MATRIX METALLOPROTEINASE-2 MEDIATES INTESTINAL IMMUNOPATHOGENESIS IN *CAMPYLOBACTER JEJUNI*-INFECTED INFANT MICE

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Increased levels of the matrix metalloproteinases (MMPs)-2 and -9 (also referred to gelatinase-A and -B, respectively) can be detected in the inflamed gut. We have recently shown that synthetic gelatinase blockage reduces colonic apoptosis and pro-inflammatory immune responses following murine *Campylobacter* (*C.*) *jejuni* infection. In order to dissect whether MMP-2 and/or MMP-9 is involved in mediating *C. jejuni*-induced immune responses, infant MMP-2$^{-/-}$, MMP-9$^{-/-}$, and wildtype (WT) mice were perorally infected with the *C. jejuni* strain B2 immediately after weaning. Whereas, at day 2 postinfection (p.i.), fecal *C. jejuni* B2 loads were comparable in mice of either genotype, mice expelled the pathogen from the intestinal tract until day 4 p.i. Six days p.i., colonic MMP-2 but not MMP-9 mRNA was upregulated in WT mice. Remarkably, infected MMP-2$^{-/-}$ mice exhibited less frequent abundance of blood in feces, less distinct colonic histopathology and apoptosis, lower numbers of effector as well as innate and adaptive immune cells within the colonic mucosa, and higher colonic IL-22 mRNA levels as compared to infected WT mice. In conclusion, these results point towards an important role of MMP-2 in mediating *C. jejuni*-induced intestinal immunopathogenesis.

Keywords: *Campylobacter jejuni,* infant mice, matrix metalloproteinase-2, gelatinases, pro-inflammatory immune responses, intestinal microbiota, IL-22, IL23, IL-18, apoptosis

Introduction

During the last decade, *Campylobacter* (*C.*) *jejuni* has been recognized as the leading cause of bacterial gastroenteritis worldwide [1, 2]. The Gram-negative bacteria are part of the commensal gut microbiota in many wild and domestic animal species. Zoonotic transmission takes place from livestock animals via consumption of contaminated meat products or water [3, 4]. Disease outcomes in humans vary considerably from mild, non-inflammatory, watery, self-limiting diarrhea to severe, inflammatory, bloody diarrhea, and abdominal pain lasting for several weeks. In some individuals, however, *C. jejuni* is also associated with the development of post-infectious sequelae such as reactive arthritis and peripheral neuropathies including the Miller–Fisher and Guillain–Barré syndromes [5, 6]. Intestinal tissues of infected patients display histological changes such as apoptosis, crypt abscesses, ulcerations, and pronounced influx of pro-inflammatory immune cell populations such as lymphocytes and neutrophils into the intestinal mucosa and lamina propria [7, 8]. Although human campylobacteriosis is of global importance, efforts to understand *C. jejuni* infection were hampered for a long time by the lack of appropriate *in vivo* models. Chicken, newborn piglets, weanling ferrets, gnotobiotic canine pups, and primates have been more or less successfully used for studying *C. jejuni*-host interactions [9, 10]. Our group has recently shown that conventionally colonized infant mice developed self-limiting acute enterocolitis like in "classical" human campylobacteriosis within one week postinfection (p.i.) when infected with *C. jejuni* immediately after weaning at the age of 3 weeks [11, 12]. Hence, infant mice are well suited as *C. jejuni* infection model to study host–pathogen interactions *in vivo*.

Matrix metalloproteinases (MMPs) form a heterogenous family of zinc- and calcium-dependent matrix-degrading endopeptidases that are tightly controlled by endogenous inhibitors, namely, tissue inhibitors of matrix metalloproteinases (TIMPs) [13–15]. According to their substrate specificity, MMPs can be grouped into colla-

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genases (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) [16]. MMPs are pivotal in embryonic development, differentiation, proliferation, and regeneration of tissues [14, 15]. A dysbalanced equilibrium between MMP activating and inhibiting factors, however, leads to a plethora of diseases such as intestinal inflammation, arthritis, atherosclerosis, or cancer [17, 18]. Expression levels of the gelatinases A and B (MMP-2 and MMP-9, respectively) were shown to be upregulated in models of intestinal inflammation [19–21] and in patients suffering from human inflammatory bowel diseases including ulcerative colitis or Crohn's disease [22–24]. However, data regarding the impact of MMPs, particularly the gelatinases, in intestinal infection, such as *C. jejuni*, are scarce. Several expression studies have shown that distinct MMPs are upregulated during infection with Gram-negative bacteria [25]. For instance, increased MMP-3 transcripts could be detected in Peyer's patches following *Salmonella* and *Yersinia* infection [26], whereas MMP-2 and MMP-9 were shown to be upregulated in *Helicobacter pylori*-infected mice [27]. Moreover, human and murine *Helicobacter*-induced gastritis were associated with increased MMP-9 levels derived from macrophages [28] and upregulated TIMP-1 and TIMP-3 expression levels in glandular epithelium and stroma [29]. Interestingly, distinct *Escherichia coli*-derived serine proteases have been shown to act as specific activatiors of pro-MMP-2 [30]. We could show previously that selective gelatinase blockage by the synthetic compound RO28-2653 (blocking both MMP-2 and MMP-9) ameliorated acute ileitis [20] as well as acute colitis in mice [21]. Furthermore, we described in a very recent study that synthetic selective gelatinase could reduce large intestinal apoptosis and pro-inflammatory immune cell responses in *C. jejuni*-infected gnotobiotic IL-10$^{-/-}$ mice [31]. This prompted us to further dissect which gelatinase in particular mediates *C. jejuni*-induced disease. To address this, we infected 3-week-old infant MMP-2$^{-/-}$, MMP-9$^{-/-}$, and corresponding wildtype (WT) mice perorally with *C. jejuni* strain B2 immediately after weaning and investigated 1) the gastrointestinal colonization properties of *C. jejuni*, 2) the clinical course of infection, 3) the colonic histopathological changes including apoptosis, 4) the abundances of immune cell populations in the colonic mucosa and lamina propria *in situ*, and, furthermore, the large intestinal expression of 5) pro-inflammatory cytokines including IL-23, IL-22, and IL-18 and, finally, of 6) gelatinases and TIMPs.

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". 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; [21]) 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 expresion, genomic DNA was isolated and disruption of either gene was confirmed by polymerase chain reaction (PCR) [21].

Immediately after weaning, 3-week-old infant mice were perorally infected with 10^9 colony forming units (CFU) of viable *C. jejuni* strain B2 in a volume of 0.3 ml phosphate buffered saline (PBS) on two consecutive days (day 0 and day 1) by gavage [32].

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, prefinal aspect), was used [31, 33].

Sampling procedures

Mice were sacrificed at day 6 p.i. by isofluran treatment (Abbott, Greifswald, Germany). Colonic *ex vivo* biopsies were removed under sterile conditions and 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 [31, 34].

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 [35]. In brief:

Colonic histopathology (max. 4 points; according to Ref. [36]): 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 [37–40]. Primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, Beverly, MA, USA, 1:200), myeloperoxidase-7 (MPO-7, # A0398, Dako, 1:500), CD3 (#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, $400\times$ magnification) were determined microscopically by a double-blinded investigator.

Quantitative analysis of C. jejuni *colonization and translocation*

Viable *C. jejuni* was detected in feces or at time of necropsy (day 6 p.i.) in luminal samples taken from the 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 (approximately 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 approximately 100 CFU per g.

Cytokine detection in supernatants of colonic ex vivo *biopsies*

Colonic *ex vivo* biopsies were cut longitudinally and washed in PBS. Strips of approximately 1 cm² colonic 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 were tested for TNF 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 [20]. Murine IL-23p19, IL-22, IL-18, MMP-2, MMP-9, TIMP-1, and TIMP-3 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".

Statistical analysis

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

Results

Given that selective gelatinase blockage has recently been shown to reduce intestinal apoptosis and pro-inflammatory immune responses in *C. jejuni*-infected mice [31], we further investigated the distinct roles of gelatinases A and B (MMP-2 and MMP-9, respectively) in murine *C. jejuni* infection in the present study. To address this, we applied the infant mouse infection model [11].

Coloniziation properties of C. jejuni *strain B2 in infant MMP-2$^{-/-}$ and MMP-9$^{-/-}$ mice and pathogenic translocation*

Immediately after weaning, conventional MMP-2$^{-/-}$, MMP-9$^{-/-}$, and WT mice were perorally infected with 10^9 CFU of viable *C. jejuni* strain B2 on two consecutive days (day 0 and day 1) by gavage. At day 2 p.i. (i.e., the first day after the latest *C. jejuni* challenge), mice harbored comparable pathogenic loads in their feces of approximately 10^4 to 10^5 CFU per gram feces *(Fig. 1a)*. One day thereafter, however, gelatinase-deficient mice had virtually expelled the pathogen from their intestinal tract given that, only in 22.2% of MMP-9$^{-/-}$ and 10.0% of MMP-2$^{-/-}$ mice, viable *C. jejuni* could be isolated from fecal samples at day 3 p.i. *(Fig. 1b)*. Conversely, 81.8% of infected WT mice still harbored *C. jejuni* at day 3 p.i. ($p < 0.05$ and $p < 0.01$ versus MMP-9$^{-/-}$ and MMP-2$^{-/-}$ mice, respectively; *Fig. 1b*). At time of necropsy (i.e., day 6 p.i.), *C. jejuni* could be cultured in 18.2% of WT and 20.0% of MMP-2$^{-/-}$ mice from fecal samples, whereas MMP-9$^{-/-}$ mice had com-
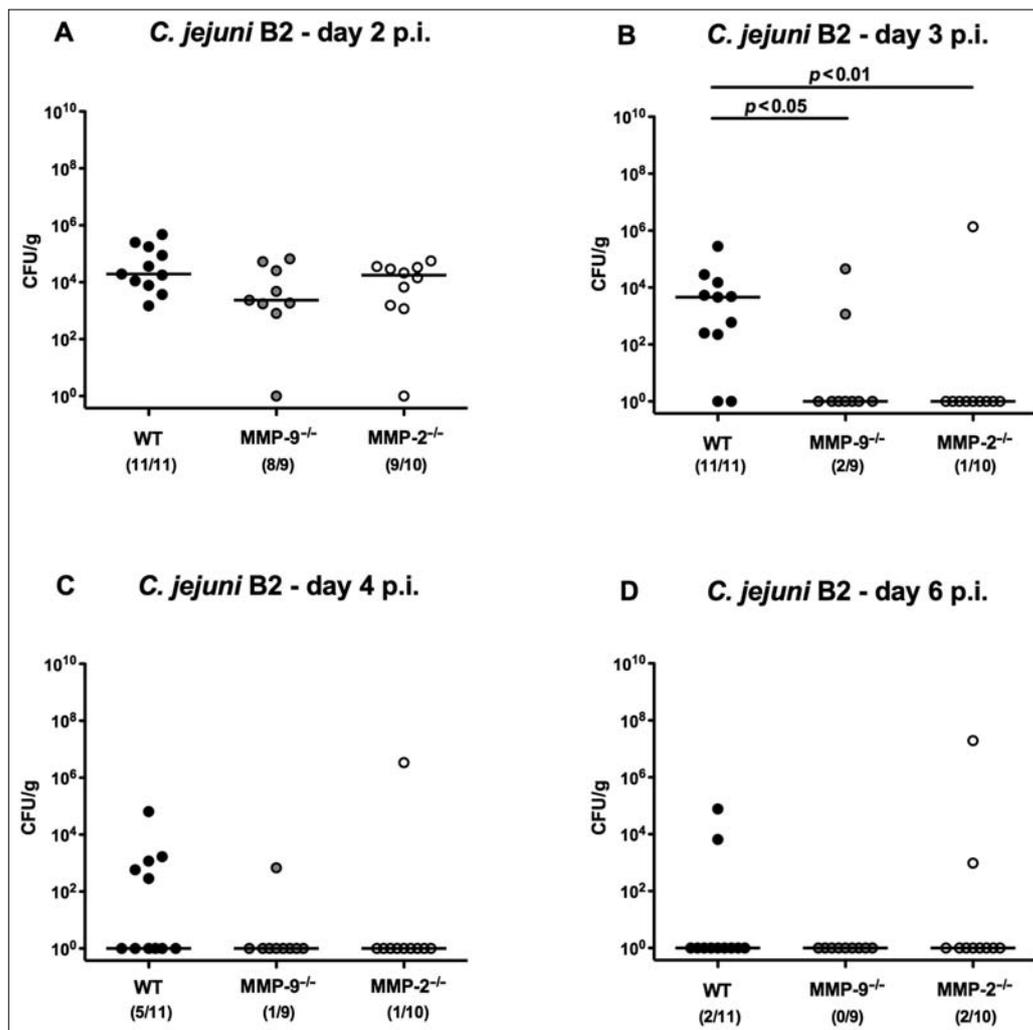
Fig. 1. Fecal loads of *C. jejuni* strain B2 in perorally infected infant mice lacking MMP-2 or MMP-9. Immediately after weaning infant wildtype (WT; black circles), MMP-9$^{-/-}$ (grey circles), and MMP-2$^{-/-}$ (white circles) mice were perorally infected with *C. jejuni* B2 by gavage. Pathogenic loads were determined in fecal samples (CFU, colony forming units per gram) at (A) day 2, (B) day 3, (C) day 4, and (D) day 6 postinfection (p.i.) by culture. Numbers of mice harboring *C. jejuni* B2 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

pletely expelled the pathogen *(Fig. 1d)*. When surveying the intestinal pathogenic colonization densities over time, fecal *C. jejuni* loads dropped significantly from day 2 to day 3 p.i. irrespective of the genotype of mice, as indicated by significantly higher *C. jejuni* loads at day 2 as compared to day 3, day 4, and day 6 p.i. ($p < 0.05$–0.001; *Fig. 2*). Furthermore, in WT mice, *C. jejuni* loads were higher in fecal samples taken at day 3 p.i. as compared to day 6 p.i. ($p < 0.05$; *Fig. 2a*). Notably, we did not observe any bacterial translocation from the intestinal tract to extra-intestinal compartments given that neither viable *C. jejuni* B2 nor any bacterial species originating from the commensal intestinal microbiota could be cultured from MLNs, spleen, liver, kidney, or cardiac blood by direct plating (not shown). Taken together, infant MMP-2$^{-/-}$, MMP-9$^{-/-}$ and WT mice harboring a conventional gut microbiota could, surprisingly, not be stably infected with the *C. jejuni* strain B2.

Macroscopic and microscopic signs of intestinal disease in infant MMP-2$^{-/-}$ and MMP-9$^{-/-}$ mice following C. jejuni *strain B2 infection*

We additionally surveyed the clinical conditions of mice including the occurrence of blood in fecal samples following *C. jejuni* infection, a hallmark of human campylobacteriosis. Despite comparable intestinal *C. jejuni* loads, gelatinase-deficient mice exhibited less frequently gross or microscopic abundances of blood in their feces at day 2 and day 4 p.i. *(Fig. 3)*. Whereas, in 63.6% of WT mice, blood-positive fecal samples could be detected at day 2 p.i., only 45.5% of MMP-9$^{-/-}$ and 9.1% of MMP-2$^{-/-}$ mice exhibited bloody feces *(Fig. 3)*. At day of necropsy, 9.1% and 11.1% of WT and MMP-9$^{-/-}$ mice, respectively, displayed abundances of blood in their feces, whereas, in none of the fecal samples derived from MMP-2$^{-/-}$ mice, could blood be detected at day 6 p.i., neither macroscopi-
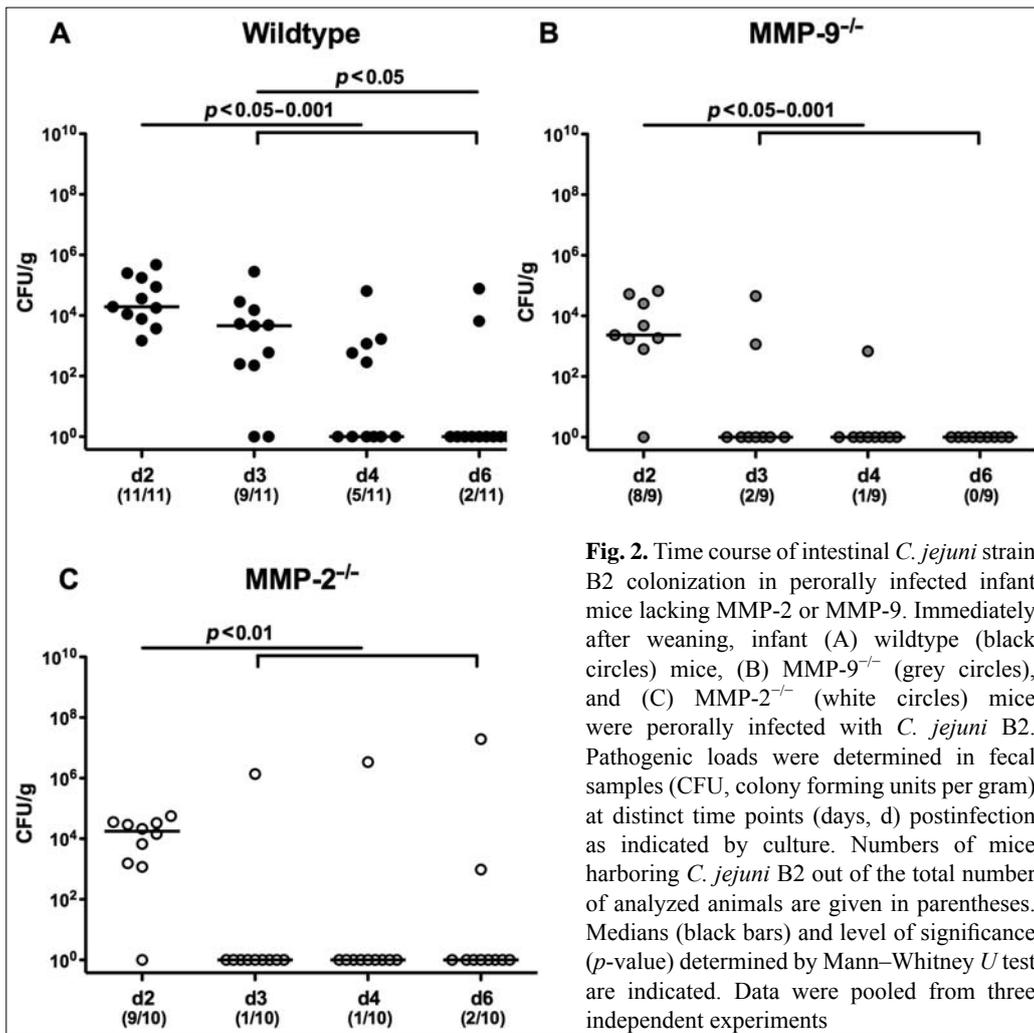
Fig. 2. Time course of intestinal *C. jejuni* strain B2 colonization in perorally infected infant mice lacking MMP-2 or MMP-9. Immediately after weaning, infant (A) wildtype (black circles) mice, (B) MMP-9$^{-/-}$ (grey circles), and (C) MMP-2$^{-/-}$ (white circles) mice were perorally infected with *C. jejuni* B2. Pathogenic loads were determined in fecal samples (CFU, colony forming units per gram) at distinct time points (days, d) postinfection as indicated by culture. Numbers of mice harboring *C. jejuni* B2 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

cally nor microscopically *(Fig. 3)*. Hence, MMP-2$^{-/-}$ mice displayed less overt clinical signs of campylobacteriosis as compared to WT control animals upon peroral *C. jejuni* B2 strain infection.

We next determined potential *C. jejuni*-induced colonic histopathological sequelae in gelatinase deficient mice. Irrespective of the genotype, infected mice displayed higher histopathological scores at day 6 p.i. as compared to naive controls (*p* < 0.01–0.001; *Fig. 4a*), despite expulsion of

the pathogen from murine intestines between days 3 and 4 p.i. *(Fig. 1)*. Of note, overall, the observed histopathological lesions were rather mild to moderate. Histopathological changes in the large intestinal mucosa were, however, less pronounced in infected MMP-2$^{-/-}$ when compared to WT mice as indicated by slightly lower histopathological scores in the former at day 6 p.i. (*p* < 0.05; *Fig. 4a*). Given that apoptosis is a commonly used diagnostic marker in the histopathological evaluation and grading of intestinal
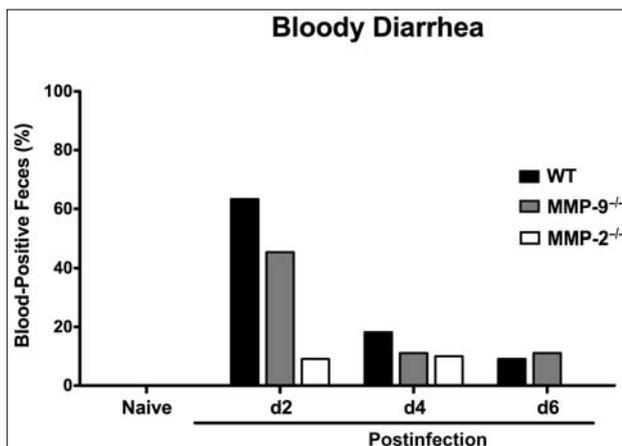

Fig. 3. Kinetic survey of occurrence of bloody diarrhea in *C. jejuni* strain B2-infected infant mice lacking MMP-2 or MMP-9. Immediately after weaning, infant wildtype (WT; black bars), MMP-9$^{-/-}$ (grey bars), and MMP-2$^{-/-}$ (white bars) mice were perorally infected with *C. jejuni* B2. Naive mice served as uninfected controls. Occurrence of bloody diarrhea was surveyed during the course of infection by microscopic (applying the Guajac method) or macroscopic detection of blood in fecal samples at distinct days (d) postinfection as indicated. Data were pooled from three independent experiments
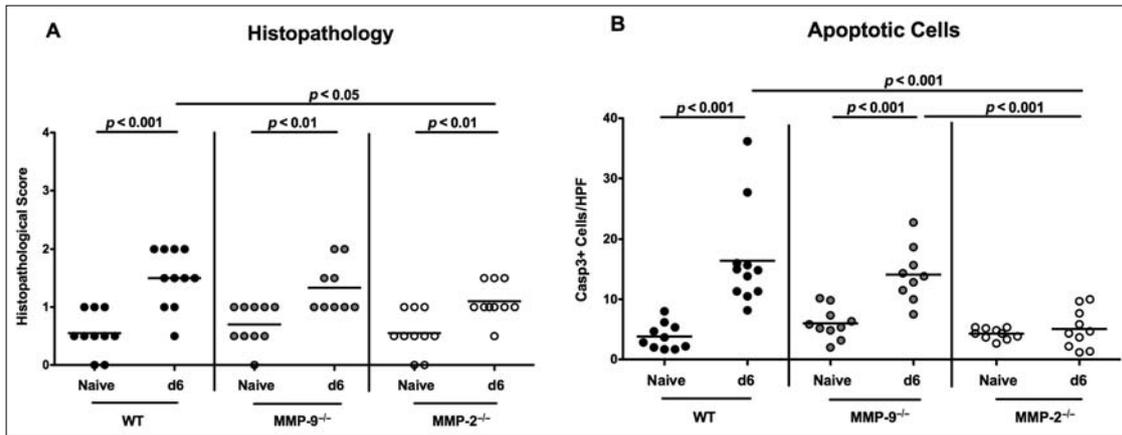
Fig. 4. Histopathological and apoptotic changes in the colonic mucosa of *C. jejuni* strain B2-infected infant mice lacking MMP-2 or MMP-9. Immediately after weaning, infant wildtype (WT; black circles), MMP-9$^{-/-}$ (grey circles), and MMP-2$^{-/-}$ (white circles) mice were perorally infected with *C. jejuni* B2. Naive mice served as uninfected controls. (A) At day six postinfection (d6 p.i.), histopathological changes of the colonic mucosa were assessed in H&E stained colonic paraffin sections applying a standardized histopathological scoring system. (B) The average number of colonic apoptotic cells (positive for caspase-3, Casp3) from at least six high power fields (HPF, 400× magnification) per animal was determined microscopically in immunohistochemically stained colonic paraffin sections at day six postinfection. Medians (black bars) and level of significance (*p*-value) determined by Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments
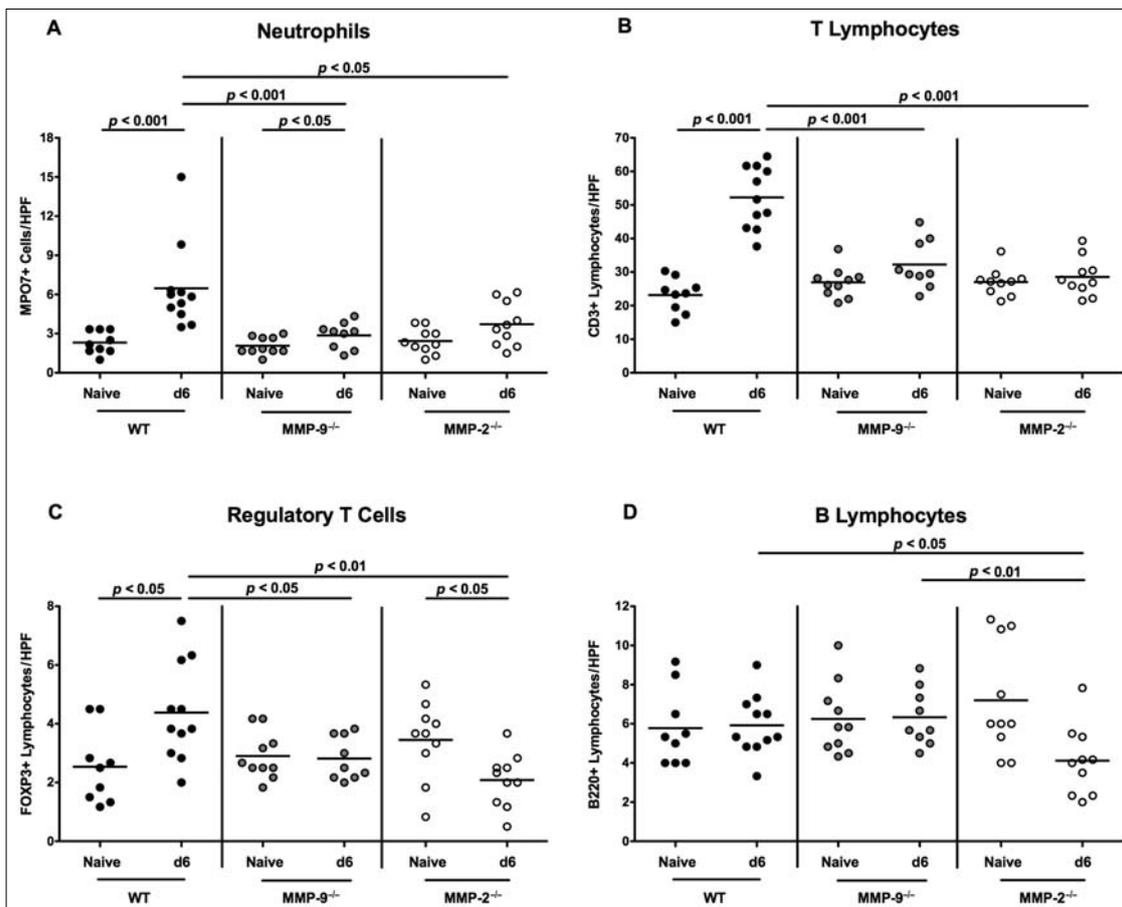

Fig. 5. Colonic immune cell responses in *C. jejuni* strain B2-infected infant mice lacking MMP-2 or MMP-9. Immediately after weaning, infant wildtype (WT; black circles), MMP-9$^{-/-}$ (grey circles), and MMP-2$^{-/-}$ (white circles) mice were perorally infected with *C. jejuni* B2. Naive mice served as uninfected controls. The average number of cells positive for (A) MPO7 (neutrophils), (B) CD3 (T Lymphocytes), (C) FOXP3 (regulatory T cells), and (D) 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 six postinfection (d6 p.i.). Medians (black bars) and significance levels as determined by the Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments

disease [32] and a hallmark of *C. jejuni*-induced enterocolitis in infected mice [11, 32], we quantitatively assessed numbers of caspase-3+ cells within the colonic epithelium of infected mice. Whereas following infection abundances of caspase-3+ cells increased in WT and MMP-9$^{-/-}$ ($p < 0.001$; *Fig. 4b*), but not MMP-2$^{-/-}$ mice, numbers of apoptotic cells within the colonic mucosa were approximately two thirds lower in MMP-2$^{-/-}$, but not MMP-9$^{-/-}$ mice as compared to WT controls at day 6 p.i. ($p < 0.001$; *Fig. 4b*). Taken together, MMP-2$^{-/-}$ mice did not only display less macroscopic (i.e., clinical) signs of campylobacteriosis but also less pronounced microscopic sequelae of *C. jejuni* infection as indicated by less severe histopathological changes and lower abundances of apoptotic cells in the colonic epithelium.

Innate and adaptive immune cell responses in infant MMP-2$^{-/-}$ and MMP-9$^{-/-}$ mice following C. jejuni strain B2 infection

It is well known that recruitment of pro-inflammatory immune cells to sites of inflammation is a key feature of enteric pathogen infection (e.g., campylobacteriosis; [32]). We therefore 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. Upon *C. jejuni* strain B2 infection, numbers of MPO-7+ cells (i.e., neutrophils) increased in large intestines of WT and MMP-9$^{-/-}$ ($p < 0.001$ and $p < 0.05$ versus naive controls, respectively; *Fig. 5a*), but not MMP-2$^{-/-}$ mice. At day 6 p.i., colonic neutrophilic numbers were lower in MMP-9$^{-/-}$ and MMP-2$^{-/-}$ mice as compared to WT animals ($p < 0.001$ and $p < 0.05$, respectively; *Fig. 5a*). Following *C. jejuni* infection, numbers of colonic CD3+ T lymphocytes and FOXP3+ regulatory T cells (Tregs) increased in WT mice only ($p < 0.001$ and $p < 0.05$, respectively; *Fig. 5b* and *c*). As for neutrophilic granulocytes, numbers of colonic T cells ($p < 0.001$; *Fig. 5b*) and Tregs ($p < 0.05$ and $p < 0.01$, respectively; *Fig. 5c*) were lower in MMP-9$^{-/-}$ and MMP-2$^{-/-}$ as compared to WT mice at day

6 p.i. Interestingly, large intestinal Tregs even decreased in MMP-2$^{-/-}$ mice upon *C. jejuni* B2 strain infection ($p < 0.05$; *Fig. 5c*). Furthermore, colonic B220+ B lymphocytes were lower in infected MMP-2$^{-/-}$ mice as compared to WT and MMP-9$^{-/-}$ animals at day 6 p.i. ($p < 0.05$ and $p < 0.01$, respectively; *Fig. 5d*). Taken together, large intestinal innate immune and effector cell responses were less distinct in *C. jejuni*-infected MMP-2$^{-/-}$ and MMP-9$^{-/-}$ mice.

Expression of intestinal pro-inflammatory cytokines, MMPs and TIMPs in infant MMP-2$^{-/-}$ and MMP-9$^{-/-}$ mice following C. jejuni strain B2 infection

We next determined expression levels of pro-inflammatory cytokines in colonic *ex vivo* biopsies derived from *C. jejuni* strain B2-infected MMP-2$^{-/-}$ and MMP-9$^{-/-}$ mice. Upon infection, secreted TNF protein levels measured in supernatants of colonic tissue samples increased in WT and MMP-2$^{-/-}$, but not MMP-9$^{-/-}$ mice ($p < 0.05$; *Fig. 6*). Furthermore, large intestinal TNF levels were higher in MMP-9$^{-/-}$ as compared to WT mice at day 6 p.i. ($p < 0.05$; *Fig. 6*).

We next determined mRNA expression levels of mediators belonging to the IL-23/IL-22/IL-18 axis during *C. jejuni* infection of gelatinase-deficient mice. Interestingly, naive MMP-2$^{-/-}$ mice exhibited higher basal IL-23p19, IL-22, and IL-18 mRNA expression levels in the colon as compared to uninfected WT mice ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; *Fig. 7*). Upon peroral *C. jejuni* strain B2 infection, IL-23p19 ($p < 0.01$ and $p < 0.001$, respectively; *Fig. 7a*) and IL-18 ($p < 0.05$; *Fig. 7c*) mRNA levels increased in WT and MMP-9$^{-/-}$, but not MMP-2$^{-/-}$ mice, whereas IL-22 mRNA expression levels were upregulated in colons derived from infected mice of either genotype ($p < 0.05$; *Fig. 7b*). In infected MMP-2$^{-/-}$ mice, colonic IL-22 mRNA levels were higher as compared to WT mice ($p < 0.05$; *Fig. 7b*), whereas, at day 6 p.i., IL-18 mRNA was upregulated as compared to MMP-9$^{-/-}$ mice ($p < 0.05$; *Fig. 7c*).

We finally adressed the question how counter-regulatory TIMPs as endogenous inhibitors of MMPs and
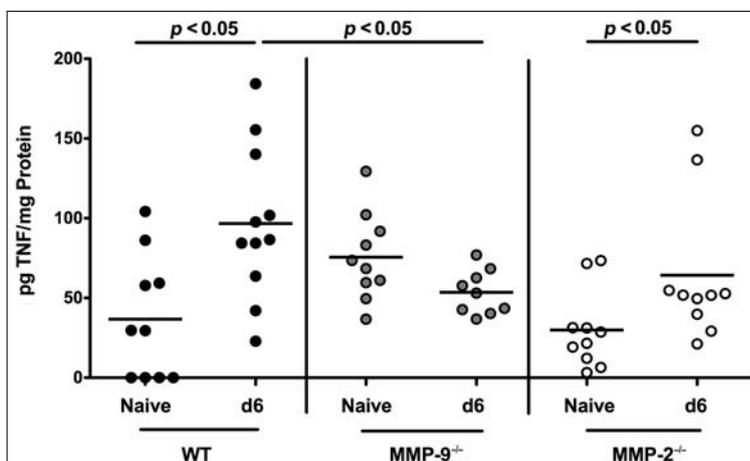

Fig. 6. Colonic TNF levels in *C. jejuni* strain B2-infected infant mice lacking MMP-2 or MMP-9. Immediately after weaning, infant wildtype (WT; black circles), MMP-9$^{-/-}$ (grey circles), and MMP-2$^{-/-}$ (white circles) mice were perorally infected with *C. jejuni* B2. Naive mice served as uninfected controls. TNF concentrations were determined in supernatants of colonic *ex vivo* biopsies at day six postinfection (d6 p.i.). Medians (black bars) and significance levels as determined by the Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments
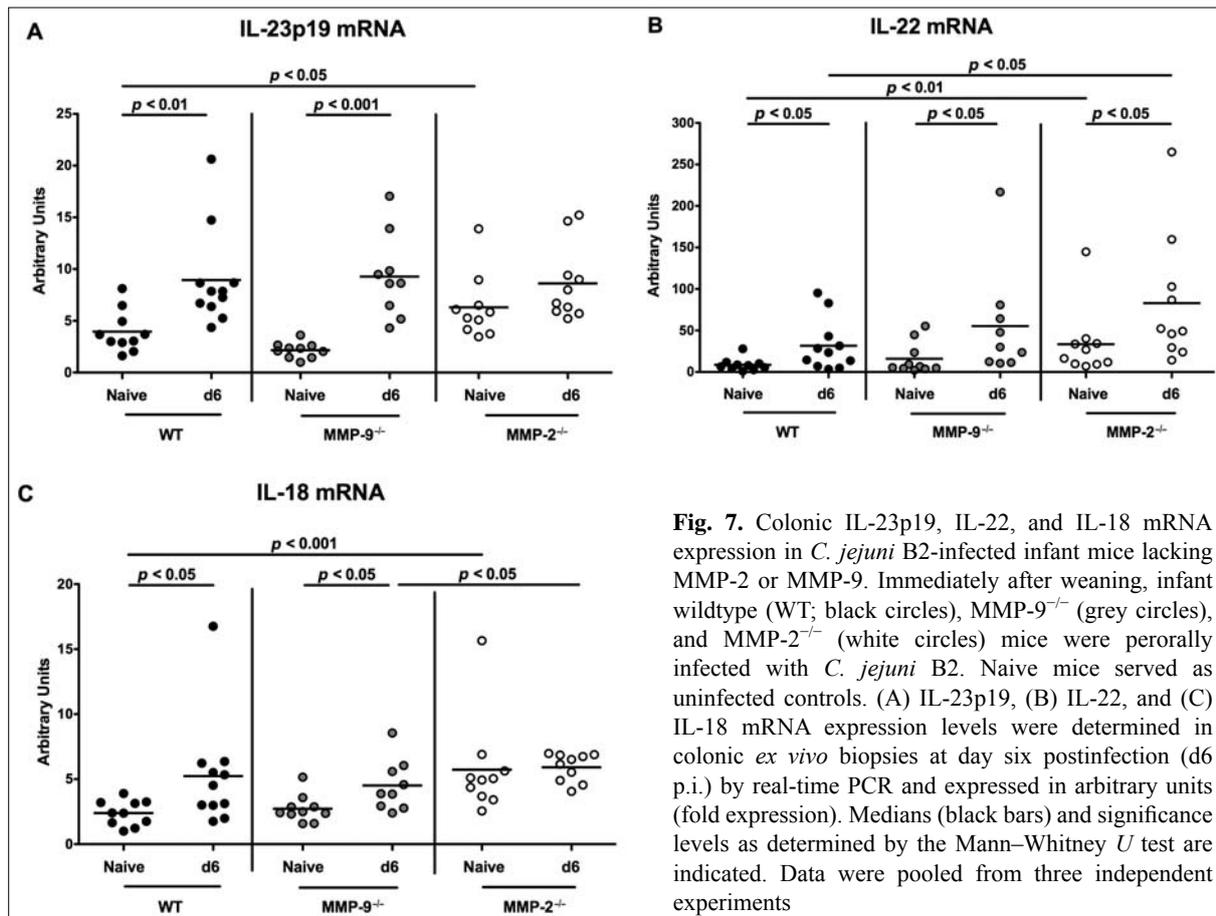
Fig. 7. Colonic IL-23p19, IL-22, and IL-18 mRNA expression in *C. jejuni* B2-infected infant mice lacking MMP-2 or MMP-9. Immediately after weaning, infant wildtype (WT; black circles), MMP-9$^{-/-}$ (grey circles), and MMP-2$^{-/-}$ (white circles) mice were perorally infected with *C. jejuni* B2. Naive mice served as uninfected controls. (A) IL-23p19, (B) IL-22, and (C) IL-18 mRNA expression levels were determined in colonic *ex vivo* biopsies at day six postinfection (d6 p.i.) by real-time PCR and expressed in arbitrary units (fold expression). Medians (black bars) and significance levels as determined by the Mann–Whitney U test are indicated. Data were pooled from three independent experiments

either gelatinase were regulated following *C. jejuni* infection of mice that were deficient for MMP-2 or MMP-9. As expected, neither MMP-2 mRNA could be detected in colonic samples derived from MMP-2$^{-/-}$ mice, nor MMP-9 in MMP-9$^{-/-}$ animals at either time point (*Fig. 8a* and *b*). Colonic MMP-2 mRNA levels were upregulated 6 days following *C. jejuni* strain B2 infection of WT as well as of MMP-9$^{-/-}$ mice ($p < 0.05$ and $p < 0.001$, respectively; *Fig. 8a*), further underlining an important role of MMP-2 in mediating *C. jejuni* infection of infant mice. Conversely, MMP-9 mRNA levels were even lower in large intestines of infected MMP-2$^{-/-}$ as compared to naive MMP-2$^{-/-}$ mice ($p < 0.001$; *Fig. 8b*). Furthermore, colonic TIMP-1 and TIMP-3 mRNA were upregulated upon *C. jejuni* infection of MMP-9$^{-/-}$ mice only ($p < 0.01$ and $p < 0.0005$, respectively; *Fig. 8c* and *d*).

Taken together, following peroral *C. jejuni* strain B2 infection, the pathogen was expelled early in the course of infection. Nevertheless, in MMP-2$^{-/-}$ mice, *C. jejuni*-induced 1) less macroscopic disease as indicated by less frequent abundance of blood in feces, 2) less microscopic sequelae of infection (as indicated by less colonic histopathology and apoptosis), which was accompanied by 3) less influx of effector as well as innate and adaptive immune cells into the colonic mucosa and lamina propria, 4) higher colonic IL-22, but (5) lower MMP-9 mRNA levels as compared to infected WT mice.

Discussion

MMP-2 and MMP-9 are key players in mediating intestinal inflammation of mice and men [19–24, 31]. Data regarding the impact of gelatinases in *C. jejuni* infections, however, are scarce. Very recently and, for the first time, we provided indirect evidence that the gelatinases are indeed important for *C. jejuni* immunopathogenesis, given that selective gelatinase blockage by the synthetic compound RO28-2653 reduced colonic pro-inflammatory immune responses and apoptosis in *C. jejuni* 81-176 strain-infected gnotobiotic IL-10$^{-/-}$ mice [31]. To address the question which gelatinase specifically mediates murine campylobacteriosis, we applied the infant mouse infection model in the present study. We here demonstrate that particularly MMP-2, rather than MMP-9, has a major impact during *C. jejuni* infection *in vivo*. Strikingly, colonic mRNA expression of MMP-2 was upregulated in both *C. jejuni* strain B2-infected WT and MMP-9$^{-/-}$ mice, whereas, conversely, MMP-9 was not – either in large intestines of WT or in MMP-2$^{-/-}$ mice at day 6 p.i. Furthermore, colonic MMP-9 mRNA was even down-regulated in infected MMP-2$^{-/-}$ mice. Remarkably, MMP-2$^{-/-}$ mice displayed less macroscopic disease as well as less microscopic sequelae of *C. jejuni* infection as indicated by less colonic histopathology and apoptosis, which was accompanied by less distinct pro-inflammatory immune cell responses. Our results presented here are well in line with our recent in-
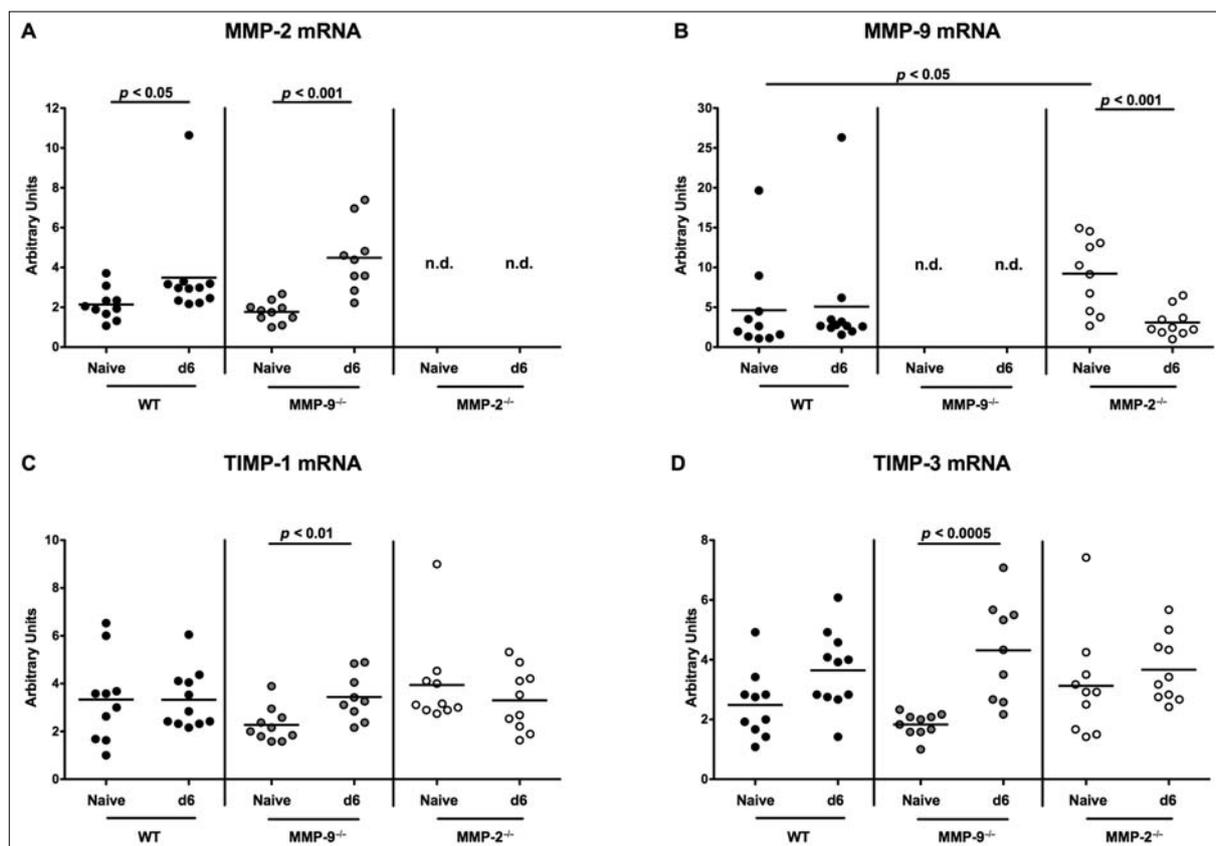
Fig. 8. Colonic MMP-2, MMP-9, TIMP-1, and TIMP-3 mRNA expression in *C. jejuni* strain B2-infected infant mice lacking MMP-2 or MMP-9. Immediately after weaning, infant wildtype (WT; black circles), MMP-9$^{-/-}$ (grey circles), and MMP-2$^{-/-}$ (white circles) mice were perorally infected with *C. jejuni* B2. Naive mice served as uninfected controls. (A) MMP-2, (B) MMP-9, (C) TIMP-1, and (D) TIMP-3 mRNA expression levels were determined in colonic *ex vivo* biopsies at day six postinfection (d6 p.i.) by real-time PCR and expressed in arbitrary units (fold expression). Medians (black bars) and significance levels as determined by the Mann–Whitney *U* test are indicated. Data were pooled from three independent experiments

testinal inflammation studies showing that MMP-2 but not MMP-9 is essentially involved in mediating acute *Toxoplasma* (*T.*) *gondii*-induced ileitis [20] and acute dextrane sodium sulphate (DSS)-induced colitis [21] in mice.

The distinct MMP-2 dependent disease outcome of *C. jejuni* infection is somewhat remarkable since the pathogen was expelled rather early in the course of infection and could not be isolated from fecal samples as early as 48 to 72 h following the latest of two consecutive peroral *C. jejuni* B2 strain infections with high loads. In our previous study with a different pathogenic strain, namely, *C. jejuni* 81-176, infant wildtype mice developed acute enterocolitis within 1 week following peroral infection immediately after weaning that resolved after another week [11]. Furthermore, after the acute episode, mice were asymptomatic carriers and shedders of *C. jejuni* for more than 100 day p.i. and exhibited intestinal and extraintestinal immune responses despite absence of any clinical signs [11, 12]. One might argue that one of the reasons why infected WT mice displayed more pronounced immunopathological sequelae than MMP-2$^{-/-}$ mice could be that the pathogenic loads in the intestinal tract of WT animals were higher as compared to those in gelatinase-deficient mice at day 3 p.i. However, before and thereafter,

the colonization densities were comparable between WT, MMP-2$^{-/-}$ and MMP-9$^{-/-}$ mice, and rather the initial hit of infection might tip the balance towards immunopathological response. The better infection-induced outcome in MMP-2$^{-/-}$ mice is well in line with results from our previous gelatinase-blocking *in vivo* study [31]. Notably, the latter was performed in a different infection model, namely, in gnotobiotic IL-10$^{-/-}$ mice. Within 1 week following *C. jejuni* infection, gnotobiotic IL-10$^{-/-}$ mice in which the intestinal microbiota was depleted by broad-spectrum antibiotic treatment develop non-self-limiting acute enterocolitis mimicking key features of campylobacteriosis in immunocompromized humans [33–35]. Like in the present study, RO28-2653-treated mice exhibited better clinical conditions including less frequent bloody diarrhea, less colonic apoptosis (but with similar histopathological changes), and lower T and B cell numbers (and a trend towards lower neutrophilic abundance) as compared to placebo control animals [31]. The better outcome in *C. jejuni*-infected MMP-2$^{-/-}$ as compared to WT mice was also associated with increased large intestinal IL-22 mRNA levels. IL-22 is a IL-10 cytokine family member and exerts antimicrobial, tissue-protective, and wound-healing responses from epithelial cells following infection and in-

flammation [41, 42]. IL-22 exerts its dichotomous actions in the gut in a tissue-depending manner. Whereas IL-22 has been shown to act as an anti-inflammatory mediator in colonic tissue, we have previously reported its pro-inflammatory properties in the small intestine. In acute *T. gondii*-induced ileitis, IL-23 caused small intestinal immunopathology via the upregulation of MMP-2 and, surprisingly, via the induction of IL-22 [20]. In our study here, IL-23 was upregulated in *C. jejuni*-infected WT and MMP-9$^{-/-}$ but not MMP-2$^{-/-}$ mice. In line with its anti-inflammatory properties in the large intestines, the elevated colonic IL-22 mRNA levels observed in MMP-2$^{-/-}$ mice here might explain the less pronounced pro-inflammatory immune responses and the better clinical and histopathological outcome at day 6 following *C. jejuni* strain B2 infection. Interestingly, basal large intestinal IL-22 level was higher in uninfected MMP-2$^{-/-}$ as compared to WT mice. We are currently further elucidating whether IL-22 directly induces MMP-2 leading to intestinal apoptosis following *C. jejuni* infection and whether IL-23 acts as a key regulator within this inflammatory scenario. Our results are supported by data derived from an *ex vivo* human gut infection model. *C. jejuni* induced increases in IL-23 and IL-22 concentrations in supernatants of *ex vivo* colonic biopsies [43]. Moreover, Malik and colleagues demonstrated that, as early as 4 days following oral infection with *C. jejuni*, IL-22 secretion was increased in MLNs and colons of IL-10$^{-/-}$ mice, whereas both T cells and innate lymphoid cells (ILCs) contributed to upregulated IL-22 levels in a time- and organ-specific fashion [44].

Conclusion

In conclusion, our study underlines the importance of gelatinases, particularly of MMP-2, in the immunopathogenesis of *C. jejuni* infection. Further studies need to unravel the regulatory pathways of MMP-2 and IL-22 following *C. jejuni* infection in order to better understand molecular mechanisms underlying human campylobacteriosis.

Competing interests

The authors declare that no competing interests exist.

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