion

In conclusion, both MMP-2 and MMP-9 are differentially involved in mediating *C. jejuni*-induced intestinal immunopathology with MMP-2 holding a more prominent and MMP-9 rather a minor role. In future studies, the regulatory pathways of MMP-2, IL-22, and IL-18 in murine *C. jejuni* infection need to be further elucidated in order to better understand molecular mechanisms underlying human campylobacteriosis.

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Competing Interests

The authors declare that no competing interests exist.

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