

CAMPYLOBACTER JEJUNI INDUCES EXTRA-INTESTINAL IMMUNE RESPONSES VIA TOLL-LIKE-RECEPTOR-4 SIGNALING IN CONVENTIONAL IL-10 DEFICIENT MICE WITH CHRONIC COLITIS

B. Otto^{1*}, L.-M. Haag^{1*}, A. Fischer¹, R. Plickert¹, A. A. Kühl², U. B. Göbel¹, M. M. Heimesaat^{1**}, ***
and S. Bereswill^{1**}

¹Department of Microbiology and Hygiene, Charité — University Medicine Berlin, Berlin, Germany

²Department of Internal Medicine, Rheumatology and Clinical Immunology / Research Center Immuno-Sciences (RCIS), Charité — University Medicine Berlin, Berlin, Germany

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Campylobacter jejuni is one of the predominant causes for foodborne bacterial infections worldwide. We investigated whether signaling of *C. jejuni*-lipoproteins and -lipooligosaccharide via Toll-like-receptor (TLR) -2 and -4, respectively, is inducing intestinal and extra-intestinal immune responses following infection of conventional IL-10^{-/-} mice with chronic colitis. At day 3 following oral infection, IL-10^{-/-} mice lacking TLR-2 or TLR-4 harbored comparable *C. jejuni* strain ATCC 43431 loads in their colon. Interestingly, infected TLR-4^{-/-} IL-10^{-/-} mice displayed less compromised epithelial barrier function as indicated by lower translocation rates of live gut commensals into mesenteric lymphnodes (MLNs), and exhibited less distinct B lymphocyte responses in their colonic mucosa as compared to naïve IL-10^{-/-} controls. Furthermore, in extra-intestinal compartments such as MLNs and spleens, abundance of myeloid cells was less distinct whereas relative percentages of activated T helper cells and cytotoxic T cells were higher in spleens and dendritic cells more abundant in MLNs of infected IL-10^{-/-} animals lacking TLR-4 as compared to IL-10^{-/-} controls. Taken together, in conventionally colonized IL-10^{-/-} mice, TLR-4, but not TLR-2, is involved in mediating extra-intestinal pro-inflammatory immune responses following *C. jejuni* infection. Thus, conventional IL-10^{-/-} mice are well suited to further dissect mechanisms underlying *Campylobacter* infections *in vivo*.

Keywords: *Campylobacter jejuni*, chronic colitis, IL-10, TLR-2, TLR-4, lipopeptide, lipopolysaccharide, host–pathogen interaction, innate and adaptive immune responses, pro-inflammatory cytokines, conventional intestinal microbiota

Abbreviations: GB, gnotobiotic; CR, colonization resistance; p.i., post infection; CFU, colony forming units; spp., species; IBD, inflammatory bowel diseases; TLR, Toll-like receptor; PAMP, pathogen-associated molecular patterns; LPS, lipopolysaccharide; LOS, lipooligosaccharide; LP, lipoprotein; MLN, mesenteric lymph nodes; DC, dendritic cell; Th1, T helper 1 cell; NaCl, sodium chloride; EDTA, ethylene diamine tetraacetic acid; FCS, fetal calf serum; NF-κB, nuclear factor ‘kappa-light-chain-enhancer’ of activated B-cells; MUC, mucin; HPRT, hypoxanthine phosphoribosyltransferase 1; PBS, phosphate buffered saline; TNF, tumor necrosis factor; IFN, interferon; MCP, monocyte chemotactic protein

Introduction

Campylobacter (C.) jejuni is one of the most common causes for acute diarrhea in humans worldwide. Campylobacteriosis is characterized by fever, watery to bloody diarrhea, and abdominal cramping [1, 2]. Although the course of disease is self-limiting in the vast majority of infections, post-infectious complications such as Guillain–Barré and Reiter’s syndrome may occur [3, 4]. *C. jejuni* infections mainly affect the colon [5, 6] and induce the recruitment of immune cells such as neutrophilic granulocytes and mononuclear cells into the lamina propria, leading to crypt

abscesses, focal ulcerations, and plasma cell proliferation [7, 8]. Despite the high prevalence of *C. jejuni* induced disease and its distinctive socioeconomic impact, molecular and cellular events leading to campylobacteriosis are still poorly understood [9]. A very recent review article from Ó Cróinín and Backert highlighted various pathogenicity factors and host cell determinants proposed to be involved in establishing *C. jejuni* infection and triggering disease [10]. Mechanisms of intestinal inflammation are scarcely investigated due to a lack of suitable vertebrate models mimicking human disease. Mice harboring a conventional gut microbiota display a physiological colonization resistance

*These authors contributed equally to this work.

**These authors contributed equally to this work.

***Corresponding author: Markus M. Heimesaat; Charité – University Medicine Berlin, CC5, Department of Microbiology and Hygiene, Campus Benjamin Franklin, Hindenburgdamm 27, D-12203 Berlin, Germany; Phone: +49-30-8445-3739; Fax: +49-30-450-524-902; E-mail: markus.heimesaat@charite.de

against *C. jejuni* preventing infection by the pathogen [4, 11]. The colonization resistance, however, can be overcome by modifying or eradicating the microbiota within the gastrointestinal tract. Already two decades ago germfree mice raised in isolators have been shown to be stably colonized following *C. jejuni* infection [12–16]. Germfree mice, however, do not represent an appropriate experimental model due to their lack of an intact immune system and the need for an extensive infrastructure in order to accomplish breeding, housing, and performing experiments under absolute sterile conditions. In mice harboring a limited enteric flora, *C. jejuni* could colonize at least 5 months [17]. In addition, gnotobiotic (GB) wildtype mice generated by quintuple antibiotic treatment of conventionally colonized mice (displaying an entirely developed immune system) for 8–10 weeks, GB mice reconstituted with a human microbiota as well as GB animals fed a Western style cafeteria diet could be stably colonized by *C. jejuni* inducing a pro-inflammatory immune response in the colonic mucosa [18, 19]. In these animals lacking any gut bacteria or harboring a modified intestinal microbiota classical symptoms of human campylobacteriosis such as bloody diarrhea, however, were missing. Therefore, we established another infection model: three-week-old infant mice, harboring a different gastrointestinal microflora when compared to adult mice 3 months of age, were infected with *C. jejuni* right after weaning and developed severe ulcerative enterocolitis with bloody diarrhea until day 7 post infection (p.i.) which was self-limiting and resolved within 2 weeks following infection [20]. In the inflamed colonic mucosa and lamina propria, a significant influx of innate and adaptive immune cells could be observed [20]. Most strikingly, in conventional adult mice in which the intestinal *Escherichia coli* loads had been artificially elevated thereby mimicking microbiota shifts seen in conditions of acute intestinal inflammation [21–24], *C. jejuni* was able to overcome colonization resistance and induced enterocolitis [25].

Toll-like receptors (TLRs) are pattern-recognition receptors of the innate immune system recognizing microbial components known as PAMPs (pathogen-associated molecular patterns) which are essential for the survival of microorganisms [26]. The lipopolysaccharide (LPS) and lipooligosaccharide (LOS) of Gram-negative bacteria are recognized by TLR-4 whereas lipoproteins, lipoteichoic acid, and peptidoglycan activate TLR-2. TLRs are expressed on various immune cells including dendritic cells (DCs), B lymphocytes and macrophages as well as epithelial cells [26]. TLRs activate the NF- κ B-pathway which in turn regulates the transcription of pro-inflammatory cytokines such as IL-12p40, TNF- α , and IL-8 [27]. Recent investigations from our group revealed that *C. jejuni* mediates immunopathology via TLR-2, -4 and -9 signaling [18, 28]. *C. jejuni* induced the secretion of pro-inflammatory cytokines such as IL-6, IL-8, IL-1 β , IL-12p70, and TNF- α in epithelial cell lines, monocytes, and DCs in culture [29, 30]. Furthermore, co-culturing these cells with T cells led to a high level of IFN- γ production which is mainly caused by a Th1-type immune response [30, 31]. In *ex vivo* colon

cultures taken from GB IL-10^{-/-} mice displaying severe enterocolitis following *C. jejuni* infection, an increased secretion of pro-inflammatory cytokines such as TNF- α , IFN- γ , and MCP-1 could be observed [28]. These mice exhibited higher counts of T and B lymphocytes in their colonic mucosa and lamina propria [28]. Recently, our group demonstrated that the induction of pro-inflammatory cytokine production following oral *C. jejuni* infection of GB mice was dependent on the detection of *C. jejuni*-LPS- and -CpG-DNA via TLR-4 and TLR-9, respectively [18]. Furthermore, GB IL-10 deficient mice lacking TLR-4 exhibited significantly less pro-inflammatory cytokines and diminished cellular responses in their colon [28]. Finally, we observed that TLR-4, signaling is essentially involved in acute inflammatory processes in murine models of acute ileitis and colitis [22, 23].

Conventionally colonized IL-10^{-/-} mice developing chronic colitis due to bacterial stimuli derived from their commensal intestinal microbiota within 5 to 6 months of age can be stably infected by *C. jejuni* at intermediate levels and develop enteritis [25, 32]. Given that TLR-2 and -4 mediate *C. jejuni* induced acute enterocolitis in gnotobiotic IL-10^{-/-} mice [28], we were interested whether signaling of *C. jejuni* lipoproteins and lipooligosaccharide, respectively, is also essentially involved in immunopathogenesis following infection of conventional IL-10^{-/-} mice with chronic colitis. In the study presented here we analyzed the activation of effector cells from the innate and adaptive immune system following *C. jejuni* infection of conventionally colonized TLR-2 IL-10 and TLR-4 IL-10 double deficient animals with chronic colitis *in vivo* and focused on extra-intestinal immune responses.

Results

C. jejuni infection of conventional IL-10^{-/-} mice lacking TLR-2 or -4 exhibiting chronic colitis

We have recently shown that 5- to 6-month-old IL-10^{-/-} mice harboring a conventional intestinal microbiota and suffering from chronic colitis, but not age-matched wildtype controls without intestinal inflammation, can be colonized by *C. jejuni* at intermediate levels for 2 weeks in approximately 70% of the cases [25]. Given that TLR-2 and -4 play pivotal roles in mediating acute enterocolitis in *C. jejuni* infected GB IL-10 deficient mice [28], we were interested whether TLR-2 and/or TLR-4 signaling were also essentially involved in *C. jejuni* infected conventional IL-10 deficient mice with chronic colitis. Interestingly, IL-10 deficient mice lacking TLR-2 or -4 as well as IL-10^{-/-} controls displayed comparable clinical as well as histopathological signs of chronic colitis before and 3 days after *C. jejuni* (ATCC 43431 strain) infection indicating that *C. jejuni* infection did not deteriorate already established chronic colitis (not shown). Furthermore, irrespective of their genotype, conventionally colonized mice harbored comparable intestinal pathogen loads in their feces at d3 p.i. (not shown).

Barrier defect and bacterial translocation in conventional *IL-10*^{-/-} mice lacking TLR-2 or -4

Next, we were interested whether *C. jejuni* infection induced translocation of commensal gut bacteria due to compromised epithelial barrier functions. Irrespective of the genotype, mRNA expression of Mucin-2 forming an integral part of the mucus significantly decreased 3 days following *C. jejuni* infection (Fig. 1a). Interestingly, uninfected *IL-10* deficient mice lacking TLR-2 or TLR-4 exhibited lower rates of translocated live bacteria originating from the commensal gut microbiota into their mesenteric lymphnodes (MLNs; 50.0 and 33.3%, respectively) as compared to *IL-10* controls (66.7%) (Fig. 1b). Three days following *C. jejuni* infection, however, bacterial translocation rates into MLNs of *IL-10* deficient mice lacking TLR-4 (47.1%), but not TLR-2 (68.4%), were lower as compared to *IL-10* deficient controls (72.2%) (Fig. 1b). Of note, live *C. jejuni* could not be cultured from the other examined extra-intestinal tissue sites at day 3 p.i. (not shown).

Colonic B lymphocyte responses in *C. jejuni*-infected conventional *IL-10*^{-/-} mice lacking TLR-2 or TLR-4

In humans, *C. jejuni* induces the recruitment of pro-inflammatory immune cell populations to sites of inflammation in the colon [5, 6]. To further elucidate whether *C. jejuni* infection of conventional *IL-10*^{-/-} mice lacking TLR-2

or -4 was accompanied by cellular immune responses, we next analyzed B220+ cells in the colonic mucosa by *in situ* immunohistochemistry. B cell numbers increased within 3 days following *C. jejuni* infection irrespective of the genotype of mice. This increase in colonic B cell numbers, however, was significantly less distinct in TLR-4^{-/-} *IL-10*^{-/-} animals (Fig. 2).

Extra-intestinal pro-inflammatory immune responses in *C. jejuni*-infected conventional *IL-10*^{-/-} mice lacking TLR-2 or TLR-4

C. jejuni infection has been recently shown to be associated with extra-intestinal immune responses [20]. Therefore, we investigated leukocytes isolated from MLNs, spleens and livers stained with respective surface markers. Following *C. jejuni* infection, only conventional TLR-4^{-/-} *IL-10*^{-/-} mice exhibited an increase of the relative percentages of CD4+ T lymphocytes (Fig. 3a) and CD11c+ dendritic cells (Fig. 3b) in their MLNs whereas B220+ B lymphocytes (Fig. 3c) and CD11b+ Gr1+ myeloid cells such as inflammatory monocytes and neutrophilic granulocytes [33–36] (Fig. 3d) had decreased until day 3 p.i. and were significantly lower as compared to MLNs taken from conventional TLR-2^{-/-} *IL-10*^{-/-} and *IL-10*^{-/-} mice. Furthermore, *IL-10* deficient mice lacking TLR-2 displayed relatively lower CD4+ cells as compared to TLR-4^{-/-} *IL-10*^{-/-} and *IL-10*^{-/-} animals (Fig. 3a).

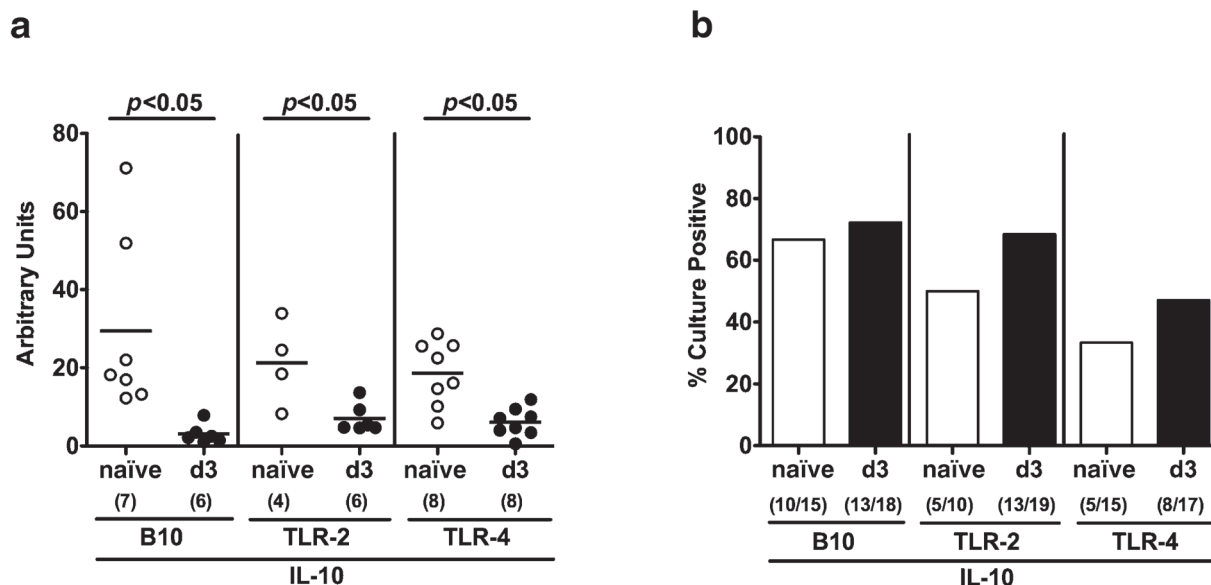


Fig. 1. Epithelial barrier function following *C. jejuni* ATCC 43431 infection of conventional *IL-10* deficient mice lacking TLR-2 or -4. Conventionally colonized *IL-10* deficient (B10 background) as well as TLR-2 *IL-10* and TLR-4 *IL-10* double deficient mice were orally infected with *C. jejuni* ATCC 43431 (solid circles or bars) and analyzed at day 3 post infection (d3) as described in Methods. Uninfected mice served as negative controls (naïve, open circles or bars). (a) RT-PCR results of Mucin-2 expression in colonic samples of individual mice are expressed as fold changes HPRT mRNA expression (arbitrary units). (b) Translocation of live commensal gut bacteria to extra-intestinal compartments at day 3 post infection was assessed by cultivation of mesenteric lymphnodes (MLN) in thioglycolate enrichment broths for maximum 7 days. Turbid broths were sub-cultivated on solid media (refer to Materials). Bars indicate mean relative rates of positive samples (in %). Absolute numbers of positive samples out of total number analyzed are given in parentheses. Means (black bars) and significance levels (*P*-values) determined by the Student's *t*-test are indicated. Data shown are representative for (a) or were pooled from three independent experiments (b)

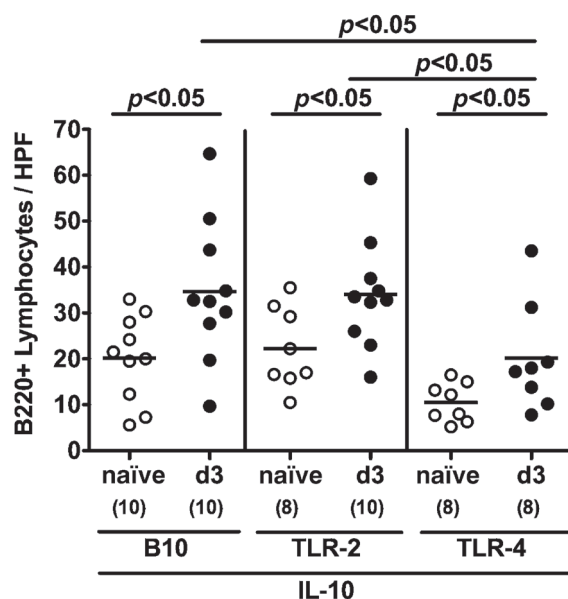


Fig. 2. Colonic B lymphocyte responses following *C. jejuni* ATCC 43431 infection of conventional IL-10 deficient mice lacking TLR-2 or -4.

Conventionally colonized IL-10 deficient (B10 background) as well as TLR-2 IL-10 and TLR-4 IL-10 double deficient mice were orally infected with *C. jejuni* ATCC 43431 (solid circles) and analyzed at day 3 post infection (d3) as described in Methods. Uninfected mice served as negative controls (naïve, open circles). The average numbers of B lymphocytes (positive for B220) from at least six high power fields (HPF, $\times 400$ magnification) per animal were determined microscopically in immunohistochemically stained colon sections. Numbers of analyzed animals are given in parentheses. Means (black bars) and levels of significance (P -values) determined by the Student's t -test are indicated. Data shown are pooled from three independent experiments

In *C. jejuni* infected IL-10^{-/-} mice lacking TLR-4, but not TLR-2, or IL-10^{-/-} control animals, an increase of CD4⁺ Th1 and activated CD4⁺ CD69⁺ Th1 lymphocytes as well as CD8⁺ cytotoxic lymphocytes could be detected in spleens at day 3 p.i. (Fig. 4a–c). Interestingly, splenic CD11b⁺ Gr1⁺ myeloid cells decreased upon *C. jejuni* infection in TLR-4^{-/-} IL10^{-/-} and IL-10^{-/-}, but not TLR-2 IL-10 double deficient animals with lowest abundance in the former at day 3 p.i. (Fig. 4d).

In the liver, however, CD11b⁺ Gr1⁺ myeloid cells increased within 3 days following *C. jejuni* infection irrespective of the genotype with significantly higher relative rates in TLR-2^{-/-} IL-10^{-/-} as compared to TLR-4^{-/-} IL-10^{-/-} and IL-10^{-/-} controls (Fig. 5).

Taken together, following oral *C. jejuni* infection conventional TLR-4 IL-10 double deficient mice displayed a less compromised epithelial barrier function as indicated by lower translocation rates of live commensal gut bacteria into MLNs, and less distinct intestinal pro-inflammatory immune responses as mirrored by lower B lymphocyte numbers within the colonic mucosa. In extra-intestinal compartments, abundance of myeloid cells (consisting of inflammatory monocytes and neutrophilic granulocytes)

was less distinct in MLNs and spleens whereas activated T helper cells and cytotoxic T cells were higher in spleens and dendritic cells more abundant in MLNs of conventional TLR-4^{-/-} IL-10^{-/-} animals as compared to TLR-2^{-/-} IL-10^{-/-} and IL-10^{-/-} mice following *C. jejuni* infection.

Discussion

Campylobacter jejuni is a leading cause of bacterial food-borne enterocolitis worldwide. Despite the increasing incidence and the related socioeconomic burden, immunopathology underlying human campylobacteriosis is not well understood. Detailed investigation is hampered by the lack of appropriate vertebrate models. In the present study, we aimed at investigating TLR-dependent immune responses to *C. jejuni* in conventionally bred IL-10 deficient mice with a conventional gut microbiota. Therefore, IL-10^{-/-} mice (in C57BL/10 background) lacking TLR-2 or -4 were infected with *C. jejuni* ATCC 43431. As shown earlier, IL-10 deficient animals in C57BL/6 background could be stably colonized by the pathogen [32]. Following *C. jejuni* infection, IL-10 deficient mice of either genotype exhibited no significant aggravation of colonic histopathology but displayed a reduction of Mucin-2 mRNA expression in the colon compared to uninfected controls. Mucin-2 acts as the first barrier invading microorganisms have to overcome and thus plays a pivotal role in colonic protection [37]. However, *C. jejuni* is able to bind to Mucin-2 [38] and to upregulate mucin-degrading enzymes [39]. The impact of mucins in contributing to resistance to *C. jejuni* infection is further underlined by the fact that Mucin-deficient mice experience higher intestinal pathogen burden, display more distinct epithelial damage as well as rapidly developing systemic infection upon *C. jejuni* infection [40].

These results are well in line with our findings that *C. jejuni* infected mice contained higher concentrations of commensal bacteria in their MLNs as compared to naïve mice. This can be explained by (1) the diminished mucus layer which facilitates the pathogen to get in close contact with the intestinal epithelium and to consecutively translocate to sub-epithelial tissue sites [41] and (2) by the earlier finding that *C. jejuni* supports translocation of commensal bacteria via lipid rafts [42]. Interestingly, in *C. jejuni*-infected TLR-4^{-/-} IL10^{-/-} mice, less frequent bacterial translocation to MLNs could be detected as compared to TLR-2^{-/-} IL10^{-/-} and IL-10 deficient controls.

Furthermore, a diminished count of myeloid cells was found in the MLNs of TLR-4^{-/-} IL-10^{-/-} mice compared to TLR-2^{-/-} IL-10^{-/-} and IL-10^{-/-} control animals. Given that myeloid cells such as inflammatory monocytes and neutrophilic granulocytes mainly act on eliminating invading microorganisms one can see these TLR-4-dependent results in coherence with the lower bacterial translocation rates into the MLNs of infected TLR-4^{-/-} IL-10^{-/-} mice.

Toll-like receptors recognize highly conserved microbial structures such as LPS and LOS (by TLR-4) as well as

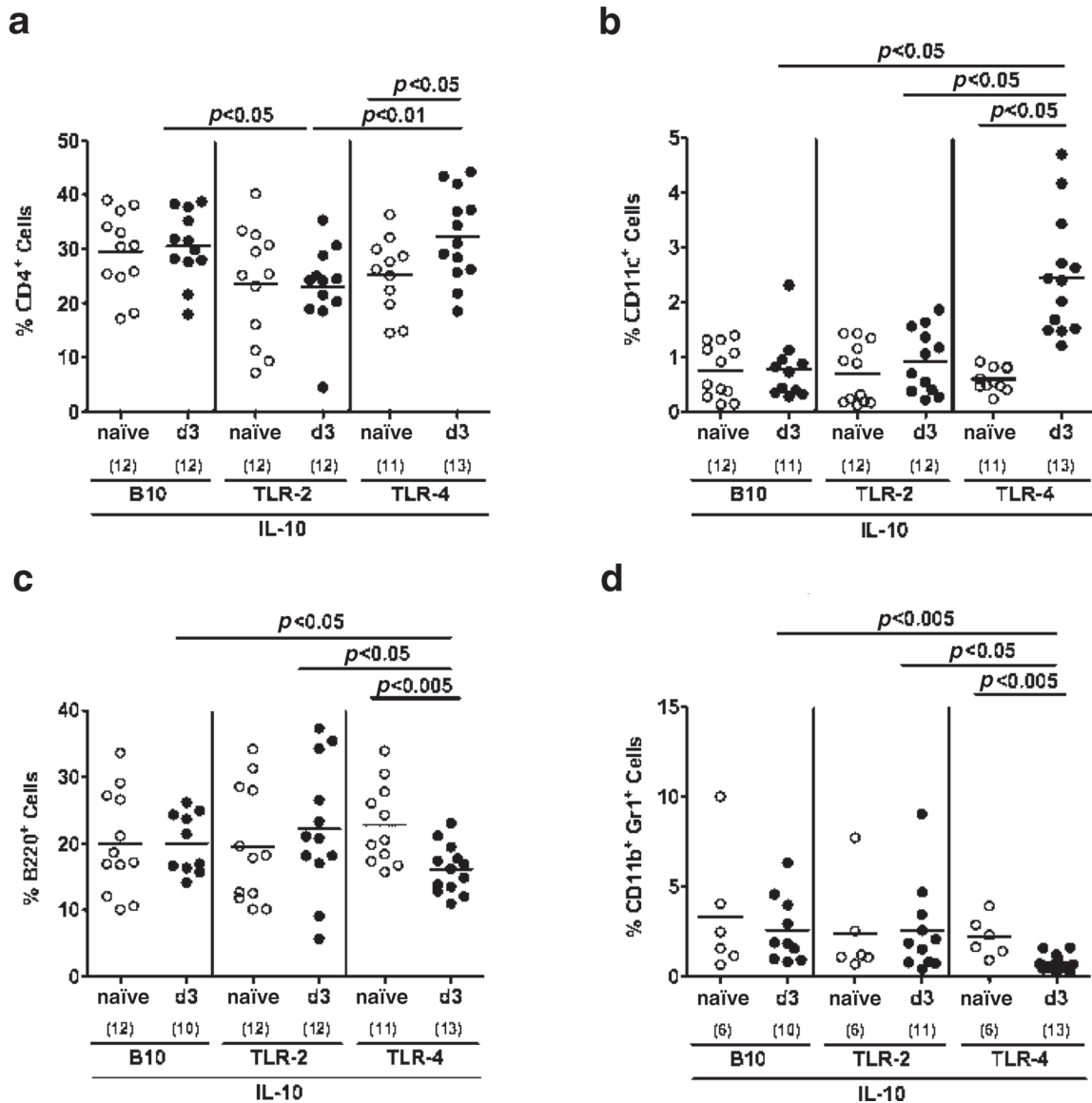


Fig. 3. Immune cell responses in mesenteric lymphnodes of *C. jejuni* ATCC 43431 infected conventional IL-10 deficient mice lacking TLR-2 or -4.

Conventionally colonized IL-10 deficient (B10 background) as well as TLR-2 IL-10 and TLR-4 IL-10 double deficient mice were orally infected with *C. jejuni* ATCC 43431 (solid circles) analyzed at day 3 post infection (d3) as described in Methods. Uninfected mice served as negative controls (naïve, open circles). Leukocytes isolated from mesenteric lymphnodes (MLN) were isolated, stained with surface markers and analyzed by flow cytometry. Relative abundance of Th1 cells (CD4+; panel a), dendritic cells (CD11c+, panel b) B lymphocytes (B220+, panel c), and myeloid cells such as inflammatory monocytes and neutrophilic granulocytes (CD11b+ Gr1+, panel d) is expressed in %. Numbers of analyzed animals are given in parentheses. Medians (black bars) and significance levels (*P*-values) determined by Student's *t*-test are indicated. Data shown are pooled from three independent experiments

lipoteichoic acid (by TLR-2), all expressed on non-immune cells (e.g. epithelial cells) and immune cells (e.g. DCs) [26]. DCs sample commensals and pathogens, get activated (e.g. by *C. jejuni*) which in turn leads to a Th1-type immune response [30, 31]. Bacteria-laden DCs migrate to MLNs and activate T and B lymphocytes. Activated B lymphocytes differentiate into plasma cells and produce IgA specific for the sampled bacteria [43, 44]. The detection of serum antibodies from infected patients indicates that *C. jejuni* induces both innate and specific (adaptive) immune responses [45]. In the colonic mucosa and lamina propria we detected an increase of B lymphocytes as early as day 3 p.i., irre-

spective of the genotype of *C. jejuni* infected mice. Like in GB TLR-4^{-/-} animals [18], infected TLR-4^{-/-} IL-10^{-/-} mice harboring a conventional intestinal microbiota displayed less colonic B lymphocytes indicating that TLR-4 is essential in recruiting adaptive immune cells into the intestinal compartment. This is supported by the fact that, even in the MLNs of TLR-4^{-/-} IL-10^{-/-} mice, B cell numbers were lower as compared to TLR-2^{-/-} IL-10^{-/-} and IL-10^{-/-} mice.

In MLNs of *C. jejuni* infected IL-10 deficient mice lacking TLR-4, but not TLR-2, we detected a higher DC abundance as compared to IL-10^{-/-} controls. *In vitro* experiments revealed that cytokine production by DC in

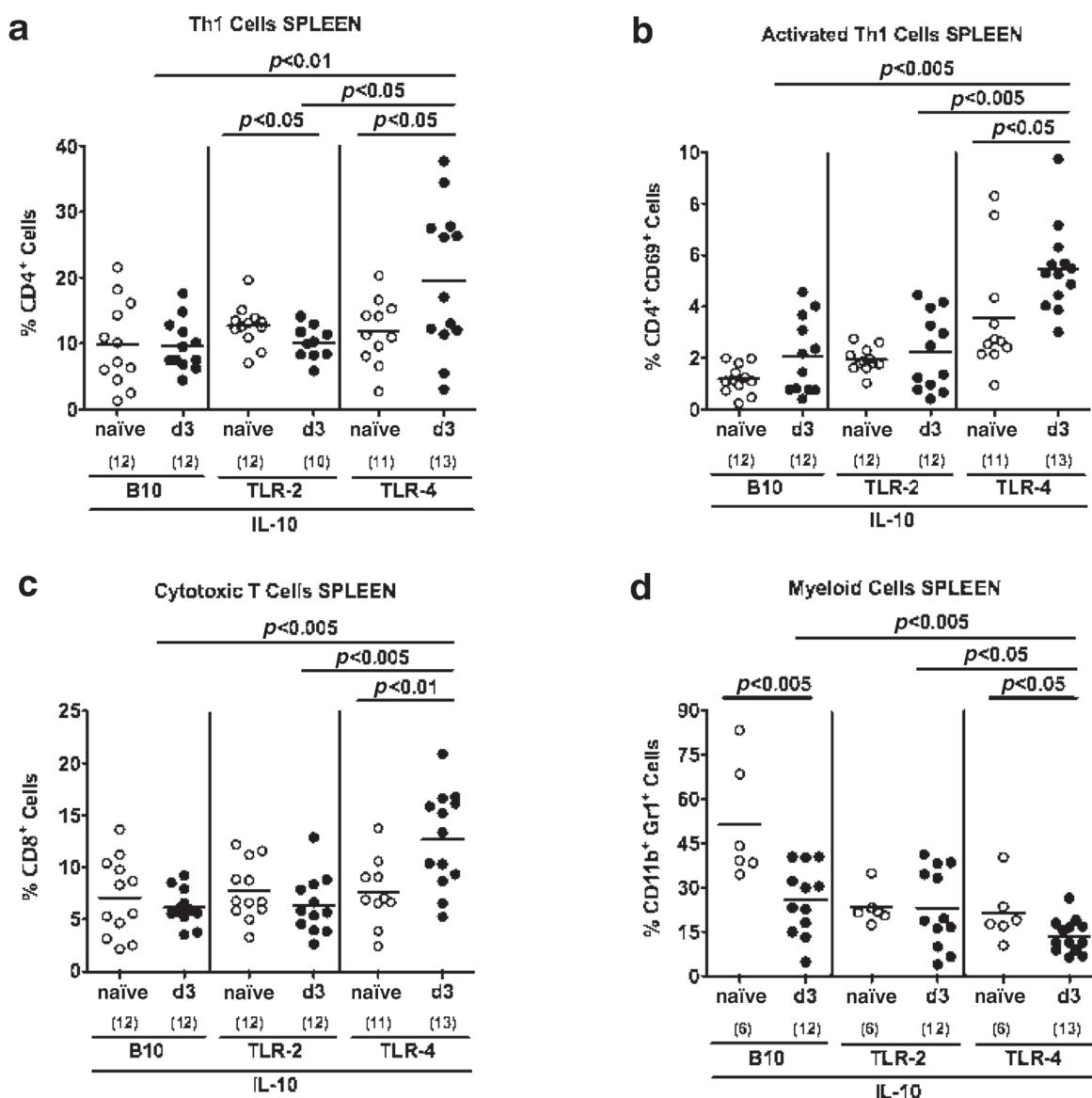


Fig. 4. Immune cell responses in spleens of *C. jejuni* ATCC 43431 infected conventional IL-10 deficient mice lacking TLR-2 or -4. Conventionally colonized IL-10 deficient (B10 background) as well as TLR-2 IL-10 and TLR-4 IL-10 double deficient mice were orally infected with *C. jejuni* ATCC 43431 (solid circles) and analyzed at day 3 post infection (d3) as described in Methods. Uninfected mice served as negative controls (naive, open circles). Splenic leukocytes were isolated, stained with surface markers, and analyzed by flow cytometry. Relative abundance of Th1 cells (CD4+; panel a), activated Th1 cells (CD4+ CD69+, panel b), cytotoxic T cells (CD8+, panel c), and myeloid cells such as inflammatory monocytes and neutrophilic granulocytes (CD11b+ Gr1+, panel d) is expressed in %. Numbers of analyzed animals are given in parentheses. Medians (black bars) and significance levels (*P*-values) determined by Student's *t*-test are indicated. Data shown are pooled from three independent experiments

response to *C. jejuni* was shown to be predominantly TLR-4-dependent [46]. Our group recently showed that GB IL-10 deficient mice lacking TLR-4 exhibited significantly less pro-inflammatory cytokines and a diminished cellular response in the colon following oral *C. jejuni* infection [28] further underlining that *C. jejuni*-LOS is a major trigger of TLR-4 activation *in vivo* [47].

In contrast, Rose et al. observed that in TLR-4^{-/-} DCs an acapsular *C. jejuni* mutant strain induced higher pro-inflammatory cytokine levels as compared to the wildtype control strain [48]. As a strategy of evading the host's immune system, Gram-negative bacteria modify cell surface structures. *C. jejuni* can adapt to its host by modification

of its LOS, flagella and capsule for instance [49]. Hereby, *C. jejuni* is able to impact TLR-4 activation and enhance antimicrobial resistance [50].

In the study presented here we were interested in examining *C. jejuni* induced immune responses not only in intestinal but also in extra-intestinal compartments. For this purpose, we focused on the spleen initiating lymphocyte differentiation, activation, and proliferation and thereby being involved in the systemic immune responses. In chickens, Li and coworkers found a more distinct systemic host response in the spleen as compared to local immune responses in the caecum following *C. jejuni* infection [51]. In our experiments we detected higher abundances of CD4+ Th1 cells,

activated CD4⁺ CD69⁺ Th1 cells and cytotoxic CD8⁺ T lymphocytes in the spleen of infected TLR-4^{-/-} IL-10^{-/-} mice as compared to TLR-2^{-/-} IL-10^{-/-} and IL-10^{-/-} controls. Both CD4⁺ and CD8⁺ T lymphocytes contribute to the protection against *C. jejuni* [30, 52]. Just as in MLNs of infected mice, abundance of myeloid cells, however, was less distinct in the spleen of TLR-4^{-/-} IL-10^{-/-} mice as compared to TLR-2^{-/-} IL-10^{-/-} and IL-10^{-/-} animals supporting further evidence for a TLR-4 dependent activation of myeloid cells such as inflammatory monocytes and neutrophilic granulocytes in IL-10^{-/-} mice following *C. jejuni* infection.

Besides its metabolic properties, the liver contributes to intestinal immune functions [53]. When the epithelial barrier function is compromised, e.g. due to a diminished mucus layer, bacterial compounds such as LPS reach the liver and induce pro-inflammatory responses [54]. In support of this, our experiments revealed a higher abundance of myeloid cells in the liver of infected animals compared to uninfected controls irrespective of their genotype. Interestingly, this *C. jejuni*-induced increase in liver myeloid cells such as inflammatory monocytes and neutrophilic granulocytes was more pronounced in TLR-2 IL-10 double deficient animals as compared to TLR-4^{-/-} IL-10^{-/-} and IL-10^{-/-} controls.

Taken together, the study presented here clearly demonstrates that, in conventionally colonized IL-10^{-/-} mice, TLR-4, but not TLR-2, is involved in mediating extra-intestinal pro-inflammatory immune responses following *C. jejuni* infection. Thus, conventional IL-10^{-/-} mice with established enterocolitis are well suited to further dissect mechanisms underlying *Campylobacter* infections *in vivo*.

Materials and methods

Ethics statement

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, Germany; Registration numbers: G0173/07 and G135/10). Animal welfare was monitored twice daily by assessment of clinical conditions.

Mice and infection

IL-10 deficient mice (in C57BL/10 background, B10) were bred and maintained in the facilities of the "Forschungsinstitut für Experimentelle Medizin" (FEM, Charité — Universitätsmedizin, Berlin, Germany), under specific pathogen-free (SPF) conditions. In order to gain double-deficient animals, TLR-2^{-/-} and TLR-4^{-/-} mice (in B10 background each) were crossed to IL-10^{-/-} mice and backcrossed at least 7 generations before use.

Six-month-old female mice of either genotype exhibiting chronic colitis were infected with approximately 10⁹ viable CFU of *C. jejuni* strain ATCC 43431 by gavage in

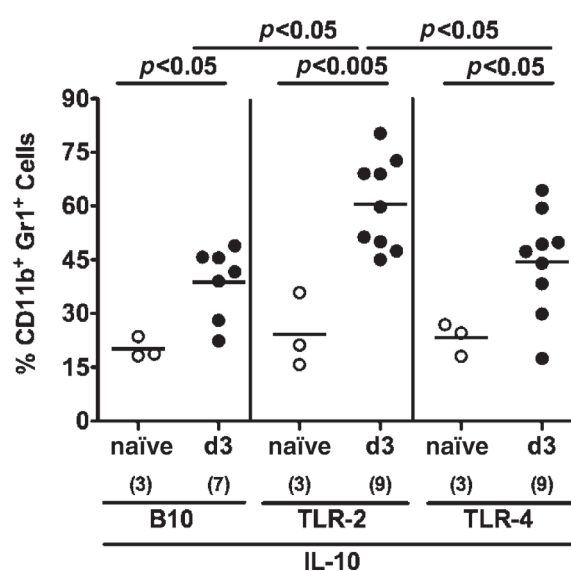


Fig. 5. Immune cell responses in livers of *C. jejuni* ATCC 43431 infected conventional IL-10 deficient mice lacking TLR-2 or -4.

Conventionally colonized IL-10 deficient (B10 background) as well as TLR-2 IL-10 and TLR-4 IL-10 double deficient mice were orally infected with *C. jejuni* ATCC 43431 (solid circles) and analyzed at day 3 post infection (d3) as described in Methods. Uninfected mice served as negative controls (naïve, open circles). Liver leukocytes were isolated, stained with surface markers, and analyzed by flow cytometry. Relative abundance of myeloid cells such as inflammatory monocytes and neutrophilic granulocytes (CD11b⁺ Gr1⁺) is expressed in %. Numbers of analyzed animals are given in parentheses. Medians (black bars) and significance levels (*P*-values) determined by Student's *t*-test are indicated. Data shown are pooled from three independent experiments

a total volume of 0.3 ml PBS on two consecutive days as described earlier in detail [18].

Clinical score

To assess clinical/macrosopic signs of *C. jejuni* induced infection on a daily basis, a standardized cumulative clinical score (maximum 12 points; modified according to Ref. [55]) addressing the occurrence of blood in feces (0 points: no blood; 2 points: microscopic detection of blood by the Guajac method using Haemocult™, Beckman Coulter/PCD, Krefeld, Germany; 4 points: 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 compromised locomotion, pre-final aspect) was used.

Sampling procedures, determination of colon length, and histopathology

Mice were sacrificed by isofluran treatment (Abbott, Germany). From each mouse, samples derived from the colon

were collected in parallel for histological, microbiological, and immunobiological analyses. Cardiac blood and tissue samples from mesenteric lymphnodes, spleen, liver, and the gastrointestinal tract were asserved under sterile conditions. Histopathological changes were determined in colon samples immediately fixed in 5% formalin and embedded in paraffin. Sections (5 μm) were stained with respective antibodies for immunohistochemistry.

Immunohistochemistry

In situ immunohistochemical analysis of colonic paraffin sections was performed as described previously [18, 23, 56]. Primary antibodies against B220 (eBioscience, San Diego, CA, USA, 1:200) were used. For each animal, the average number of positive stained cells within at least six independent high power fields (HPF, $\times 400$ magnification) was determined microscopically and subjected to statistical analysis as indicated.

Real time-PCR

RNA was isolated from organs using the RNeasy Mini Kit (Qiagen, Hilden, Germany). mRNA was reverse transcribed and analyzed in triplicate assays by TaqMan PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) as described previously [57]. For detection of murine mucin-2 (MUC-2), assays including double-fluorescent probes in combination with assays for the murine housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT) were purchased from Applied Biosystems. Expression levels were calculated relative to the HPRT expression (expressed as normalized ratio and indicated as Arbitrary Units).

Isolation of lymphocytes and flow cytometry

Mesenteric lymphnodes were removed and subsequently minced through a 70- μm filter. For intracellular staining, cells were stimulated for 6 h with 50 ng/ml 12-O-tetradecanoylphorbol-13 acetate (Sigma, Missouri, USA), 750 ng/ml ionomycin (Sigma) and Golgi Stop (BD Biosciences, San Diego, USA) at 37 °C.

Stainings and cell sorting with anti-CD4 (L3T4 clone APC conjugated), anti-CD69 (FITC-conjugated), anti-CD8 (53-6.7 clone pacific blue conjugated), anti-B220 (PE conjugated), anti-CD11c (APC conjugated), anti-CD11b (FITC-conjugated), and anti-Gr1 (APC-conjugated) were performed. Cells were analyzed with a FACSCalibur Flow Cytometer (BD Biosciences; Invitrogen, Karlsruhe, Germany).

In order to isolate splenic lymphocytes, spleens were removed and homogenized through a nylon mesh sieve

(70 μm). Cell suspensions were washed with PBS, centrifuged at 350 g for 10 min, and supernatants were taken. For lysis of erythrocytes, 1-ml distilled water was added. After 45 s 1.8% NaCl was added, and the cells were centrifuged again. Supernatants were resolved in PBS containing 5% FCS/0.4% EDTA 2 mM. Staining and cell sorting were performed as mentioned above.

Liver lymphocytes were isolated following perfusion of mice through the portal vein using PBS. Livers were then removed and homogenized through a nylon mesh sieve (70 μm). Cell suspensions were washed with PBS, centrifuged at 50 g for 1 min, and supernatants were taken. This procedure was repeated four times. Afterwards, supernatants were pooled, washed using RPMI 1640 containing 5% FCS and penicillin/streptomycin, and centrifuged through a 40%/70% Percoll gradient. Cells were collected from the interface, washed with RPMI 1640, and used for flow cytometry analysis. Staining and cell sorting were performed as mentioned above.

Microbiota analyses

Cultural analyses, biochemical identification and molecular detection of luminal bacterial communities from colon as well as feces were performed as previously described [18, 21–23]. For bacterial translocation experiments, mesenteric lymphnodes were transferred into thioglycollate enrichment broths under sterile conditions and cultivated for 7 days. Turbid broths were streaked onto solid culture media and cultivated under aerobic, microaerophilic, and obligate anaerobic conditions in order to detect translocated live commensal gut bacteria or *C. jejuni* followed by species identification via biochemical and molecular means [18]. Given that bacterial growth in enrichment broths cannot be quantitated, the relative rates of translocating bacteria are expressed in %.

Statistical analysis

Mean values, medians, standard deviations, and levels of significance were determined applying the two-tailed Student's *t*-test as indicated. Two-sided probability (*P*) values ≤ 0.05 were considered significant. All experiments were repeated at least twice.

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Author contributions

S. Bereswill, A. Fischer, U. B. Göbel, L.-M. Haag, R. Plickert, and M. M. Heimesaat conceived and designed the experiments.

A. Fischer, L.-M. Haag, B. Otto, R. Plickert, and M. M. Heimesaat performed the experiments.

A. Fischer, L.-M. Haag, B. Otto, A. A. Kühl, and M. M. Heimesaat analyzed the data.

A. A. Kühl contributed reagents/materials/analysis tools.

B. Otto, L.-M. Haag, S. Bereswill, A. Fischer, and M. M. Heimesaat wrote the paper.

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Competing interests

The authors have declared that no competing interests exist.

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