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DISSERTATION

Molekulare Mechanismen der durch *Campylobacter jejuni* induzierten intestinalen und extra-intestinalen Immunpathologien bei gnotobiotischen IL-10-defizienten Mäusen

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1. Zusammenfassung

1.1 Abstrakt

Obwohl *Campylobacter* (*C.*) *jejuni*-Infektionen weltweit eine hohe Prävalenz zeigen, sind die zugrunde liegenden immunpathologischen Mechanismen noch nicht vollständig verstanden. Bisher gab es kaum geeignete *in* vivo-Modelle, um die *C. jejuni*-Infektion des Menschen zu imitieren und so die Mechanismen der Infektion näher zu analysieren. So entwickelte unsere Arbeitsgruppe mit gnotobiotischen (sekundär abiotischen) IL-10-defizienten (IL-$10^{-/-}$) -Mäusen ein neues *in vivo*-Infektionsmodell, um die *C. jejuni*-Wirtsinteraktionen zu untersuchen. Unabhängig vom verwendeten Stamm kolonisierte *C. jejuni* nach peroraler Infektion den Darm gnotobiotischer IL-$10^{-/-}$ Mäuse stabil. Ähnlich wie bei immunkompromittierten Patienten entwickelten infizierte Mäuse innerhalb einer Woche eine nicht-selbstlimitierende ulzerative Enterokolitis.
In der vorliegenden Arbeit werden Untersuchungen zu drei Proteinen bzw. Proteinklassen zusammengefasst, die maßgeblich an der Pathogenität von *C. jejuni* beteiligt sind. Wir untersuchten hierbei zum Einen die Bedeutung der Serinprotease HtrA (high temperature requirement A), welche bei der zellulären Invasion und Transmigration von *C. jejuni* eine wichtige Rolle *in vitro* spielt und konnten zeigen, dass Mäuse, welche oral mit einem *htrA*-Gen-defizienten *C. jejuni*-Stamm infiziert worden waren, weniger Immunpathologie im Darm aufwiesen als die entsprechenden Kontrollgruppen. Ähnliche Ergebnisse erzielten unsere Experimente mit einem *cj0268c*-„Knockout“-Stamm. Auch dem Cj0268c-Protein konnte mittels *in vitro*-Versuchen bereits eine maßgebliche Rolle für die Adhäsion und Invasion von *C. jejuni* in Wirtszellen zugeschrieben werden. Ein weiterer Fokus der Versuche lag auf der Rolle der Matrixmetalloproteinasen (MMPs) -2 und -9 (Gelatinasen A und B) während der *C. jejuni*-Infektion. Die Expression dieser Endopeptidasen ist bei Darmentzündungen unterschiedlicher Genese bei Mensch und Maus erhöht. So konnten wir in vorausgehenden Studien bereits nachweisen, dass die Inhibition der MMP-2 und -9 mit der synthetischen Substanz RO28-2653 in Modellen der akuten Dünn- und Dickdarmentzündung zu abgeschwächter Krankheitssymptomatik führte.

Daher untersuchten wir die mögliche Beeinflussung der akuten *C. jejuni*-Enterokolitis durch RO28-2653 und konnten zeigen, dass die synthetische Hemmung von MMP-2 und -9 auch im Rahmen der *C. jejuni*-induzierten Enterokolitis im gnotobiotischen IL-10$^{-/-}$-Mausmodell zu einer weniger stark ausgeprägten Immunpathologie führte.

Zusammenfassend konnten wir die Bedeutung von drei verschiedenen Proteinen bzw. Proteinklassen (HtrA, Cj0268c sowie MMP-2 und -9) als wichtige, die Pathogenität von *C. jejuni* vermittelnde Faktoren im Mausmodell nachweisen.

Abstract

Campylobacter (*C.*) *jejuni* infections exhibit a high prevalence worldwide, but the underlying immunopathological mechanisms are not entirely understood. Until now *in vivo* models mimicking human C. *jejuni* infections and hence allowing for detailed analyses of pathogenic-host interactions are scarce. Previously, we have generated the gnotobiotic (secondary abiotic) IL-10-deficient (IL-10$^{-/-}$) mouse model for investigating *C. jejuni* infection *in vivo*. Upon peroral infection, the intestinal tract of gnotobiotic IL-10$^{-/-}$ mice could be stably colonized by the pathogen in high loads irrespective of the applied strain. Subsequently, mice developed non-selflimiting ulcerative enterocolitis within one week postinfection. In the present work, *in vivo* studies of three distinct groups of proteins or protein classes that are primarily attributed to the *C. jejuni* pathogenicity have been summarized. Namely, we analysed the role of the serineprotease htrA (high temperature requirement A), which plays an important role in the cellular invasion and transmigration of *C. jejuni in vitro* and could demonstrate that following infection with a *C. jejuni* strain lacking the *htrA* gene, mice displayed less pronounced intestinal immunopathological sequelae as compared to parental (wildtype) strain infection. Similar results (i.e. less immunopathology) could be obtained upon infection with a *C. jejuni* strain lacking *cj0268c*, a gene encoding a protein playing a significant role in pathogenic invasion *in vitro*. Furthermore, we focussed on the role of the matrix metalloproteinases (MMPs) -2 and -9 (also referred to gelatinases A and B, respectively) during *C. jejuni* infection, given that MMP-2 and -9 are highly expressed in inflamed intestinal tissues of mice and men and selective gelatinase inhibition by the synthetic component RO28-2653 resulted in less distinct acute small and large intestinal inflammation of mice. Following gelatinase blockage, pro-inflammatory

immune responses in colons of *C. jejuni* infected gnotobiotic IL-$10^{-/-}$ mice were less pronounced as compared to placebo controls.

Taken together, the three distinct proteins or protein classes under investigation (namely HtrA, Cj0268c and MMP-2- and -9) are involved in *C. jejuni* induced immunopathology *in vivo*.

1.2 Einführung

1.2.1 *Campylobacter jejuni*

Infektionen durch Bakterien der Gattung *Campylobacter* sind weltweit verbreitet. Vor allem in den Industrienationen ist die Prävalenz von *Campylobacter*-Infektionen steigend [1,2,3]. Seit 2007 ist *Campylobacter* der häufigste gemeldete bakterielle Durchfallerreger in Deutschland, mit einem Häufigkeitsgipfel im Sommer. Im Jahr 2014 wurden insgesamt 70.972 *Campylobacter*-Enteritis-Fälle gemeldet [3]. Dies entspricht einer Inzidenz von 87,9 Erkrankungen pro 100.000 Einwohner und stellt im Vergleich zum Vorjahr eine Zunahme der Erkrankungen um 11,5 % dar. Bei 69 % der *Campylobacter*-Enteritiden lagen genauere Angaben zur Spezies vor: Hierbei entfielen 69 % auf *C. jejuni*. Es handelt sich bei *Campylobacter* um gramnegative, bewegliche Stäbchen, welche ein mikroaerobes Wachstumsmilieu bevorzugen. Weiterhin zählen *Campylobacter* zu den thermophilen Erregern und vermehren sich bei Temperaturen ≤ 25°C kaum. Bislang wurden ca. 20 Spezies dieser Gattung identifiziert, von denen *C. jejuni*, *C. coli* und *C. lari* die wichtigsten humanpathogenen Arten darstellen [3].
C. jejuni zählt zur kommensalen Darmflora vieler Säugetiere und Vögel und findet sich auch bei Nutztieren. Nicht ausreichend gegarte und kontaminierte Lebensmittel, v.a. Geflügelfleisch, stellen neben kontaminiertem Oberflächenwasser demnach die wichtigste Infektionsquelle für den Menschen dar [3,4,5]. Nach einer Inkubationszeit von zwei bis fünf, in Einzelfällen von bis zu zehn Tagen, stellt sich die *Campylobacter*-Infektion nach einer kurzen Prodromalphase mit Kopf- und Gliederschmerzen sowie Fieber, klinisch als akute Enteritis mit breiig-wässrigen bis blutigen Diarrhoen,

Abdominalkrämpfen und Fieber dar, kann jedoch auch asymptomatisch verlaufen [3]. Bei immunkompetenten Patienten verläuft die Erkrankung in der Regel innerhalb einer Woche selbstlimitierend; allerdings treten bei 5-10% der unbehandelten Patienten Rezidive auf. Bei immungeschwächten Erkrankten hingegen können seltene Komplikationen wie das Guillian-Barré- bzw. Miller-Fisher-Syndrom sowie reaktive Arthritiden folgen [6-8]. Histologisch zeigt durch *C. jejuni* entzündlich verändertes Gewebe Apoptosen, Kryptenabszesse, Ulzerationen und Ansammlungen von proinflammatorischen Immunzellen wie Lymphozyten, Makrophagen und neutrophilen Granulozyten in der Mukosa und Lamina propria des Dickdarms. Die Therapie beinhaltet vor allem die orale Flüssigkeits- und Elektrolytsubstitution. Bei Risikopatienten und besonders schweren Verläufen sollte eine antibiotische Therapie mit Makroliden oder Fluorchinolonen eingeleitet werden [3].

1.2.2 Untersuchte vermittelnde Proteine der *C. jejuni*-induzierten Immunpathologie im gnotobiotischen IL-10$^{-/-}$-Mausmodell

C. jejuni besitzt die Fähigkeit, an Zellen des menschlichen Gastrointestinaltraktes zu binden, einzudringen und in der Folge Gewebeschäden zu verursachen [9]. Hierfür nutzt *C. jejuni* eine Vielzahl von bereits bekannten Adhäsionsmolekülen wie J1pA, PEB1, CadF und FlpA [10-14]. Ein kürzlich entdeckter Virulenzfaktor von *C. jejuni* ist das HtrA (high temperature requirement A) -Protein [15-18]. HtrA ist eine Serinprotease mit Chaperon-Aktivität, welche die Virulenz einer Vielzahl von Bakterien beeinflusst [19-22]. HtrA-Proteine befinden sich im Periplasma verschiedener bakterieller Spezies [16,17]. Dort formieren sie sich zu proteolytisch aktiven Multimeren, die für die intrazelluläre Kontrolle der korrekten Faltung von Proteinen unerlässlich sind [16,17,23,24]. Kürzlich konnte gezeigt werden, dass HtrA nicht nur intrazellulär wirkt, sondern während einer Infektion (bspw. mit *Helicobacter* (*H.*) *pylori* oder *C. jejuni*) auch aktiv in den Extrazellularraum sezerniert werden kann um an spezifische Adhäsionsmoleküle zu binden [18,25]. Weiterhin wurde gezeigt, dass *C. jejuni* mittels HtrA und dessen Bindung an E-Cadherin *in vitro* unmittelbar Zell-Zell-Verbindungen öffnen kann. Ohne funktionsfähiges HtrA fehlt dem Bakterium die Fähigkeit, E-Cadherin zu binden und folglich in Zellen einzudringen [18]. Weil das HtrA-Protein normalerweise unter Stress fehlgefaltete Proteine abbaut, führte die Abwesenheit des *htrA*-Gens bei

verschiedenen Bakterien zu verminderter Stresstoleranz [15,16,22]. Da bisher vor allem *in vitro*-Versuche mit dem HtrA-Protein durchgeführt wurden, untersuchten wir den Einfluss von HtrA *in vivo* mittels eines Deletionsmutanten-Stamms in unserem gnotobiotischen IL-10$^{-/-}$-Mausmodell. Hierbei infizierten wir gnotobiotische IL-10$^{-/-}$-Mäuse entweder mit dem *C. jejuni* Wildtyp (WT) -Stamm NCTC11168 oder der Deletions-Mutante *C. jejuni* NCTC11168Δ*htrA* und fokussierten uns auf die Kolonisationsfähigkeit und Translokation des Bakteriums, die Klinik der infizierten Tiere, die intestinale Immunpathologie, sowie mögliche systemische und extra-intestinale Immunantworten.

Weiterhin führten wir Untersuchungen zur Rolle des Proteins Cj0268c während der *C. jejuni*-Infektion im Mausmodell durch. Anhand von *in vitro*-Versuchen konnte bereits gezeigt werden, dass dieses Protein eine wichtige Rolle für die Zellinvasion durch *C. jejuni* spielt, da es für die Bindung des Bakteriums an verschiedene Wirtszellarten benötigt wird [26-28]. Darüber hinaus konnte die Relevanz von Cj0268c für die Motilität von *C. jejuni*, dessen Resistenz gegenüber Gallensalzen und für die bakterielle Stabilität und „Fitness" dargestellt werden [28]. Auch für andere Bakterien, bspw. *Escherichia* (*E.*) *coli* stellt Cj0268c einen wichtigen Adhärenzfaktor dar und ist im Periplasma lokalisiert [28]. Für unsere *in vivo*-Versuche infizierten wir gnotobiotische IL-10$^{-/-}$-Mäuse peroral entweder mit dem WT-Stamm NCTC1168, der Deletionsmutante NCTC11168::*cj0268c* oder der komplementären Version NCTC11168::*cj0268c*-comp-*cj0268c* und analysierten in der Folge die Kolonisationskapazitäten der jeweiligen Stämme, den klinischen Zustand der Mäuse und die intestinalen sowie extra-intestinalen pro-inflammatorischen Zell- und Zytokin-Antworten.

Außerdem untersuchten wir die Bedeutung der Matrixmetalloproteinasen (MMPs) -2 und -9 im Verlauf der *C. jejuni*-Infektion gnotobiotischer IL-10$^{-/-}$-Mäuse. MMPs bilden eine heterogene Familie Zink- und Calcium-abhängiger Endopeptidasen, die für viele physiologische Gewebeumbau-Funktionen von Bedeutung sind und einer strikten Regulation durch Zytokine und endogene „tissue inhibitors of matrix metalloproteinases" (TIMPs) unterliegen. Eine gestörte Balance zwischen Aktivatoren und Inhibitoren der MMP-Aktivität kann in verschiedene Pathologien wie etwa Arteriosklerose, Arthritis oder Tumore münden [29-32]. Sowohl in unterschiedlichen intestinalen

Inflammationsmodellen als auch bei Patienten, die unter chronisch entzündlichen Darmerkrankungen leiden, konnte eine erhöhte Aktivität der Gelatinasen A und B (MMP-2 und -9) nachgewiesen werden [2, 33-37].
Wir konnten bereits in früheren Untersuchungen zeigen, dass die Behandlung von Mäusen mit akuter *Toxoplasma gondii*-induzierter Ileitis und „Dextran Sulphate Sodium“ (DSS) -induzierter Kolitis mit synthetischem RO28-2653, einem selektiven Gelatinaseinhibitor, zu verminderter klinischer Symptomatik führte [33,34]. Daher untersuchten wir eine mögliche Beeinflussung der Krankheitsaktivität von *C. jejuni*-infizierten IL-$10^{-/-}$-Mäusen durch Gelatinaseinhibition mittels RO28-2653.
Auch in diesen Versuchsreihen eruierten wir die Kolonisierungsdichten von *C. jejuni* sowie die induzierten proinflammatorischen Immunzell- und Zytokinantworten im Darm und in extra-intestinalen Kompartimenten. Infiziert wurden die Tiere mit dem Patienten-Referenzstamm *C. jejuni* 81-176*.* Als Vergleichsgruppe diente eine Placebogruppe, die ebenfalls mit *C. jejuni* 81-176 infiziert, aber statt mit RO28-2653 mit reiner Phosphat-gepufferter Salzlösung (phosphate buffered saline, PBS) behandelt wurde.

1.2.3 Gnotobiotische IL-$10^{-/-}$-Mäuse als *in vivo*-Modell

Das Fehlen geeigneter *in vivo*-Infektionsmodelle, die einen Entzündungszustand imitieren, wie er beim Menschen beobachtet wird, erschwerte bisher das Verständnis der Pathogenese der *C. jejuni*-Infektion. Die in der Vergangenheit angewandten Tiermodelle (z.B. Hühner, Ferkel, Frettchen, Hundewelpen) scheiterten an zu hohen Kosten sowie eingeschränkter Reproduzierbarkeit und damit einhergehenden inhomogenen Versuchsergebnissen [38,39]. In unserer Arbeitsgruppe konnte kürzlich gezeigt werden, dass gnotobiotische IL-$10^{-/-}$-Mäuse unabhängig vom verwendeten Bakterienstamm ein zuverlässiges *C. jejuni*-Infektionsmodell darstellen [40]. IL-10 ist ein wichtiges regulatorisches Zytokin, welches als Suppressor für Makrophagen, Th1-Zellen und Natürliche Killerzellen fungiert [41,42] und vor allem von Th2-Zellen produziert wird [43]**.** Ferner ist IL-10 für die Aktivierung von B-Zellen und somit für die Produktion von Antikörpern durch das angeborene Immunsystem von Bedeutung [42]. Es handelt sich demnach um ein anti-inflammatorisches Zytokin, welches über verschiedene Mechanismen wie das Herunterregulieren von MHC-II-Molekülen, Interferon (IFN) -γ, Tumornekrosefaktor (TNF) -α und die Interleukinproduktion durch

Makrophagen (IL-1, IL-6, IL-12) die Immunantwort auf intrazelluläre Pathogene reguliert [44]. Somit beeinflusst die Expression von IL-10 im Gastrointestinaltrakt die Reaktion des Immunsystems auf Antigenstimuli von Kommensalen und Pathogenen gleichermaßen [44,45]. IL-10$^{-/-}$-Mäuse wurden bereits vielfach erfolgreich in der *Helicobacter*- und teilweise auch in der *Campylobacter*-Forschung eingesetzt [46-49]. Werden IL-10$^{-/-}$-Mäuse unter konventionellen Bedingungen aufgezogen, entwickeln sie innerhalb von drei bis vier Monaten spontan eine chronische Kolitis [45]. Dies ist durch eine überschießende Th-1-Typ-Immunantwort auf Antigene der konventionellen bakteriellen Mikrobiota zu erklären, welche normalerweise über IL-10 und dessen regulierende Funktion auf pro-inflammatorische Zytokine unterdrückt wird. Werden konventionelle IL-10$^{-/-}$-Mäuse jedoch unter strengen hygienischen, nämlich spezifisch Pathogen-freien (SPF) Bedingungen gehalten, wird die Kolitis-Entstehung verhindert bzw. verlangsamt [45, 50-52].
Die konventionelle Darmmikrobiota der in unseren Versuchen verwendeten Mäuse wurde mittels Fünffach-Antibiose im Trinkwasser nahezu vollständig eradiziert, um die Entwicklung der Kolitis zu verhindern und der natürlichen Kolonisationsresistenz gegenüber *C. jejuni* entgegen zu wirken [40,53]. Wir konnten zeigen, dass *C. jejuni* nach oraler Infektion im Darm dieser Mäuse stabil ansiedeln konnte und die Tiere - ähnlich wie etwa immunsupprimierte Patienten - binnen einer Woche eine akute, nicht-selbstlimitierende, ulzerative Kolitis entwickelten [40,54].

1.3 Zielsetzung

Trotz der großen Relevanz von *Campylobacter*-Infektionen werden die molekularen Grundlagen der Immunpathogenese von *C. jejuni*-Wirts-Interaktionen bisher nur unzureichend verstanden. Um diesen Mechanismen weiter nachzugehen, untersuchten wir verschiedene Proteine, die zur Invasivität und somit Pathogenität von *C. jejuni* entscheidend beitragen. Diese Arbeit stellt Ausschnitte aus drei bereits veröffentlichten Publikationen zu den oben beschriebenen Proteinen bzw. Proteinklassen dar und soll einen Überblick über die bisher erlangten Erkenntnisse schaffen.

1.4 Methodik

In diesem Teil werden die für sämtliche drei zugrunde liegenden Publikationen gleichermaßen verwendeten Methoden dargestellt. Detailliertere Angaben können dem Methodenteil der jeweiligen Publikation entnommen werden.

1.4.1 Ethische Erklärungen

Alle Tierversuche wurden gemäß den Bestimmungen des deutschen Tierschutzgesetzes durchgeführt (2010/63/EU) und vom Landesamt für Gesundheit und Soziales, Berlin (LaGeSo, Berlin) unter den Registrierungsnummern G0173/07 und G0123/12 genehmigt. Der Gesundheitszustand der Tiere wurde in Zusammenschau mit definierten Versuchsabbruchkriterien mindestens zweimal täglich überprüft.

1.4.2 Versuchstiere und Haltung

IL-10$^{-/-}$-Mäuse (C57BL/10-Hintergrund) wurden in der Forschungseinrichtung für Experimentelle Medizin (FEM) der Charité Berlin gezüchtet und unter SPF-Bedingungen aufgezogen. Mittels einer fünffachen antibiotischen Behandlung über das Trinkwasser wurden die Tiere ab der dritten Woche *post partum* für ca. 12 Wochen in sterilen Käfigen behandelt, um die Darmmikrobiota zu eradizieren. Hierfür wurde ein Gemisch aus Ampicillin (1 mg/l; Ratiopharm), Vancomycin (500 mg/l; Cell Pharm), Ciprofloxacin (200 mg/l; Bayer Vital), Imipinem (250 mg/l; MSD) und Metronidazol (1 g/l; Fresenius) im Trinkwasser *ad libitum* verabreicht. Bereits ab der dritten Woche nach Beginn der antibiotischen Behandlung wurden wöchentlich Faecesproben von jedem Tier in eine Thioglycolat-Anreicherungsbouillion (Oxoid, Wesel, Germany) überführt und bebrütet. Hierdurch konnte das Vorhandensein auch nur geringster Mengen von Darmbakterien ausgeschlossen und der Erfolg der antimikrobiellen Behandlung bestätigt werden [40,55].

1.4.3 Infektion mit *C. jejuni*

Um zu verhindern, dass die antimikrobiellen Substanzen mit der Ansiedlung von *C. jejuni* im Darm interagierten, wurde die Antibiotikalösung drei Tage vor der Infektion

durch sterilisiertes Leitungswasser ersetzt. Die Mäuse wurden daraufhin ein- bzw. zweimalig mit 10^9 Kolonie-bildenden Einheiten (KBE) *C. jejuni* in 0,3 ml PBS mittels Gavage peroral infiziert [39,40].

1.4.4 Quantitative Analysen von *C. jejuni* im Faeces und bakterielle Translokationen

Um die Mengen lebender *C. jejuni* im Gastrointestinaltrakt zu bestimmen, wurden zu definierten Zeitpunkten während des Versuchs Faecesproben und zum Versuchsende luminale Inhalte aus Magen, Duodenum, Ileum und Kolon entnommen und diese mittels Kultur quantitativ analysiert [40,55]. Um die Translokation lebender Bakterien aus dem Darm in extra-intestinale Kompartimente zu bestimmen, wurden die jeweiligen Organe ganz oder teilweise entnommen, in sterilem PBS homogenisiert und auf entsprechenden Agar-Nährmedien bebrütet.

1.4.5 Klinischer Zustand der Mäuse

Um den klinischen Zustand der Versuchstiere im Verlauf der Infektion bewerten zu können, wurden diese vor Infektion sowie zu festgelegten Zeitpunkten nach der Infektion anhand eines standardisierten klinischen „Scores“ beurteilt. Der maximal zu erreichende Punktwert dieses „Scores“ lag bei 12. Zu den Kriterien zählte das Auftreten von Blut im Stuhl (0 Punkte: kein Blut, 2 Punkte: Blut mikroskopisch detektierbar (Gujak-Test, Beckman Coulter/PCD, Krefeld), 4 Punkte: Blut makroskopisch sichtbar), die Stuhlkonsistenz (0 Punkte: geformt, 2 Punkte: weich, 4 Punkte: flüssig) und der klinische Aspekt der Tiere (0 Punkte: unbeeinträchtigt, 2 Punkte: struppiges Fell, weniger körperliche Aktivität, 4 Punkte: Isolation, keine körperliche Aktivität, präfinal).

1.4.6 Probengewinnung und Darmlängenbestimmung

Mittels Isofluran-Begasung wurden die Mäuse zu einem definierten Zeitpunkt (6 bzw. 7 Tage *post infectionem*, p.i.) getötet. Herzblut und Gewebeproben von Organen und aus dem Gastrointestinaltrakt wurden unter sterilen Bedingungen entnommen. Die Proben wurden für histopathologische, immunhistochemische und mikrobiologische Untersuchungen parallel aufbereitet. Weiterhin wurde die absolute Länge des

Dickdarms vom Colon ascendens bis zum Rektum sowie des Dünndarms vom Duodenum bis zum ileozökalen Übergang mittels Lineal bestimmt.

1.4.7 Histopathologie und Immunhistochemie

Die für die immunhistochemischen Untersuchungen vorgesehenen Kolonproben wurden in 5%-igem Formalin fixiert, in Paraffin eingebettet und in 5 μm dünne Schichten geschnitten, die daraufhin mit spezifischen Antikörpern gefärbt wurden [39,40,53,56,57]. Für jedes Tier wurde die durchschnittliche Anzahl der jeweiligen Immunzellen *in situ* ermittelt, indem die positiv gefärbten Zellen in jeweils sechs hochauflösenden Feldern („High Power Fields“, HPF; 0,287 mm^2; 400-fache Vergrößerung) von zwei unabhängigen Untersuchern gezählt wurden. Verwendet wurden Antikörper gegen Caspase-3 für apoptotische Zellen (Asp175, Cell Signaling, USA, 1:200), Ki67 für proliferierende Zellen (TEC3, Dako, Denmark, 1:100), F4/80 für Makrophagen (#14-1801, clone BM8, e Bioscience, 1:50), Myeloperoxidase-7 für neutrophile Granulozyten (MPO-7, #A398, Dako, 1:500), CD3 für T-Lymphozyten (M-20, Santa Cruz, dilution 1:1000), FOXP3 für regulatorische T-Zellen (FJK-16s, eBioscience, 1:100) und B220 für B-Lymphozyten (eBioscience, San Diego, CA, USA, 1:200). Der Schweregrad der histopathologischen Veränderungen wurde in Hämatoxylin-Eosin (HE) -gefärbten Kolonschnitten mithilfe eines standardisierten „Scores“ befundet [54].

1.4.8 Zytokinmessungen in Überständen von *ex vivo* Biopsien aus Kolon und extra-intestinalen Organen

Biopsien des Kolons wurden längs geschnitten und in sterilem PBS ausgewaschen. Die Kolon- bzw. entsprechenden Organproben (1 cm^2) wurden in 24-well-Platten (Nunc, Wiesbaden) in jeweils 500 μl serumfreiem RPMI-1640 Medium mit Penicillin (100 U/ml) und Streptomycin (100 μg/ml, PAA Laboratories) für 18 Stunden bei 37°C kultiviert. Im Anschluss wurden die verschiedenen pro-inflammatorischen Zytokine in den Überständen mittels „Inflammation Cytometric Bead Assay“ (CBA; BD Biosciences) und FACS Canto II (BD Biosciences) quantifiziert.

1.4.9 Statistische Analysen

Graphische Darstellungen erfolgten mit dem Programm GraphPad Prism 5.0a (GraphPad Software, LaJolla, USA). Die Analysen der LightCycler- Ergebnisse wurden mit Hilfe des Programms Relquant 1.0 (Roche Molecular Biochemicals, Basel, Schweiz) durchgeführt. Mit schwarzem Balken sind jeweils Mittelwert oder Median dargestellt. Signifikante Unterschiede wurden mit dem Mann-Whitney-U-Test berechnet. Wahrscheinlichkeitswerte (P-Werte) $\leq 0,05$ wurden als signifikant angenommen. In den Abbildungslegenden ist angegeben, wie häufig das jeweilige Experiment wiederholt wurde.

1.5 Ergebnisse

In den drei hier zusammengefassten Publikationen konnte gezeigt werden, dass *C. jejuni* unabhängig vom verwendeten Stamm nach peroraler Infektion im Gastrointestinaltrakt gnotobiotischer IL-$10^{-/-}$-Mäusen stabil ansiedeln konnte. Somit waren für alle durchgeführten Untersuchungen vergleichbare Ausgangsbedingungen geschaffen. Unter dem Punkt 1.5 wurden jeweils Daten zur Kolonisierung von *C. jejuni*, der klinischen Symptomatik der Mäuse und exemplarisch gezählten aussagekräftigen Immunzellpopulationen im Dickdarm aufgeführt, um die Bedeutung der untersuchten Proteine bzw. Proteinklassen bei der murinen *Campylobacteriose* darzustellen.
Es wurden die Abbildungen der Originalpublikationen verwendet.

1.5.1 Auszüge der Untersuchungsergebnisse zur vermittelnden Rolle von HtrA in der *C. jejuni*-induzierten Enterokolitis im gnotobiotischen IL-$10^{-/-}$-Mausmodell

Nachdem die Darmflora der IL-$10^{-/-}$-Mäuse mittels Fünffach-Antibiose eradiziert worden war, erfolgte an zwei aufeinanderfolgenden Tagen (Tag 0, 1) die perorale Infektion mit 10^9 KBE *C. jejuni* NCTC11168 WT oder der Mutante *C. jejuni* NCTC11168Δ*htrA*. In Abb. 1 ist zu sehen, dass 6 Tage p.i. bei allen infizierten Mäusen - unabhängig von der *htrA*-Genexpression in *C. jejuni* - eine vergleichbar stabile Kolonisierung mit dem jeweiligen *C. jejuni*-Stamm in Magen, Duodenum, Ileum und Kolon erreicht werden konnte.

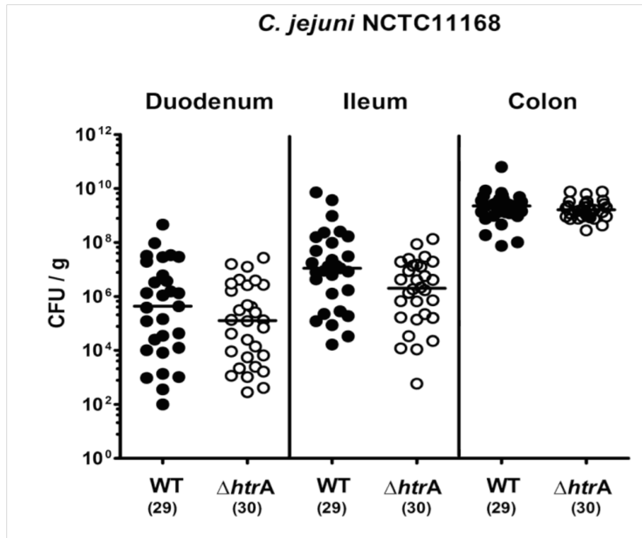

Abb. 1 Kolonisierung von *C. jejuni* NCTC11168-WT und *C. jejuni* NCTC11168Δ*htrA* entlang des Gastrointestinaltraktes gnotobiotischer IL-10$^{-/-}$-Mäuse (Tag 6 p.i.). Schwarze Balken symbolisieren den Median, die Anzahl der analysierten Tiere ist in Klammern angegeben. Es handelt sich um gepoolte Daten aus fünf unabhängigen Versuchen. CFU (Colony Forming Units) = KBE (Kolonie-bildende Einheiten)

Sechs Tage nach Infektion zeigten die mit dem *C. jejuni* NCTC11168Δ*htrA*-Stamm infizierten Mäuse signifikant weniger allgemeine Krankheitsaktivität (p < 0,0005; Abb. 2 A) und weniger häufig blutige Diarrhoen (p < 0,001; 50% vs. 89,7%; Abb. 2B) im Vergleich zu den mit dem WT-Stamm *C. jejuni* NCTC11168 infizierten Tieren.

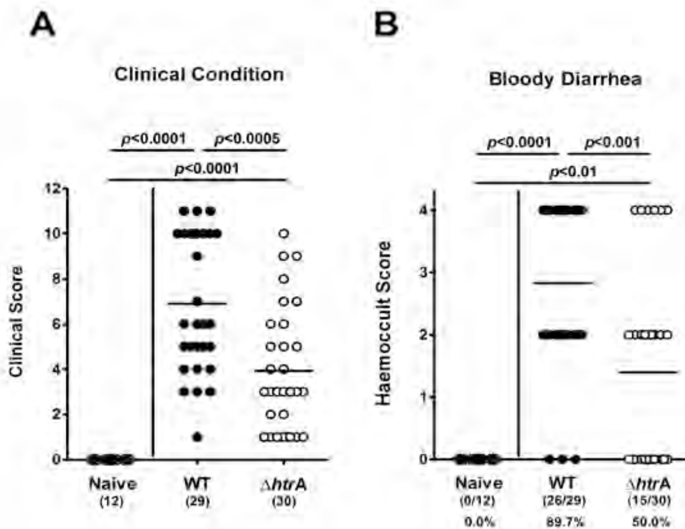

Abb. 2 Klinischen Symptomatik gnotobiotischer IL-10$^{-/-}$-Mäuse nach Infektion mit *C. jejuni* NCTC11168-WT oder *C. jejuni* NCTC11168Δ*htrA* (Tag 6 p.i.). Dargestellt sind die allgemeine Krankheitsaktivität (A) und das Auftreten von Blut im Stuhl (B) 6 Tage nach *C. jejuni*-Infektion. Schwarze Balken symbolisieren den Mittelwert, die Anzahl der analysierten Tiere ist in Klammern angegeben. In Abb. 2B unten befindet sich weiterhin die absolute und relative Häufigkeit von Hämoccult-positiven Mäusen. Es handelt sich um gepoolte Daten aus drei unabhängigen Versuchen.

Abb. 3 zeigt die Anzahl der in sechs hochauflösenden Feldern („High Power Fields“, HPF; 400-fache Vergrößerung) durchschnittlich gezählten apoptotischen sowie

proliferierenden Zellen. In den Dickdärmen der mit dem Mutantenstamm *C. jejuni* NCTC11168Δ*htrA* infizierten Gruppe wurden am sechsten Tag nach Infektion signifikant weniger apoptotische ($p < 0,0001$; Abb. 3A) und signifikant mehr proliferierende Zellen ($p < 0,05$; Abb. 3B) im Vergleich zu den mit dem WT-Stamm infizierten Tieren gezählt. Somit war die *htrA*-Gendefizienz von *C. jejuni* mit weniger Zelluntergang und mehr Zellregeneration nach der Infektion verbunden.

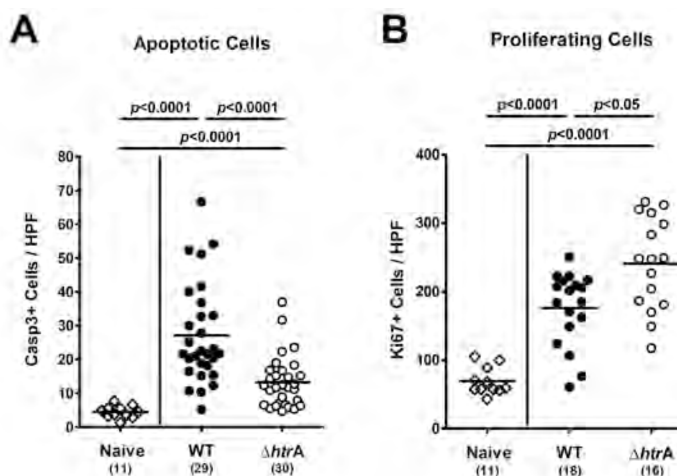

Abb. 3 Apoptotische (A) und proliferierende Zellen (B) im Kolon gnotobiotischer IL-10$^{-/-}$ Mäuse nach Infektion mit *C. jejuni* NCTC11168-WT oder *C. jejuni* NCTC11168Δ*htrA* (Tag 6 p.i.). Schwarze Balken symbolisieren den Mittelwert, die Anzahl der analysierten Tiere ist in Klammern angegeben. Es handelt sich um gepoolte Daten aus fünf unabhängigen Experimenten.

1.5.2 Auszüge der Untersuchungsergebnissen zur vermittelnden Rolle von Cj0268c in der *C. jejuni*-induzierten Enterokolitis im gnotobiotischen IL-10$^{-/-}$-Mausmodell

Mittels Fünffach-Antibiose wurde die kommensale Mikrobiota von IL-10$^{-/-}$-Mäusen eradiziert. Die so generierten gnotobiotischen Tiere wurden an zwei Tagen (Tag 0,1) mit 10^9 lebenden Bakterien der Mutante *C. jejuni* NCTC11168::*cj0268c*, der komplementären Version NCTC11168::*cj0268c*-comp-*cj0268c* oder dem WT-Stamm peroral infiziert (Tag 0, 1). Abb. 4 zeigt, dass die jeweiligen *C. jejuni*-Isolate in allen Abschnitten des Gastrointestinaltraktes vergleichbar stabil ansiedeln konnten. Die Abwesenheit des *cj0268c*-Gens hatte demnach keinen Einfluss auf die Kolonisationskapazität von *C. jejuni* im Darm gnotobiotischer IL-10$^{-/-}$-Mäuse.

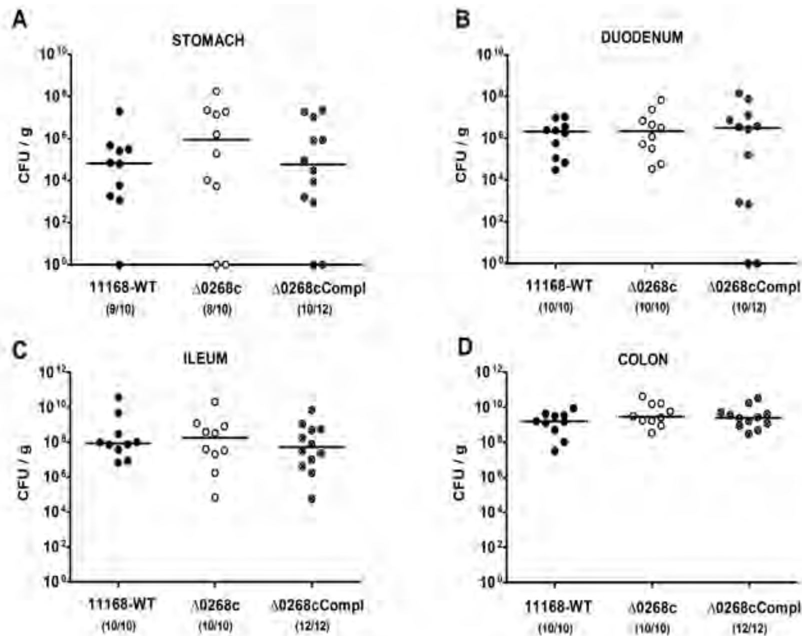

Abb. 4 Kolonisierung von *C. jejuni* NCTC11168-WT, *C. jejuni* NCTC11168::*cj0268c* und *C. jejuni* NCTC11168::*cj0268c*-comp-*cj0268c* entlang des Gastrointestinaltraktes gnotobiotischer IL-10$^{-/-}$-Mäuse (Tag 6 p.i.). Schwarze Balken symbolisieren den Median, die Anzahl der analysierten Tiere ist in Klammern angegeben. Es handelt sich um gepoolte Daten aus drei unabhängigen Experimenten.
CFU (Colony Forming Units) = KBE (Kolonie-bildende Einheiten)

In Mausmodellen ist eine Darmentzündung häufig mit Darmverkürzung vergesellschaftet [33,40,55]. Während der Sektion an Tag 6 p.i. wurden daher die absoluten Längen von Dünn- und Dickdarm erfasst. Bei den Mäusen, welche mit der *C. jejuni*-Mutante NCTC11168::*cj0268c* infiziert worden waren, konnten ungefähr um 10% längere Dünn- und um ca. 20% längere Dickdärme als bei den mit dem WT-Stamm *C. jejuni* NCTC11168 ($p < 0,05$; Abb. 5A und 5B) und den mit der Komplementanten *C. jejuni* NCTC11168::*cj0268c*-comp-*cj0268c* infizierten Mäuse ($p < 0,01$; Abb. 5A; $p < 0,05$; Abb. 5B) gemessen werden. Dies spricht für eine weniger stark ausgeprägte Entzündungsreaktion im Darm der IL-10$^{-/-}$-Mäuse nach Infektion mit der *cj0268c*-Mutante.

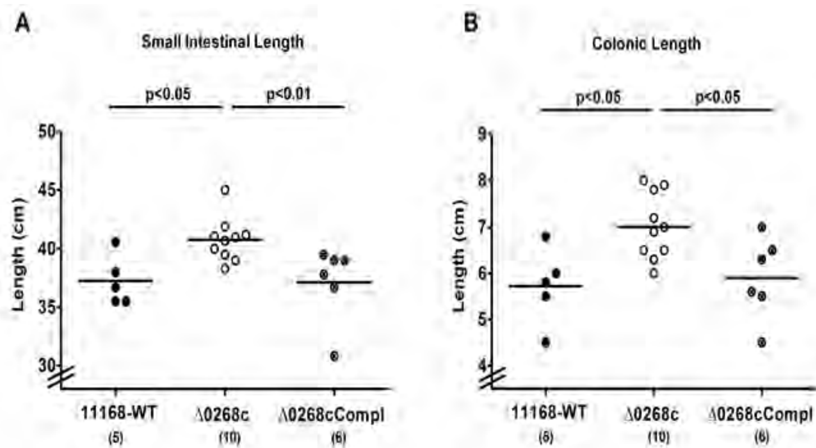

Abb. 5 Darmlängen gnotobiotischer IL-10$^{-/-}$-Mäuse nach Infektion mit *C. jejuni* NCTC11168-WT, *C. jejuni* NCTC11168::*cj0268c* oder *C. jejuni* NCTC11168::*cj0268c*-comp-*cj0268c* (Tag 6 p.i.). Schwarze Balken symbolisieren den Mittelwert, die Anzahl der analysierten Tiere ist in Klammern angegeben. Es handelt sich um gepoolte Daten aus drei unabhängigen Experimenten.

In Abb. 6 ist die Anzahl der durchschnittlich gezählten apoptotischen Zellen und T-Lymphozyten (Marker CD3) dargestellt. In der Kolonmukosa der mit der Mutante *C. jejuni* NCTC11168::*cj0268c* infizierten Gruppe wurden signifikant weniger apoptotische Zellen ($p < 0{,}05$; Abb. 6A) und T-Lymphozyten ($p < 0{,}01$ vs. WT und $p < 0{,}05$ vs. Komplementante; Abb. 6B) im Vergleich zu den mit dem WT-Stamm bzw. der Komplementanten infizierten Mäuse gezählt. Dieses Ergebnis steht mit weniger programmiertem Zelltod und einer reduzierten T-Zell-gesteuerten *C. jejuni*-induzierten pro-inflammatorischen Immunantwort bei Fehlen des *cj0268c*-Gens im Einklang.

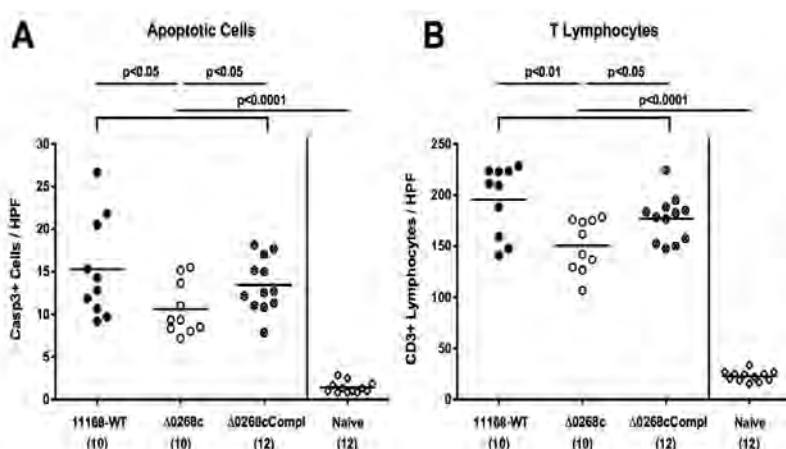

Abb. 6 Apoptotische Zellen (A) und T-Lymphozyten (B) im Dickdarm gnotobiotischer IL-10$^{-/-}$- Mäuse nach Infektion mit *C. jejuni* NCTC11168-WT, *C. jejuni* NCTC11168::*cj0268c* oder *C. jejuni* NCTC11168::*cj0268c*-comp-*cj0268c* (Tag 6 p.i.). Schwarze Balken symbolisieren den Mittelwert, die Anzahl der analysierten Tiere ist in Klammern angegeben. Es handelt sich um gepoolte Daten aus drei unabhängigen Experimenten.

1.5.3 Auszüge der Untersuchungsergebnisse zur vermittelnden Rolle von MMP-2 und MMP-9 in der *C. jejuni*-induzierten Enterokolitis im gnotobiotischen IL-$10^{-/-}$-Mausmodell

Auch bei gnotobiotischen IL-$10^{-/-}$-Mäusen, die mit *C. jejuni* 81-176 infiziert und dann von Tag 1 bis 6 p.i. mit dem synthetischen selektiven Gelatinasehemmer RO28-2653 bzw. Placebo („PLC“: steriles PBS) behandelt wurden, konnte in beiden Gruppen eine vergleichbare pathogene Kolonisierung entlang des Gastrointestinaltraktes beobachtet werden (Abb. 7).

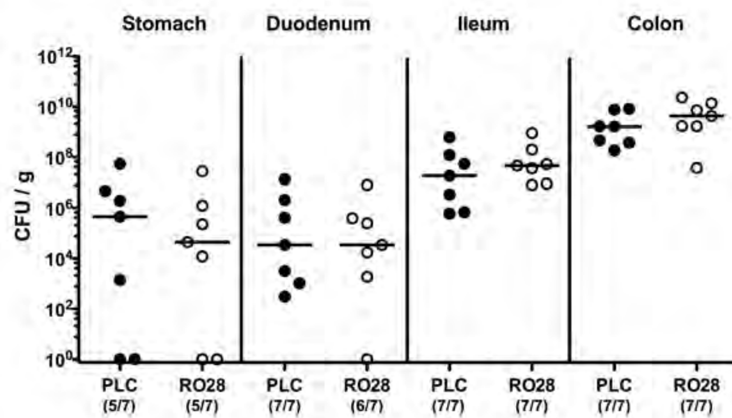

Abb. 7 Kolonisierung von *C. jejuni* 81-176 entlang des Gastrointestinaltraktes gnotobiotischer IL-$10^{-/-}$-Mäuse nach Behandlung mit RO28-2653 oder PLC (Tag 7 p.i.). Schwarze Balken symbolisieren den Median, die Anzahl der analysierten Tiere ist in Klammern angegeben. Es handelt sich um gepoolte Daten aus zwei unabhängigen Experimenten. CFU (Colony Forming Units) = KBE (Kolonie-bildende Einheiten).

Abb. 8 zeigt den klinischen Zustand der mit *C. jejuni* 81-176 infizierten gnotobiotischen IL-$10^{-/-}$-Mäuse unter Behandlung mit RO28-2653 bzw. PLC an Tag 7 p.i.. Durch die Gabe des selektiven Gelatinaseinhibitors wurde - verglichen mit der Placebogruppe - der klinische Zustand im Allgemeinen sowie das Auftreten blutiger Durchfälle im Speziellen signifikant reduziert ($p < 0,05$; Abb. 8).

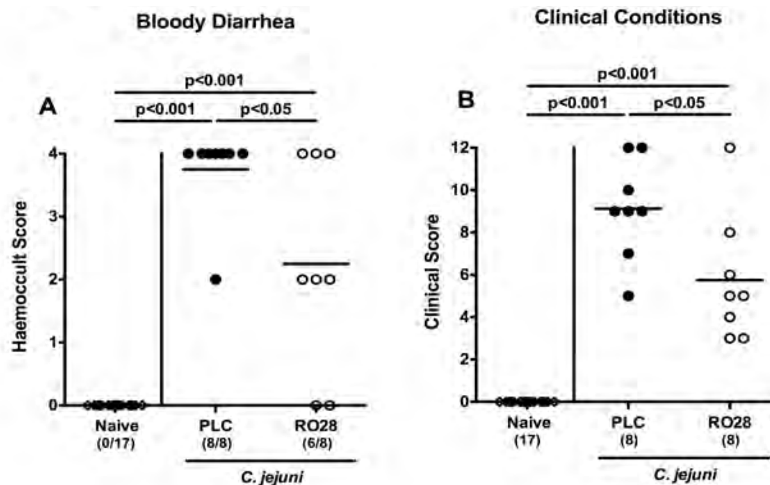

Abb. 8 Klinische Symptomatik gnotobiotischer IL-10$^{-/-}$-Mäuse nach Infektion mit *C. jejuni* 81-176 und Behandlung mit RO28-2653 oder PLC (Tag 7 p.i.). Dargestellt sind das Auftreten von blutigen Durchfällen (A) sowie die allgemeine Krankheitsaktivität (B). Schwarze Balken symbolisieren den Mittelwert, die Anzahl der analysierten Tiere ist in Klammern angegeben. Es handelt sich um gepoolte Daten aus zwei unabhängigen Experimenten.

Die anti-inflammatorische Wirksamkeit von RO28-2653 bei der *C. jejuni*-vermittelten Immunpathologie zeigte sich anhand der Quantifizierung apoptotischer und proliferierender Zellen in der Kolonmukosa infizierter Mäuse. So ist in Abb. 9 die Anzahl der in mindestens 6 hochauflösenden Feldern durchschnittlich gezählten Caspase-3- und Ki67-positiv gefärbten Zellen dargestellt. Interessanterweise wiesen *C. jejuni*-infizierte und mit RO28-2653 behandelte IL-10$^{-/-}$-Mäuse im Vergleich zur Placebo-Kontrollgruppe signifikant weniger apoptotische ($p < 0{,}01$; Abb. 9A) und mehr proliferierende Zellen ($p < 0{,}05$; Abb. 9B) in der Kolonmukosa an Tag 7 p.i. auf.

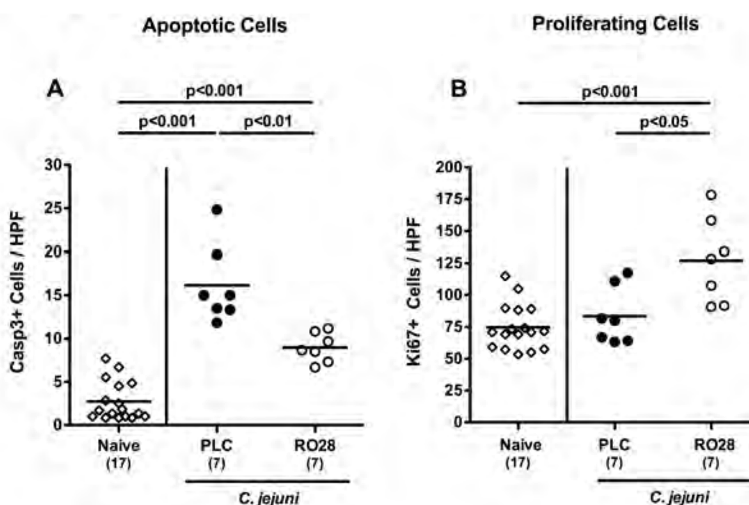

Abb. 9 Apoptotische (A) und proliferierende Zellen (B) im Dickdarm gnotobiotischer IL-10$^{-/-}$-Mäuse nach Infektion mit *C. jejuni* 81-176 und Behandlung mit RO28-2653 oder PLC (Tag 7 p.i.). Schwarze Balken symbolisieren den Mittelwert, die Anzahl der analysierten Tiere ist in Klammern angegeben. Es handelt sich um gepoolte Daten aus zwei unabhängigen Experimenten.

1.6. Diskussion

Die in dieser Arbeit zusammengefassten *in vivo*-Studien zeigen, dass sowohl HtrA und Cj0268c als auch die Gelatinasen MMP-2 und -9 die *C. jejuni*-induzierte Immunpathologie im gnotobiotischen IL-$10^{-/-}$-Mausmodell vermitteln. Dies wurde anhand von klinischen Beobachtungen der Tiere und Quantifizierung definierter Immunzellpopulationen bzw. dem Vorhandensein apoptotischer und proliferierender Zellen in der Kolonmukosa *in situ* dargestellt. Apoptose dient als aussagekräftiger diagnostischer Marker für die histopathologische Bewertung von intestinalen Pathologien [58] und ist vor allem bei der *C. jejuni*-induzierten Enterokolitis von Bedeutung [40]. Das Ki67-Protein stellt einen geeigneten Marker für Zellproliferation dar, da es während der aktiven Zellzyklus-Phasen (G(1), S, G(2)), nicht aber während der Arrestphase ruhender Zellen (G(0)) an der Zelloberfläche exprimiert wird [59] und zeigte in den dargestellten Studien eine Kompensation des durch *C. jejuni* verursachten apoptotischen Zelluntergangs durch Zunahme der Zellproliferation an.
Weder die Abwesenheit der Gene *htrA* bzw. *cj0268c* im *C. jejuni*-Stamm NCTC11168, noch die Behandlung *C. jejuni* 81-176-infizierter IL-$10^{-/-}$-Mäuse mit dem selektiven Gelatinaseinhibitor RO28-2653 beeinträchtigte die intestinale Kolonisationsfähigkeit von *C. jejuni*. Darüber hinaus konnte eine mögliche antimikrobielle Wirksamkeit des Gelatinaseinhibitors RO28-2653 *in vitro* ausgeschlossen werden.

Die Bedeutung von HtrA bei der *C. jejuni*-vermittelten Immunpathologie im gnotobiotischen IL-$10^{-/-}$-Mausmodell

Zahlreiche gastrointestinale Pathogene nutzen die Serinprotease HtrA, um die natürliche Barriere im Darm zu überwinden und in Wirtszellen eindringen zu können [19-22]. Kürzlich konnte durch *in vitro*-Versuche gezeigt werden, dass von *C. jejuni* sezerniertes HtrA einen wichtigen Pathogenitätsfaktor darstellt, da das Protein an der zellulären Invasion sowie Transmigration durch Epithelzellen mittels Spaltung von E-Cadherin und Öffnung von Zell-Zell-Verbindungen beteiligt ist [18,20,25,60]. Humphreys et al. konnten zeigen, dass es nach oraler im Gegensatz zur intravenösen Infektion mit einer *htrA*-Mutante von *Salmonella* (*S.*) *typhimurium* nicht zur bakteriellen Translokation in Leber und Milz kam [61]. Dieser Befund legt nahe, dass HtrA zum

Überwinden der intestinalen Barriere von Bedeutung ist. Weiterhin erwiesen sich *htrA*-Mutanten von *S. typhimurium, Listeria monocytogenes* und *Yersinia enterocolitica* im Vergleich zu WT-Stämmen als anfälliger für oxidativen Stress [62-64]. Dies ist ein Indiz für die Bedeutung von HtrA für die Stabilität und „Fitness" der Bakterien. HtrA verfügt sowohl über eine Chaperon- als auch über eine Protease-Aktivität, wobei die Chaperon-Aktivität mittels korrekter Faltung von extramembranösen Adhäsionsmolekülen vor allem für die Bindung von *C. jejuni* an Epithelzellen und die Protease-Aktivität für die Invasion in Wirtszellen verantwortlich ist [16,17].

Wie bereits erwähnt, beeinflusste die Inaktvierung des *htrA*-Gens in unseren Versuchen die Kolonisationsfähigkeit von *C. jejuni* in gnotobiotischen IL-$10^{-/-}$-Mäusen nicht. Bæk et al. zeigten jedoch, dass *C. jejuni* die Chaperon-Aktivität des HtrA-Proteins für die effiziente Bindung an Epithelzellen benötigt. Wenn man nun davon ausgeht, dass die effiziente Bindung von *C. jejuni* an Darmepithelzellen eine Voraussetzung für die stabile Kolonisierung mit dem Pathogen darstellt, erscheint die in unseren Versuchen gezeigte stabile Kolonisierung mit dem „Knockout"-Stamm *C. jejuni* NCTC11168Δ*htrA* widersprüchlich zu den Befunden von Bæk et al.. Die von Bæk et al. verwendeten INT-407-Zellen bildeten allerdings keine polaren Zellschichten, während in Mäusedärmen sehr wohl polare Zellen vorkommen. Somit handelt es sich bei den geschilderten *in vitro*- und *in vivo*-Versuchen um verschiedene Ausgangsbedingungen auf Rezeptorebene [16,17]. Weiterhin beeinflussen neben HtrA auch viele andere Proteine die Adhäsions- und Invasionsfähigkeit von *C. jejuni* [10-14].

An Tag 6 p.i. war die ulzerierende Enterokolitis bei den Mäusen, die mit der *htrA*-Mutante infiziert worden waren, weniger stark ausgeprägt als bei den mit dem WT-Stamm infizierten Kontrolltieren. Weiterhin waren nicht nur lokale (d.h. intestinale), sondern auch systemische Immunantworten nach Infektion mit dem Mutanten-Stamm *C. jejuni* NCTC11168Δ*htrA* weniger stark ausgeprägt, was an signifikant geringeren Serum-Konzentrationen von pro-inflammatorischen Zytokinen wie TNF-α und IL-6 im Vergleich zu den WT-infizierten Mäusen zu sehen war (Daten nicht dargestellt, siehe Publikation auf Seite 42).

Wie im Kolon wurden in der mit dem Mutanten-Stamm infizierten Gruppe auch geringere TNF-α - und NO-Konzentrationen in den Nieren sowie niedrigere NO-Spiegel in den Lebern an Tag 6 p.i. im Vergleich zu den WT-infizierten gnotobiotischen IL-$10^{-/-}$-Mäusen gemessen (Daten nicht dargestellt, siehe Publikation auf Seite 43,44).

Obwohl die von uns erhobenen Daten für einen protektiven Effekt der *htrA*-Gendefizienz von *C. jejuni* im Darm sprechen, wurden auf systemischer Ebene diskrepante Befunde erhoben. So waren unerwarteterweise die Konzentrationen von IFN-γ, IL-6 und TNF-α in Milz und Leber an Tag 6 nach Infektion mit dem Mutanten-Stamm höher als nach Infektion mit dem WT-Stamm - jedoch mit beträchtlichen Standardabweichungen innerhalb der mit dem Mutanten-Stamm infizierten Gruppe (Daten nicht dargestellt, siehe Publikation auf Seite 44), was die nicht unerhebliche interindividuelle Variabilität der Immunreaktion auf eine Infektion widerspiegelt. Aufgrund der signifikant höheren Konzentrationen von IFN-γ, IL-6 und TNF-α in den Milzen nach Infektion mit dem Mutanten-Stamm im Vergleich zum WT-Stamm könnte vermutet werden, dass durch die Präsentation von *C. jejuni* NCTC11168Δ*htrA* im Darm oder durch lösliche bakterielle Faktoren in weitaus stärkerem Maße Immunzellen (wie z.B. dendritische Zellen, Lymphozyten) in der Milz aktiviert wurden, welche die Darminfektion bekämpften und somit protektiv wirkten. Es sollten weitere Studien durchgeführt werden, um diesem Phänomen nachzugehen und die molekularen Mechanismen der *C. jejuni*-*htrA*-abhängigen systemischen Immunantwort näher zu beleuchten.

In weiteren Experimenten mit unserem Jungtier-Infektionsmodell konnten wir einen ähnlichen HtrA-abhängigen Effekt nach *C. jejuni*-Infektion ermitteln. Hierbei wurden drei Wochen junge konventionell gehaltene WT-Mäuse nach dem Absetzen von der Mutter entweder mit dem Mutanten-Stamm *C. jejuni* NCTC11168Δ*htrA* oder dem WT-Stamm *C. jejuni* NCTC11168 peroral infiziert [65]. Interessanterweise wiesen die mit dem Mutanten-Stamm infizierten Mäuse an Tag 7 p.i. signifikant weniger apoptotische Zellen in der Kolonmukosa auf als die mit dem WT-Stamm kolonisierten Mäuse. Weiterhin konnte auch in dieser Versuchsreihe ein Anstieg der proliferierenden Zellen nach Infektion mit dem Mutanten-Stamm im Vergleich zu den naiven und den WT-Stamm-infizierten Kontrolltieren beobachtet werden [65].

Die Bedeutung von Cj0268c bei der *C. jejuni*-vermittelten Immunpathologie im gnotobiotischen IL-10$^{-/-}$-Mausmodell

Wie Tareen et al. anhand von *in vitro*-Versuchen mit dem Referenzstamm *C. jejuni* NCTC11168 zeigen konnten, ist das *C. jejuni*-Protein Cj0268c von großer Relevanz für die Adhäsion und Invasivität des Pathogens [26,28]. Die Bedeutung von Cj0268c wurde auch dadurch verdeutlicht, dass die *cj0268c*-Gendefizienz die Invasionsfähigkeit des Bakteriums unabhängig von der verwendeten Zelllinie (menschliche oder von Hühnern stammende Darmzellen) gleichermaßen *in vitro* beeinflusste [28]. Weiterhin konnten Tareen et al. mittels heterologer Expression des *cj0268c*-Gens in einem *E. coli*-Stamm und daraus folgernder verstärkter Invasion von kultivierten Darmzellen zeigen, dass Cj0268c keine weiteren *C. jejuni*-Proteine für die Wirtszell-Interaktionen benötigte [28]. Anhand unserer Versuche mit gnotobiotischen IL-10$^{-/-}$-Mäusen konnten wir erstmalig die vermittelnde Bedeutung von Cj0268c bei der *Campylobacter*-Infektion *in vivo* veranschaulichen. Nach Infektion mit dem *C. jejuni cj0268c*-„Knockout“-Stamm waren im Vergleich zum WT-Stamm die IFN-γ und IL-6-Konzentrationen in *ex vivo* Biopsien des Kolons, sowie die IFN-γ-Spiegel in mesenterialen Lymphknoten (MLN) um ca. 50% reduziert (Daten nicht dargestellt, siehe Publikation auf Seite 54). In vorherigen Arbeiten konnten wir bereits zeigen, dass eine ausgeprägte *Campylobacteriose* im gnotobiotischen IL-10$^{-/-}$-Mausmodell mit erhöhten Konzentrationen von pro-inflammatorischen Zytokinen wie IFN-γ und IL-6 in Organkulturüberständen von Kolon und MLN einhergeht [40,53,66], was die Signifikanz der in dieser Studie erhobenen Befunde untermauert. Da T-Lymphozyten eine wichtige Rolle bei der Induktion und Aufrechterhaltung der *C. jejuni*-induzierten Immunpathologie in Mäusen spielen, indem sie mittels Zytokinausscheidung weitere Immunzellen anlocken [39,40,53,57,66], könnte spekuliert werden, dass die verminderten pro-inflammatorischen Zytokinantworten nach Infektion mit dem Mutanten-Stamm im Vergleich zum WT-Stamm auf eine weniger stark ausgeprägte Rekrutierung von T-Zellen in den Darm bei Fehlen des *cj0268c*-Gens hinweisen. Eine mögliche Erklärung für die verminderte T-Zell-Rekrutierung könnte der geringere bakterielle Reiz durch *C. jejuni* NCTC11168::*cj0268c* darstellen, da das Bakterium bei *cj0268c*-Gendefizienz aufgrund reduzierter Invasionsfähigkeit [26,28] die Darmbarriere weniger gut überwinden kann.

Unabhängig vom verwendeten *C. jejuni*-Stamm zeigten die IL-10$^{-/-}$-Mäuse überraschenderweise in etwa vergleichbare Durchfallsymptomatik. Dies steht im Widerspruch zu der weniger stark ausgeprägten Immunpathologie im Gastrointestinaltrakt gnotobiotischer IL-10$^{-/-}$-Mäuse nach Infektion mit dem Mutanten-Stamm *C. jejuni* NCTC11168::*cj0268c* im Vergleich zum WT-Stamm. Der fehlende Unterschied in der klinischen Symptomatik könnte darauf zurückgeführt werden, dass das Bild einer Erkrankung stets die Summe der Effekte darstellt, die aus verschiedenen immunpathologischen Mechanismen resultieren. Weiterhin ist Cj0268c nicht der einzige Faktor, der eine Rolle für die Adhäsion und Invasion und folglich die Induktion der Immunpathologie durch *C. jejuni* spielt [10-14]. Je nach Immunstatus des Wirts und Ausprägung der Pathogenitätsfaktoren des Bakteriums unterliegt das klinische Bild der *Campylobacteriose* außerdem einer starken interindivuellen Variabilität.

Die Bedeutung von MMP-2 und -9 bei der *C. jejuni*-vermittelten Immunpathologie im gnotobiotischen IL-10$^{-/-}$-Mausmodell

Die Gelatinasen A und B spielen eine wichtige Rolle bei chronisch entzündlichen Darmentzündungen von Mensch und Maus [33-37,65,67] und unterliegen einem „proinflammatorischen Teufelskreis“. So rekrutieren Gelatinasen Immunzellen in den Gastrointestinaltrakt, woraufhin biologisch aktive Mediatoren wie IL-1, IL-6 und TNF-α von der Oberfläche der Makrophagen freigesetzt werden. Diese Zytokine können wiederum die MMP-Expression in Immun-, Epithel- und Parenchym-Zellen induzieren [68,69]. Die pharmakologische Blockade der Gelatinasen scheint somit als Interventionsstrategie derartiger Immunpathologien im Darm sinnvoll. Nebenwirkungen synthetischer MMP-Inhibition wie Muskelschmerzen, Arthralgien und Wundheilungsstörung finden sich vor allem im Zusammenhang mit nicht-selektiver Hemmung der Kollagenasen (MMP-1, -8, -13) und Stromelysine (MMP-3, -10, -12), nicht aber bei selektiver Gelatinasehemmung [68,69]. Weder in unseren bisherigen Studien zur Anwendung von RO28-2653 in murinen Darmentzündungsmodellen [33,34], noch in einem Ratten-Modell zum Prostatakarzinom oder in toxikologischen Studien an Affen und Ratten [70] konnten nennenswerte, limitierende Nebenwirkungen von RO28-2653 beobachtet werden; selbst mit wesentlich höheren therapeutische Dosen als in unseren Versuchen gewählt.

In unserer Studie zeigten die mit dem selektiven Gelatinaseinhibitor RO28-2653 therapierten Mäuse an Tag 7 p.i. sowohl makroskopisch (signifikant weniger blutige Durchfälle) als auch mikroskopisch (reduzierte Anzahl von Apoptosen in der Kolonmukosa) eine verminderte Krankheitsaktivität im Vergleich zu der Placebo-Gruppe. Diese Ergebnisse stehen im Einklang mit denen unserer vorherigen Studien, in welchen wir den Einfluss von RO28-2653 bei akuter Dünndarm- sowie Dickdarm-Entzündung untersuchten [33,34]. So führte die Behandlung mit RO28-2653 in beiden Inflammationsmodellen (akute *Toxoplasma gondii*-induzierte Ileitis und akute DSS-Kolitis) zu weniger starkem Gewichtsverlust und Darmverkürzung sowie zu einer deutlichen Verbesserung des klinischen Erscheinungsbildes der Mäuse [33,34], was, wie auch in unserem *C. jejuni*-Mausmodell, mit einem verminderten Einstrom von Immunzellen wie T- und B-Lymphozyten, Makrophagen, Monozyten und neutrophilen Granulozyten in die Darmmukosa und Lamina propria verbunden war [33,34].

Um zu eruieren, welche der beiden Gelatinasen-A und -B bei der *C. jejuni*-induzierten Kolitis zur Vermittlung der Immunpathologie beiträgt, infizierten wir im Rahmen einer weiterführenden Studie drei Wochen junge, konventionell besiedelte MMP-2$^{-/-}$- und MMP-9$^{-/-}$- sowie WT-Mäuse unmittelbar nach dem Absetzen von der Mutter mit *C. jejuni* (B2-Stamm) [71]. Bei den mit *C. jejuni* B2 infizierten MMP-2$^{-/-}$-Mäusen fanden sich weniger starke Krankheitszeichen (z.B. blutige Durchfälle) sowie weniger stark ausgeprägte histopathologische Veränderungen inklusive weniger Apoptosen im Kolonepithel als bei den infizierten WT- und MMP-9$^{-/-}$-Mäusen. Weiterhin zeigten sich im Kolon infizierter MMP-2$^{-/-}$- Mäuse deutlich verminderte pro-inflammatorische Immunzellantworten im Vergleich zu infizierten MMP-9$^{-/-}$- oder WT-Mäusen. Da sowohl in intestinalen Inflammationsmodellen als auch bei Patienten, die unter chronisch entzündlichen Darmerkrankungen leiden, eine erhöhte Aktivität der Gelatinasen A und B gezeigt werden konnte [2, 33-37, 65], untersuchten wir in dieser Studie auch die mRNA-Expression von MMP-2 und -9 während der *C. jejuni*-Infektion. In den Dickdärmen der infizierten MMP-9$^{-/-}$- und WT-Mäuse fand sich im Vergleich zu entsprechenden nicht-infizierten Kontrolltieren eine gesteigerte MMP-2 mRNA-Expression. Im Gegensatz dazu war die Expression von MMP-9-mRNA bei infizierten MMP-2$^{-/-}$- und WT-Mäusen vergleichbar. Die hier beschriebenen Befunde zeigen, dass MMP-2 einen wichtigen Faktor bei der *C. jejuni*-induzierte Enterokolitis im Jungtier-

Mausmodell darstellt [71]. Dies steht im Widerspruch zu einer anderen Versuchsreihe, in der Garg et al. anhand von histologischen und klinischen Daten zeigten, dass MMP-2 im Rahmen einer Kolitis protektiv wirkte und zur Aufrechterhaltung der epithelialen Darmbarriere beitrug [31], während MMP-9 die Kolitis verstärkte [32]. Die im Vergleich zu den von uns erhobenen Daten unterschiedlichen Ergebnisse sind möglicherweise auch auf die verschiedenen verwendeten Infektionsmodelle zurückzuführen: So generierten Garg et al. ihre Befunde im murinen DSS-Kolitis- und Trinitrobenzensulfonsäure (TNBS) -Modell sowie im *Salmonella*-Infektionsmodell [31,32]. Garg et al. schlussfolgerten, dass die MMP-9-vermittelte Immunpathologie insgesamt stärker wiegt als die protektive Funktion von MMP-2 und befürworteten daher die selektive Gelatinase-Blockade als mögliche Interventionsstrategie in der Kolitis-Therapie.

Die Zusammenschau der Daten zeigt, dass die drei untersuchten Proteine bzw. Proteinklassen eine wichtige vermittelnde Rolle im Rahmen der *Campylobacter*-Enteritis *in vivo* spielen. Die Befunde wurden in einem perakuten *C. jejuni*-induzierten Inflammationsmodell erhoben, wobei nicht behandelte Tiere innerhalb einer Woche eine schwere ulzerierende Enterokolitis entwickeln, die zum Tode führt. Die anti-inflammatorischen Effekte durch Gendefizienz von *htrA* oder *cj0268c* bzw. die selektive Blockade von Gelatinasen sprechen für die biologische Relevanz der signifikanten Befunde. Die geschilderten Ergebnisse gestatten somit wertvolle Einblicke in die molekularen Mechanismen der Pathogen-Wirtsinteraktion und ermöglichen zukünftig deren weitere Charakterisierung. Der synthetische selektive Gelatinaseinhibitor RO28-2653 stellt zudem eine potentielle Behandlungsoption für Darmentzündungen bspw. im Rahmen der *Campylobacteriose* dar.

1.7 Literaturverzeichnis

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2. Eidesstattliche Versicherung

„Ich, Marie Elisabeth Alutis, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema „Molekulare Mechanismen der durch *Camplyobacter jejuni* induzierten intestinalen und extra-intestinalen Immunpathologien bei gnotobiotischen IL-10-defizienten Mäusen“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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Datum Unterschrift

Anteilserklärung an den erfolgten Publikationen

Marie Elisabeth Alutis hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1:

Heimesaat MM, Alutis M, Grundmann U, Fischer A, Tegtmeyer N, Böhm M, Kühl AA, Göbel UB, Bereswill S.

The role of serine protease HtrA in acute ulcerative enterocolits and extra-intestinal immune responses during *Campylobacter jejuni* infection of gnotobiotic IL-10 deficient mice. Front Cell Infect Microbiol 2014

Beitrag im Einzelnen: Festlegung des Studiendesigns, Versuchsplanung und -vorbereitung, Tierarbeit mit antibiotischer Behandlung, Infektion, täglicher Tiervisite, „Scoring“ und Sektion; mikrobiologische Auswertungen, Analysen der entnommenen Proben, Anfertigung und Beurteilung histologischer Schnitte, statistische Aufarbeitung der Daten, Hilfe bei der redaktionellen Überarbeitung des Manuskripts.

Publikation 2:

Heimesaat MM, Lugert R, Fischer A, Alutis M, Kühl AA, Zautner AE, Tareen AM, Göbel UB, Bereswill S.

Impact of *Campylobacter jejuni cj0268c* knockout mutation on intestinal colonization, translocation, and induction of immunopathology in gnotobiotic IL-10 deficient mice. PLoS One 2014

Beitrag im Einzelnen: Festlegung des Studiendesigns, Versuchsplanung und -vorbereitung, Tierarbeit mit antibiotischer Behandlung, Infektion, täglicher Tiervisite, „Scoring“ und Sektion; mikrobiologische Auswertungen, Analysen der entnommenen Proben, Anfertigung und Beurteilung histologischer Schnitte, statistische Aufarbeitung der Daten, Hilfe bei der redaktionellen Überarbeitung des Manuskripts.

Publikation 3:

Alutis ME, Grundmann U, Fischer A, Kühl AA, Bereswill S, Heimesaat MM.

Selective gelatinase inhibition reduces apoptosis and pro-inflammatory immune cell responses in *Campylobacter jejuni*-infected gnotobiotic IL-10 deficient mice. Eur J Microbiol Immunol 2014

Beitrag im Einzelnen: Aufstellung der Forschungsfrage, Festlegung des Studiendesigns, Versuchsplanung und -vorbereitung, Tierarbeit mit antibiotischer Behandlung, Infektion, täglicher Tiervisite, „Scoring“ und Sektion; mikrobiologische Auswertungen, Analysen der entnommenen Proben, Anfertigung und Beurteilung histologischer Schnitte, statistische Aufarbeitung der Daten, Literaturrecherche, Verfassung des Manuskripts.

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Heimesaat MM, Lugert R, Fischer A, Alutis M, Kühl AA, Zautner AE, Tareen AM, Göbel UB, Bereswill S. Impact of *Campylobacter jejuni cj0268c* knockout mutation on intestinal colonization, translocation, and induction of immunopathology in gnotobiotic IL-10 deficient mice. PLoS One 2014;9:e90148.

Alutis ME, Grundmann U, Fischer A, Kühl AA, Bereswill S, Heimesaat MM. Selective gelatinase inhibition reduces apoptosis and pro-inflammatory immune cell responses in *Campylobacter jejuni*-infected gnotobiotic IL-10 deficient mice. Eur J Microbiol Immunol 2014;4:213-22.

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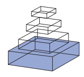

The role of serine protease HtrA in acute ulcerative enterocolitis and extra-intestinal immune responses during *Campylobacter jejuni* infection of gnotobiotic IL-10 deficient mice

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Campylobacter jejuni infections have a high prevalence worldwide and represent a significant socioeconomic burden. *C. jejuni* can cross the intestinal epithelial barrier as visualized in biopsies derived from human patients and animal models, however, the underlying molecular mechanisms and associated immunopathology are still not well understood. We have recently shown that the secreted serine protease HtrA (high temperature requirement A) plays a key role in *C. jejuni* cellular invasion and transmigration across polarized epithelial cells *in vitro*. In the present *in vivo* study we investigated the role of HtrA during *C. jejuni* infection of mice. We used the gnotobiotic IL-10$^{-/-}$ mouse model to study campylobacteriosis following peroral infection with the *C. jejuni* wild-type (WT) strain NCTC11168 and the isogenic, non-polar NCTC11168Δ*htrA* deletion mutant. Six days post infection (p.i.) with either strain mice harbored comparable intestinal *C. jejuni* loads, whereas ulcerative enterocolitis was less pronounced in mice infected with the Δ*htrA* mutant strain. Moreover, Δ*htrA* mutant infected mice displayed lower apoptotic cell numbers in the large intestinal mucosa, less colonic accumulation of neutrophils, macrophages and monocytes, lower large intestinal nitric oxide, IFN-γ, and IL-6 as well as lower TNF-α and IL-6 serum concentrations as compared to WT strain infected mice at day 6 p.i. Notably, immunopathological responses were not restricted to the intestinal tract given that liver and kidneys exhibited mild histopathological changes 6 days p.i. with either *C. jejuni* strain. We also found that hepatic and renal nitric oxide levels or renal TNF-α concentrations were lower in the Δ*htrA* mutant as compared to WT strain infected mice. In conclusion, we show here that the *C. jejuni* HtrA protein plays a pivotal role in inducing host cell apoptosis and immunopathology during murine campylobacteriosis in the gut *in vivo*.

Keywords: ulcerative enterocolitis, colonization resistance, innate immunity, host-pathogen-interaction, bacterial translocation, intestinal immunopathology, extra-intestinal immune responses, systemic immune responses

INTRODUCTION

Campylobacter species are classical zoonotic pathogens, living predominantly as commensals in the gastrointestinal tract of a wide range of birds and mammals, including agriculturally important animals (Young et al., 2007; van Putten et al., 2009; Dasti et al., 2010; Gaynor and Szymanski, 2012). Thus, contaminated animal food products serve as a major source of *Campylobacter* infections in humans (Alter et al., 2011; Oyarzabal and Backert, 2011). The most prevalent *Campylobacter* species in human disease is *C. jejuni*, which represents the leading cause of bacterial infections in the gut and acute diarrheal disease worldwide (Friedman et al., 2000; Young et al., 2007; Mukhopadhya et al., 2011). Disease outcome in humans varies from mild, non-inflammatory, self-limiting diarrhea to severe, inflammatory, bloody diarrhea lasting for several weeks (Young et al., 2007; Oyarzabal and Backert, 2011). In addition, in a minority of infected persons, *C. jejuni* can be associated with the development of reactive arthritis and peripheral neuropathies, the Miller–Fisher and Guillain–Barrè syndromes, respectively (Nachamkin et al., 2008; Szymanski and Gaynor, 2012). *In vivo* and *in vitro* research studies performed in the last two decades revealed that *C. jejuni* exhibits various remarkable properties during infection. An important feature of *C. jejuni* is their ability to bind to and enter human gut epithelial cells causing intestinal tissue damage (Ó'Cróinín and Backert, 2012). *C. jejuni* adherence to epithelial cells has been shown to involve a variety of proposed

and confirmed outer membrane adhesins, including JlpA, PEB1, CadF, FlpA among others (Pei et al., 1998; Konkel et al., 2005; Poly and Guerry, 2008; Novik et al., 2010; Eucker and Konkel, 2012). *C. jejuni* invasion of cultured INT-407 and other cell lines has been observed to induce rearrangements of the host cytoskeleton by small Rho GTPases, Rac1, and Cdc42, which are directly linked to bacterial uptake (Krause-Gruszczynska et al., 2007a, 2011; Boehm et al., 2011; Eucker and Konkel, 2012). *C. jejuni* can also cross the intestinal epithelial barrier as visualized in biopsies derived from human patients (Backert et al., 2013). Efforts with rodent and chicken infection model systems have been made to study pathogenicity mechanisms of *C. jejuni in vivo*, but each animal system has diverse limitations. Currently, disease manifestation such as gastroenteritis mimicking human camplylobacteriosis can be achieved in distinct infection models such as conventionally colonized infant wild-type (WT) and gnotobiotic IL-$10^{-/-}$ mice (Gaynor and Szymanski, 2012; Haag et al., 2012a,b). Notably, when infecting with a *C. jejuni* B2 strain (which is well known for its effective colonization properties) immediately after weaning, approximately 90% of conventionally colonized 3-weeks-old infant mice developed self-limiting enterocolitis within 6–8 days resolving within 2 weeks post infection (p.i.) (Haag et al., 2012b). However, when infecting with other *C. jejuni* strains than B2 such as strains 81–176 or 11168, a huge variability in colonization and disease development could be observed in the infant mouse model. Irrespective of the *C. jejuni* strain, however, gnotobiotic IL-$10^{-/-}$ mice get readily colonized by the pathogen at high loads following peroral infection. Very similar to immunocompromised patients, infected mice develop non-self limiting wasting ulcerative enterocolitis within 1 week p.i. (Haag et al., 2012a; Heimesaat et al., 2014). Importantly, the intestinal inflammation induced by *C. jejuni* in mice and humans is aggravated by *C. jeuni* lipooligosaccharide (LOS) via Toll-like-receptor-4 (TLR-4), as we could show previously (Haag et al., 2012a).

We and others have recently reported that the HtrA (high temperature requirement A) protein of *C. jejuni* is a novel virulence factor (Brøndsted et al., 2005; Bæk et al., 2011a,b; Boehm et al., 2012; Hoy et al., 2012). Bacterial HtrA proteins represent a class of conserved heat shock induced serine proteases with additional chaperone activity, which were shown to have a significant impact on the virulence capabilities of various bacterial pathogens (Ingmer and Brøndsted, 2009; Backert et al., 2013; Frees et al., 2013; Skorko-Glonek et al., 2013). For example, it was demonstrated that growth of the Δ*htrA* mutant was severely impaired at 44°C as compared to WT *C. jejuni* and tolerance of the mutant bacteria against oxygen stress is strongly reduced (Brøndsted et al., 2005). In many different bacterial species, HtrA proteins are localized in the periplasm, where they form proteolytically active multimers with crucial function in the intracellular protein quality control machinery (Clausen et al., 2002, 2011). The class of HtrA proteins typically consists of a signal peptide, a trypsin-like serine protease domain and one or two PDZ [post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (ZO-1)] domains for protein-protein interactions (Kim and Kim, 2005). For long time it was assumed that HtrA family members are strictly acting intracellularly within the bacteria. However, very recently we have discovered a remarkably new feature of HtrA during infection. In various ε-proteobacteria such as *H. pylori* and *C. jejuni*, HtrA is actively secreted into the extracellular environment, where it cleaves the cell surface adhesion protein and tumor-suppressor E-cadherin (Hoy et al., 2010, 2012; Boehm et al., 2012, 2013). Infection experiments with *C. jejuni in vitro* indicated that HtrA can open the cell-to-cell junctions in cell monolayers by cleaving-off the ∼90-kDa extracellular NTF domain of E-cadherin (Boehm et al., 2012; Hoy et al., 2012). Deletion of the *htrA* gene leads to a defect in E-cadherin shedding and transmigration of *C. jejuni* across monolayers of polarized human MKN-28 epithelial cells *in vitro* (Boehm et al., 2012). However, the potential relevance of the *htrA* gene for the interaction of *C. jejuni* with the host immune system has not been investigated so far.

To address this important question, we applied in the present study the *C. jejuni* infection model system of gnotobiotic IL-$10^{-/-}$ mice. Here we investigated the role of *C. jejuni* HtrA in (i) colonization capacity, (ii) translocation, (iii) clinical outcome, (iv) intestinal inflammation, and (v) extra-intestinal sequelae including systemic immune responses following infection of gnotobiotic IL-$10^{-/-}$ mice with the *C. jejuni* WT strain NCTC11168 and the isogenic knockout mutant strain NCTC11168Δ*htrA*.

MATERIALS AND 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, Germany; registration numbers G0123/12). Animal welfare was monitored twice daily by assessment of clinical conditions.

C. JEJUNI STRAINS AND GENETIC COMPLEMENTATION OF *HTRA*

For genetic complementation of *htrA*, we used the pCam-148 chromosomal *C. jejuni* complementation vector kindly provided by Dr. Dennis Linton (University Manchester, UK). Briefly, pCam-148 contains a 2,178 bp fragment of *C. jejuni* NCTC11168 genomic DNA (position 205,297 to 207,475) cloned into the *Sma*I restriction site of plasmid pUC18. pCam-148 contains a singular *Spe*I restriction site in the *C. jejuni* sequence within the pseudogene downstream of Cj0208. We used this *Spe*I site to introduce three additional restriction sites (*Not*I, *Mlu*I, and *Nru*I) using the primer annealing approach. Subsequently, we amplified by PCR a 1,694 bp fragment of the *htrA* gene of *C. jejuni* NCTC11168, including 200-bp upstream and 75-bp downstream sequences using the primers HtrA-1 5′GTTATATTTCCTTAAAAATTTTTAC and HtrA-2 5′AGTTTTCCTTTATTTAAACTTAAT. The resulting PCR product was cloned into the pSB-249 vector containing flanking *Not*I and *Mlu*I sites, respectively. The *htrA* gene was then further subcloned into the *Not*I and *Mlu*I sites of pCam-148. As a selection marker, we used a 795-bp kanamycin-resistant Aph cassette with its own promoter from plasmid pRYSK12 (kindly provided by Dr. Sabine Kienesberger, University Graz, Austria). This Aph cassette was cloned into the *Mlu*I and *Nru*I restriction sites of pCam-148 next to the NCTC11168 *htrA* gene. The resulting *htrA* complementation vector was called

pSB-250. pSB-250 was then transformed into the *C. jejuni* NCTC11168Δ*htrA* deletion mutant (Boehm et al., 2012, 2013) and called NCTC11168Δ*htrA/htrA*. Correct integration of *htrA* in the *C. jejuni* chromosome was confirmed by PCR and standard sequencing. Expression of HtrA proteins was verified by Western blotting.

GROWTH OF *C. JEJUNI* STRAINS ON MH AGAR PLATES

C. jejuni NCTC11168 WT, NCTC11168Δ*htrA* and NCTC11168Δ*htrA/htrA* were grown overnight on Müller-Hinton (MH) agar plates at 37°C under microaerobic conditions using CampyGen gas packs (Oxoid, Wesel, Germany). Bacterial cells were harvested using brain heart infusion broth, and the OD_{600} was adjusted to 0.1. Subsequently, serial dilutions were made, and 10 μl volumes of the 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions were spotted onto three MH agar plates, which were incubated under microaerobic conditions using for 3 days at 42 or 44°C, or at 42°C in the presence of 18% O_2 as described (Brøndsted et al., 2005). Experiments were repeated at least three times.

CASEIN ZYMOGRAPHY

Bacterial lysates, culture supernatants or recombinant HtrA were separated under non-reducing conditions in gels containing casein. Subsequently, gels were renatured in 2.5% Triton-X-100 and equilibrated in developing buffer (Boehm et al., 2012; Hoy et al., 2012). Caseinolytic activity was visualized by staining with 0.5% Coomassie Blue R250.

MICE

IL-$10^{-/-}$ knockout mice (in C57BL/10 background, B10) were bred and maintained under specific pathogen-free (SPF) conditions in the facilities of the "Forschungsinstitut für Experimentelle Medizin" (FEM, Charité - Universitätsmedizin, Berlin, Germany). To eradicate the commensal gut flora, mice were transferred to sterile cages and treated by adding a mix of ampicillin (1 g/L; Ratiopharm), vancomycin (500 mg/L; Cell Pharm), ciprofloxacin (200 mg/L; Bayer Vital), imipenem (250 mg/L; MSD), and metronidazole (1 g/L; Fresenius) to the drinking water *ad libitum* starting at 3 weeks of age right after weaning (Heimesaat et al., 2006; Haag et al., 2012a). Age matched female mice were subjected to the quintuple antibiotic treatment for approximately 4 months before the infection experiments.

C. JEJUNI INFECTION OF MICE

Mice were infected with 10^9 viable colony forming units (CFU) of the *C. jejuni* parental strain NCTC11168 WT strain or the isogenic mutant strain NCTC11168Δ*htrA* by gavage in a total volume of 0.3 mL PBS on 2 consecutive days (day 0 and 1) as described (Haag et al., 2012a,b).

CLINICAL SCORING

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 points: no blood; 2 points: microscopic detection of blood using Haemoccult, Beckman Coulter / PCD, Krefeld, Germany; 4 points: overt 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 (Haag et al., 2012a,b).

SAMPLING PROCEDURES, DETERMINATION OF COLONIC LENGTH, AND HISTOPATHOLOGY

Mice were sacrificed by isofluran treatment (Abbott, Germany). Cardiac blood and tissue samples from mesenteric lymph nodes (MLNs), spleen, liver, kidneys, and intestinal tract (duodenum, ileum, and colon) were removed under sterile conditions. Absolute large intestinal lengths were determined by measuring the distance from the ascending colon leaving the caecum to the rectum by a ruler and expressed in cm. Intestinal samples from each mouse were collected in parallel for histopathological, immunohistochemical, microbiological, and immunological analyses. Immunohistopathological changes were determined in samples derived from colon, liver and kidney that were immediately fixed in 5% formalin and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (H&E), examined by light microscopy (magnification 100× and 400×) and histopathological changes quantitatively assessed applying respective histopathological scoring systems by two independent double-blinded investigators. In brief:

Colonic Histopathology (max. 4 points; according to Paclik et al., 2008): 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.

Hepatic Histopathology (max. 9 points; modified Ishak score, Ishak et al., 1995): Lobular inflammation: 0: normal; 1: minimal inflammation (few inflammatory infiltrates); 2: mild inflammation (increased inflammatory cells, but less pyknotic necrosis); 3: moderate inflammation (marked increase in inflammatory cells and lots of pyknotic necroses); 4: severe inflammation (necrosis); 5: severe inflammation (plus bridging necroses). Portal inflammation: 0: normal; 1: mild inflammation (<1/3 of portal tracts); 2: moderate inflammation (ca. 1/2 of portal tracts); 3: severe inflammation (>2/3 of portal tracts); 4: severe inflammation (plus portal inflammation disperse into parenchyma).

Renal Histopathology (max. 4 points; according to Appel et al., 1978): 0: normal glomerulus; 1: focal and mild hypercellularity (normal = 3 per segment); 2: multifocal and moderate hypercellularity with capillary dilatation and mild hyalinosis; 3: diffuse hypercellularity (>50% of the tuft) and capillary aneurysm; 4: extensive sclerosis/crescents (>3 cell layer), tuft obliteration, collapse.

IMMUNOHISTOCHEMISTRY

In situ immunohistochemical analysis of 5 μm thin colonic paraffine sections was performed as described previously (Heimesaat et al., 2010, 2013; Bereswill et al., 2011; Haag et al., 2012a,b).

Primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, USA, 1:200), Ki67 (TEC3, Dako, Denmark, 1:100), CD3 (M-20, Santa Cruz, dilution 1:1000), myeloperoxidase-7 (MPO-7, # A0398, Dako, 1:500), F4/80 (# 14-4801, clone BM8, eBioscience, 1:50), FOXP3 (FJK-16s, eBioscience, 1:100), and B220 (eBioscience, San Diego, CA, USA, 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 two independent double-blinded investigators and subjected to statistical analysis as indicated below.

QUANTITATIVE ANALYSIS OF *C. JEJUNI* AND BACTERIAL TRANSLOCATION INTO OTHER ORGANS

Live *C. jejuni* were detected at time of necropsy (day 6 p.i.) in luminal samples taken from the duodenum, ileum, or colon diluted in sterile PBS by culture as described earlier (Bereswill et al., 2011). To quantify bacterial translocation into different organs, MLNs, spleen, liver, and kidney were homogenized in sterile PBS and analyzed by cultivating in dilution series on karmali agar (Oxoid, Wesel, Germany) in a microaerobic atmosphere at 37°C for at least 48 h (Heimesaat et al., 2013). In addition, 0.5 mL of cardiac blood was streaked out immediately on karmali agar. The respective weights of luminal fecal or tissue samples were determined by the difference of the sample weights before and after asservation.

CYTOKINE DETECTION IN SERUM SAMPLES AND CULTURE SUPERNATANTS OF *EX VIVO* BIOPSIES TAKEN FROM COLON, MESENTERIC LYMPH NODES, SPLEEN, LIVER, AND KIDNEY

Colonic biopsies and kidneys were cut longitudinally and the former washed in PBS. MLNs or strips of approximately 1 cm^2 colon and liver tissue and additionally half of spleen and kidney were placed in 24-flat-bottom well culture plates (Nunc, Wiesbaden, Germany) containing 500 μL serum-free RPMI 1640 medium supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL; PAA Laboratories). After 18 h of incubation at 37°C, culture supernatants as well as serum samples were analyzed for IFN-γ, TNF-α, and IL-6 by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences) on a BD FACS Canto II flow cytometer (BD Biosciences). Nitric oxide (NO) was determined by Griess reaction as described earlier (Heimesaat et al., 2006).

QUANTITATIVE REAL-TIME PCR (QRT-PCR)

RNA was isolated from colonic tissues using the RNeasy Mini Kit (Qiagen). mRNA was reversed transcribed and analyzed in triplicate assays by TaqMan PCR using a sequence detection system (ABI Prism 7700; Applied Biosystems) as described previously (Wolk et al., 2002; Munoz et al., 2009). For detection of murine MUC-2 assays including double-fluorescent probes in combination with assays for the mouse housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) were purchased from Applied Biosystems. Expression levels were calculated relative to the HPRT expression.

ANTIBODIES AND WESTERN BLOTTING

C. jejuni cell pellets were lysed and proteins were separated by SDS-PAGE (Krause-Gruszczynska et al., 2007b; Wiedemann et al., 2012). The polyclonal rabbit α-HtrA antibody was raised against a conserved peptide corresponding to amino acid (aa) residues 288–301: C-QGDTKKAYKNQEGA. The α-CiaB antibody was generated against the epitope 597–610 (C-EIDNSGEFERYKKK) and the α-MOMP antibody against aa residues 400–413 (C-NLDQGVNTNESADH) in the corresponding proteins, respectively. All three peptides were conjugated to *Limulus polyphemus* haemocyanin carrier protein, and two rabbits each were immunized by Biogenes GmbH (Berlin, Germany) using standard protocols (Tegtmeyer et al., 2013). The resulting antiserum was affinity-purified and the specificity against the proteins in *C. jejuni* was confirmed by Western blotting (Tegtmeyer et al., 2011; Backert and Hofreuter, 2013). Horseradish peroxidase-conjugated anti-rabbit polyvalent sheep immunoglobulin was used as secondary antibody (DAKO Denmark A/S, DK-2600 Glostrup, Denmark). Blots were developed with ECL Plus Western blot reagents (GE Healthcare, UK limited Amersham Place, UK) as described (Conradi et al., 2012; Hirsch et al., 2012).

STATISTICAL ANALYSIS

Mean values, medians, and levels of significance were determined using Mann-Whitney-U-test. Two-sided probability (*P*) values ≤ 0.05 were considered significant. All experiments were repeated various times as indicated in the corresponding figure legends.

RESULTS

HTRA DOES NOT AFFECT THE COLONIZATION CAPACITY OF *C. JEJUNI* IN GNOTOBIOTIC IL-10$^{-/-}$ MICE

In order to eradicate the chronic colitogenic stimulus derived from the commensal intestinal microbiota, IL-10$^{-/-}$ mice were pre-treated with a quintuple antibiotic regimen for approximately 4 months starting immediately after weaning (Haag et al., 2012a). The resulting gnotobiotic IL-10$^{-/-}$ mice were then perorally infected with 10^9 CFU of either *C. jejuni* NCTC11168 WT or isogenic *htrA* mutant (NCTC11168Δ*htrA*) strain, each grown to stationary phase on 2 consecutive days (day 0 and 1). Control blots demonstrate that equal amounts of *C. jejuni* were infected per sample and HtrA is not expressed in the Δ*htrA* mutant as expected (**Figure 1A**). We also confirmed that our Δ*htrA* mutant is non-polar because genetic complementation experiments with the corresponding WT gene restored (i) expression of proteolytically active HtrA multimers (**Figures S1A,B**), (ii) *C. jejuni* growth at high temperature (44°C) (**Figures S2A,B**) and (iii) growth under high oxygen stress conditions (**Figures S2A,C**). Six days following peroral challenge all mice harbored comparable pathogen loads with either strain in the duodenum, ileum and colon, which is indicative for an uncompromised colonization capacity of the Δ*htrA* mutant strain *in vivo* (**Figure 1B**). After 6 days 12.5% of IL-10$^{-/-}$ mice infected with either the Δ*htrA* mutant or the *C. jejuni* WT strain contained viable pathogens also in MLNs as shown by culture. Notably, bacterial presence in extra-intestinal compartments such as spleen, liver, kidney, or blood could not be observed with either strain based on CFU determination (data not shown).

IMPACT OF HTRA ON ACUTE ENTEROCOLITIS IN *C. JEJUNI* INFECTED GNOTOBIOTIC IL-10$^{-/-}$ MICE

Six days p.i. with the *C. jejuni* NCTC11168 WT strain, gnotobiotic IL-10$^{-/-}$ mice were decisively compromised by acute enterocolitis as indicated by wasting clinical symptoms, diarrhea and occurrence of blood in liquid feces in up to 90% of cases (**Figures 2A,B**). In contrast, mice infected with the Δ*htrA* mutant displayed significantly less severe clinical symptoms ($p < 0.0005$; **Figure 2A**) and lower frequency of bloody diarrhea as compared to controls infected with the WT strain (50.0 vs. 89.7%, respectively; $p < 0.001$, **Figure 2B**). Given that intestinal inflammation results in a significant shortening of the intestines (Heimesaat et al., 2006; Bereswill et al., 2011; Haag et al., 2012a), we further assessed the colonic lengths upon *C. jejuni* infection. At day 6 p.i., IL-10$^{-/-}$ mice infected with the Δ*htrA* mutant displayed longer colons as compared to WT strain infected control animals ($p < 0.05$; **Figure 2C**). These results provide first evidence that HtrA aggravates the inflammatory outcome of *C. jejuni* infection. This was further confirmed by histopathological analysis of paraffin embedded colonic sections. Microscopical investigations of H&E-stained tissues revealed that Δ*htrA* mutant infected mice displayed significantly lower histopathological scores as compared to mice infected with the *C. jejuni* WT strain at day 6 p.i. ($p < 0.0001$, **Figure 2D**). The Δ*htrA* mutant induced rather mild inflammatory changes whereas *C. jejuni* WT strain infected mice exhibited acute enterocolitis characterized by ulcerations of and bleeding into the colonic mucosa as well as by diffuse mucosal and submucosal leukocytic infiltrates, loss of goblet cells and crypt drop-outs (**Figure 2D**).

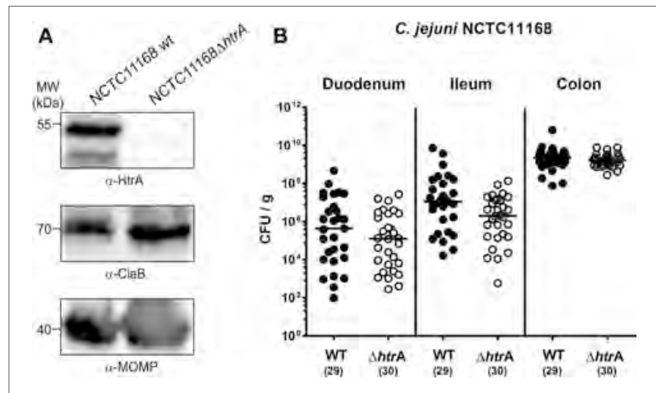

FIGURE 1 | *C. jejuni* colonization along the intestinal tract of gnotobiotic IL-10$^{-/-}$ mice is independent of *htrA* gene expression. **(A)** Control Western blots for the expression of indicated proteins in the two *C. jejuni* strains used in this infection study. **(B)** Gnotobiotic IL-10$^{-/-}$ mice were generated by broad-spectrum antibiotic treatment and perorally infected with *C. jejuni* NCTC11168 wild-type strain (WT, closed circles) or mutant strain NCTC11168Δ*htrA* (Δ*htrA*, open circles). The pathogen densities in distinct compartments of the intestinal tract were determined by quantification of live *C. jejuni* in luminal samples taken from duodenum, ileum, and colon at day 6 p.i. by cultural analysis (CFU, colony forming units). Medians (black bars) are indicated and numbers of analyzed animals given in parentheses. Data shown were combined from five independent experiments.

HTRA TRIGGERS *C. JEJUNI*-MEDIATED INTESTINAL IMMUNE RESPONSES

The role of *C. jejuni* HtrA in intestinal inflammation was next assessed by microscopical quantification of apoptotic and proliferating cells as well as infiltrating immune cells. This was achieved by specific immunohistochemical stainings of colonic paraffin sections. Six days following *C. jejuni* WT strain infection, gnotobiotic IL-10$^{-/-}$ mice displayed a multifold increase of apoptotic cells, neutrophils, macrophages and monocytes, B and T lymphocytes as well as regulatory T cells (Treg) in the colonic mucosa and lamina propria ($p < 0.001 - 0.0001$ as compared to naive control animals; **Figure 3**). The induction of apoptosis and the increase

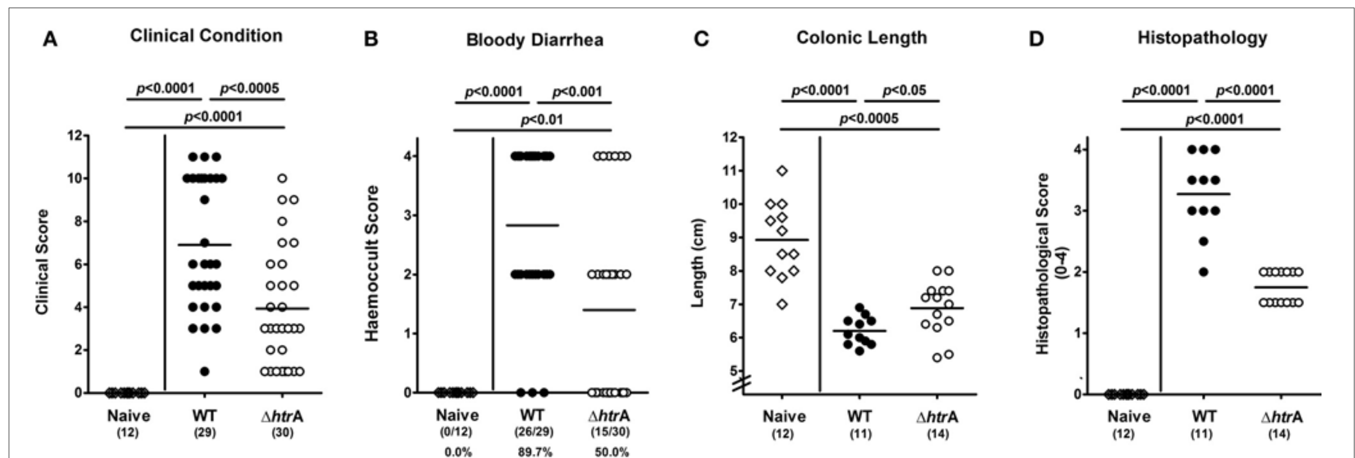

FIGURE 2 | Role of *C. jejuni htrA* gene in acute enterocolitis following infection of gnotobiotic IL-10$^{-/-}$ mice. Six days following *C. jejuni* infection **(A)** disease activity and **(B)** occurrence of blood in fecal samples was assessed by applying respective standardized clinical scoring system. Furthermore, **(C)** large intestinal lengths (in cm) were measured and **(D)** histopathological changes applying a standardized histopathological score in H&E-stained colonic paraffin sections were determined at necropsy. Means (black bars), levels of significance (*P*-values) determined by the Mann-Whitney-U-test, and numbers of analyzed animals (in parentheses) or absolute and relative (in %) numbers of positive samples out of the total number are indicated. Data shown were pooled from three independent experiments.

in inflammatory immune cells, however, were significantly less pronounced in animals infected with the *C. jejuni* Δ*htrA* mutant except for the T cell and Treg populations ($p < 0.05 - 0.0001$ as compared to animals infected with the *C. jejuni* WT strain; **Figure 3**). Given that Ki67 comprises a nuclear protein that is associated with and necessary for cellular proliferation (Scholzen and Gerdes, 2000), we stained colonic paraffin sections against Ki67 to detect proliferative measures of the colonic epithelium counteracting cellular destruction during the inflammatory process. Interestingly, the Δ*htrA* mutant induced significantly higher numbers of $Ki67^+$ proliferating cells as compared to the WT strain ($p < 0.05$; **Figure 3B**).

The *C. jejuni* induced colonic immune cell responses were accompanied by increased expression of pro-inflammatory cytokines in the large intestine. Until day 6 p.i., levels, of IFN-γ and IL-6 and, in addition, NO were multifold increased in colonic *ex vivo* biopsies upon *C. jejuni* infection. Cytokines increased to a significantly lower extent in gnotobiotic IL-$10^{-/-}$ mice infected with the Δ*htrA* mutant as compared to WT strain infected mice ($p < 0.05 - 0.01$; **Figures 4A–C**). In addition, *C. jejuni* infected

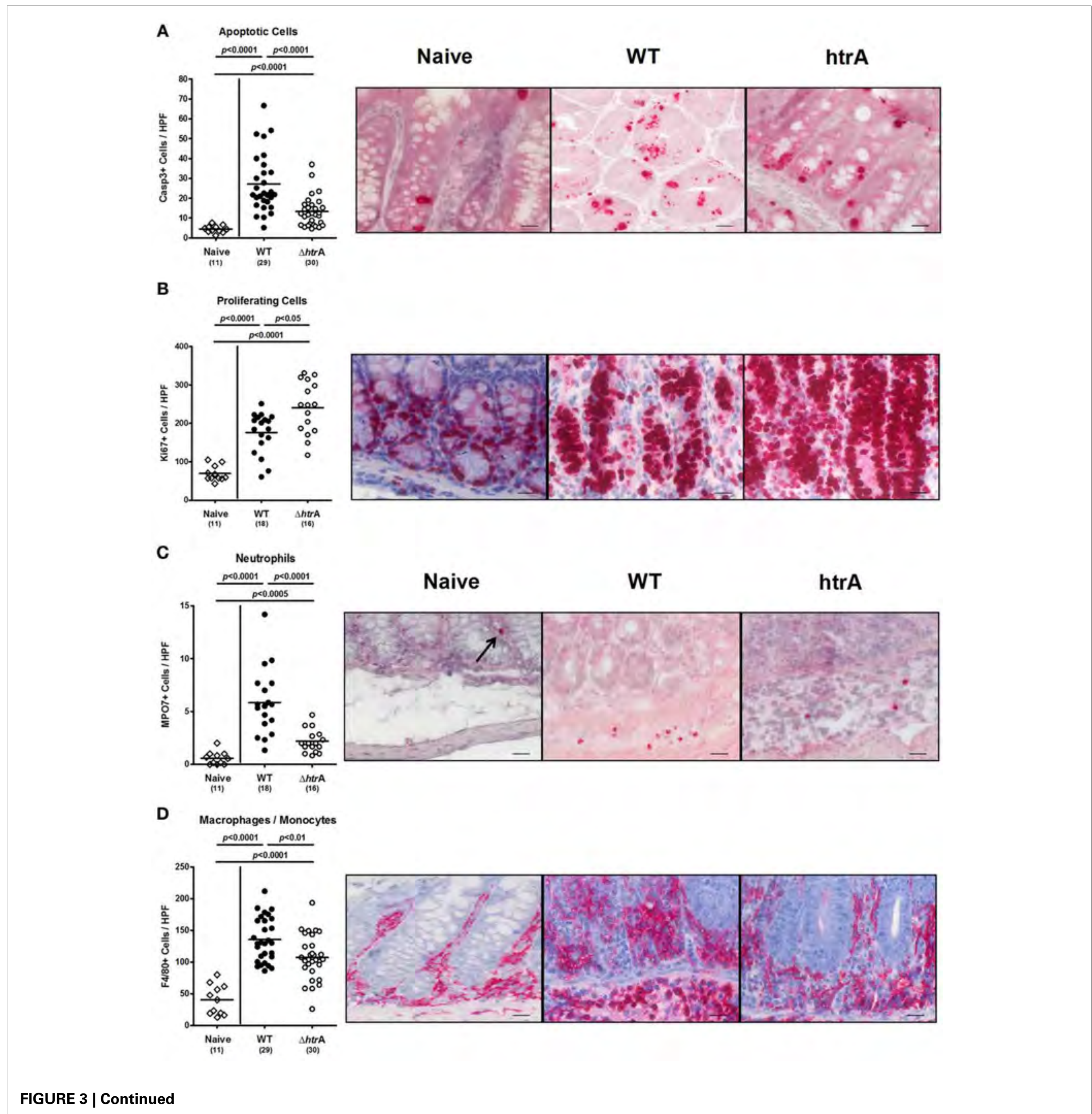

FIGURE 3 | Continued

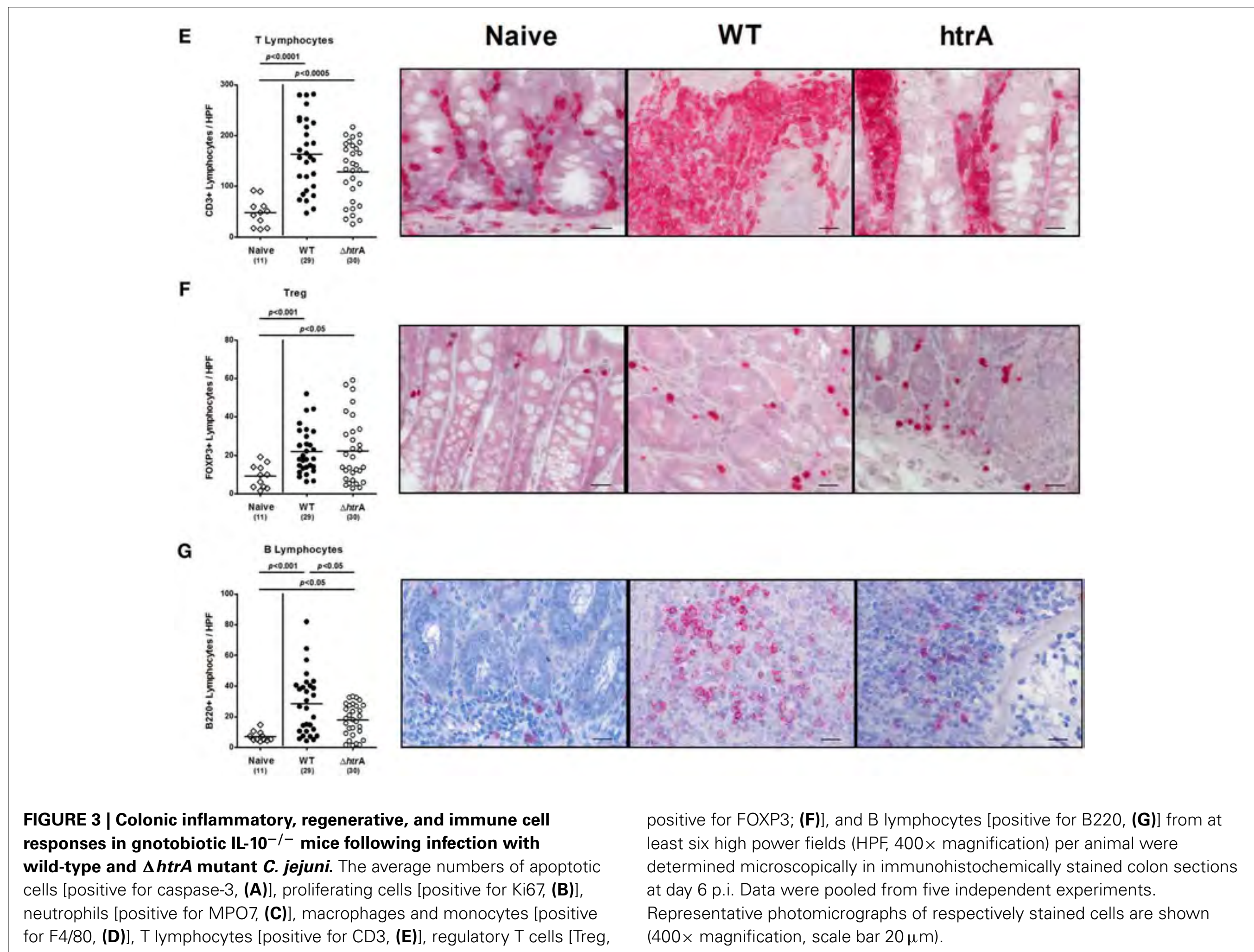

FIGURE 3 | Colonic inflammatory, regenerative, and immune cell responses in gnotobiotic IL-10$^{-/-}$ mice following infection with wild-type and Δ*htrA* mutant *C. jejuni*. The average numbers of apoptotic cells [positive for caspase-3, **(A)**], proliferating cells [positive for Ki67, **(B)**], neutrophils [positive for MPO7, **(C)**], macrophages and monocytes [positive for F4/80, **(D)**], T lymphocytes [positive for CD3, **(E)**], regulatory T cells [Treg, positive for FOXP3; **(F)**], and B lymphocytes [positive for B220, **(G)**] from at least six high power fields (HPF, 400× magnification) per animal were determined microscopically in immunohistochemically stained colon sections at day 6 p.i. Data were pooled from five independent experiments. Representative photomicrographs of respectively stained cells are shown (400× magnification, scale bar 20 μm).

mice displayed increased NO secretion into draining MLNs at day 6 p.i. as compared to uninfected controls. This increase, however, was significantly lower in mice infected with the Δ*htrA* mutant vs. the parental WT strain infected animals ($p < 0.05$; **Figure 4D**).

Given that a proper mucus layer is a pivotal barrier protecting the intestinal epithelium from intestinal pathogens, we next determined the mRNA expression levels of mucin-2 (MUC-2), which is mainly secreted from goblet cells in the epithelial lining of the large intestine (Allen et al., 1998; Naughton et al., 2014). Six days following *C. jejuni* infection of IL-10$^{-/-}$ mice, colonic mucin-2 mRNA was significantly downregulated by *C. jejuni* WT strain, but to a lesser extent by the Δ*htrA* knockout mutant ($p < 0.05$; **Figure 4E**). Taken together, the less severe clinical, histopathological and inflammatory outcome of enterocolitis in mice infected with the *C. jejuni* Δ*htrA* mutant strain was paralleled by a higher mucin-2 expression level as compared to control animals infected with the parental strain.

HTRA IS ALSO INVOLVED IN THE INDUCTION OF EXTRA-INTESTINAL PRO-INFLAMMATORY IMMUNE RESPONSES IN *C. JEJUNI* INFECTED GNOTOBIOTIC IL-10$^{-/-}$ MICE

We next investigated potential systemic pro-inflammatory immune responses upon *C. jejuni* infection. At day 6 p.i. with either strain, TNF-α and IL-6 serum levels were increased as compared to naïve controls, but less distinctly in Δ*htrA* mutant strain infected mice ($p < 0.005$ and $p < 0.05$, respectively; **Figure 5**). Hence, *C. jejuni* htrA knockout mutation not only reduces intestinal but also systemic inflammatory responses upon infection. Unexpectedly, 6 days following infection with the Δ*htrA* gene mutant but not the WT strain, IFN-γ, TNF-α, and NO levels were up-regulated in splenic *ex vivo* biopsies ($p < 0.05 - 0.0005$; **Figures 6A–C**). Furthermore, secretion of IL-6 into spleens were higher following Δ*htrA* mutant infection as compared to the parental strain at day 6 p.i. ($p < 0.0001$, **Figure 6D**).

We have recently shown that following long-term *C. jejuni* infection (i.e., more than 100 days p.i.) conventionally colonized infant mice exhibited pro-inflammatory immune responses at extra-intestinal locations such as liver and kidneys (Haag et al., 2012b; Heimesaat et al., 2013). We were therefore interested to investigate whether extra-intestinal sequelae might arise even after relatively short-term *C. jejuni* infection of gnotobiotic IL-10$^{-/-}$ mice suffering from acute enterocolitis. To address this idea, we assessed inflammatory changes in H&E-stained paraffin sections of liver and kidneys. Six days following *C. jejuni* infection with either strain mice displayed only minimal to mild lobular

FIGURE 4 | Importance of *C. jejuni htrA* gene expression in intestinal pro-inflammatory cytokine responses and mucin-2 expression induced during infection of gnotobiotic IL-10$^{-/-}$ mice. Colonic **(A)** nitric oxide, **(B)** IFN-γ, and **(C)** IL-6 levels, **(D)** nitric oxide concentrations in mesenteric lymph nodes (MLN), and **(E)** large intestinal mucin-2 (MUC-2) mRNA expression levels were determined in respective *ex vivo* biopsies taken at day 6 p.i. Data shown were combined from three independent experiments.

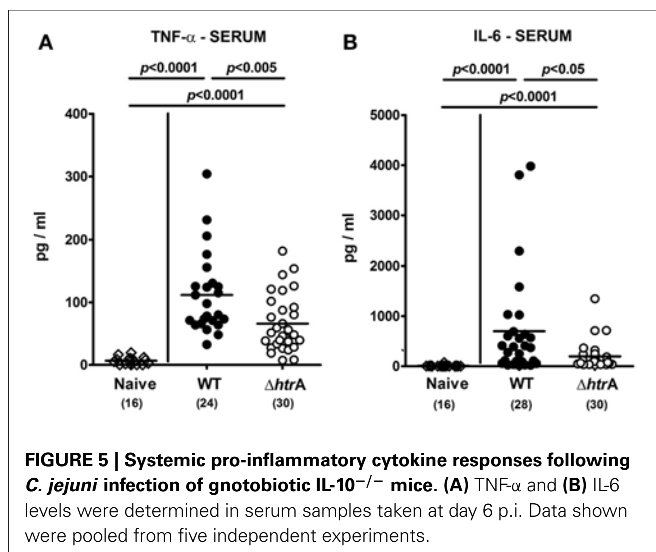

FIGURE 5 | Systemic pro-inflammatory cytokine responses following *C. jejuni* infection of gnotobiotic IL-10$^{-/-}$ mice. (A) TNF-α and **(B)** IL-6 levels were determined in serum samples taken at day 6 p.i. Data shown were pooled from five independent experiments.

and/or portal inflammatory infiltrates in livers and mild focal hypercellularity in kidneys (**Figure 7**).

We next determined pro-inflammatory cytokine responses in *ex vivo* biopsies from liver and kidneys upon *C. jejuni* infection of gnotobiotic IL-10$^{-/-}$ mice. Six days following *C. jejuni* infection hepatic IFN-γ, TNF-α, and IL-6 concentrations increased ($p < 0.05 - 0.0001$; **Figures 8A–C**), but to even higher levels upon Δ*htrA* knockout mutant as compared to parental *C. jejuni* WT strain infection ($p < 0.05$; **Figures 8A–C**). Notably, the standard deviation in the cohort infected with the Δ*htrA* gene knockout mutant was rather high. Interestingly, hepatic NO levels increased 6 days following WT infection, but not during Δ*htrA* mutant strain infection ($p < 0.05$; **Figure 8D**), which helds also true for NO secretion in *ex vivo* biopsies taken from kidneys ($p < 0.05$; **Figure 8E**). Furthermore, TNF-α levels increased in kidneys of *C. jejuni* infected IL-10$^{-/-}$ mice ($p < 0.0001$; **Figure 8F**), but less distinctly in the Δ*htrA* knockout mutant infected group ($p < 0.05$; **Figure 8F**). Taken together, even as early as 6 days following *C. jejuni* infection pro-inflammatory immune responses could be detected in extra-intestinal compartments such as liver and kidneys that were even more pronounced in livers, but less distinct in kidneys of Δ*htrA* mutant as compared to parental WT strain infected IL-10$^{-/-}$ mice suffering from acute enterocolitis.

DISCUSSION

The mucosa in the intestine of mammals forms a tight barrier, which protects against commensals and other microbes

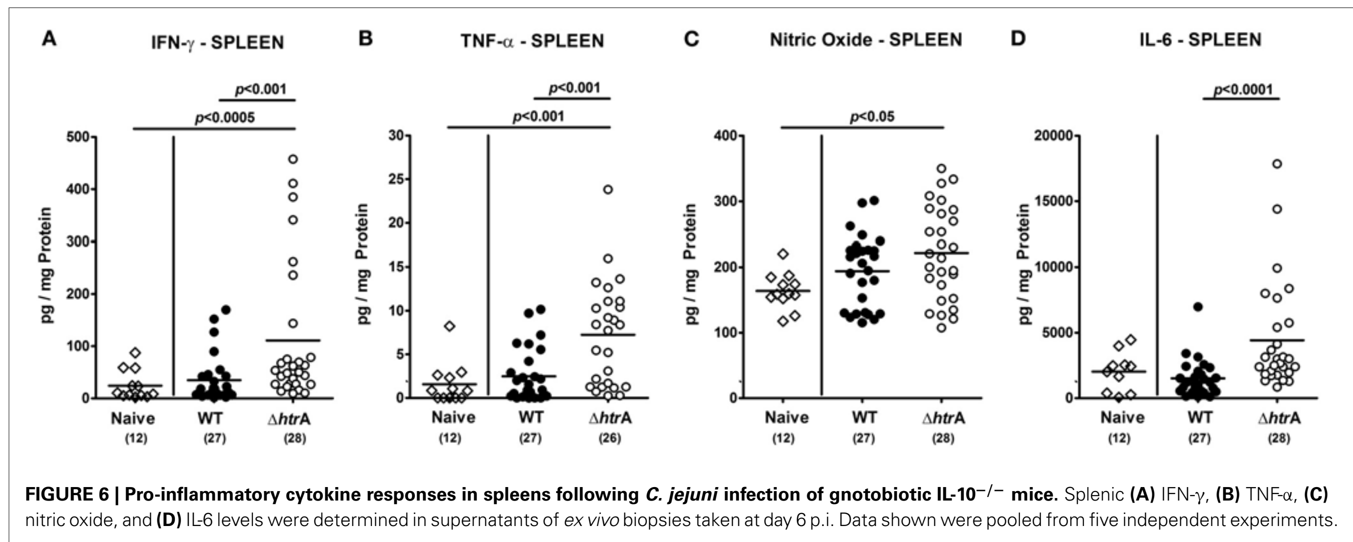

FIGURE 6 | Pro-inflammatory cytokine responses in spleens following *C. jejuni* infection of gnotobiotic IL-10$^{-/-}$ mice. Splenic **(A)** IFN-γ, **(B)** TNF-α, **(C)** nitric oxide, and **(D)** IL-6 levels were determined in supernatants of *ex vivo* biopsies taken at day 6 p.i. Data shown were pooled from five independent experiments.

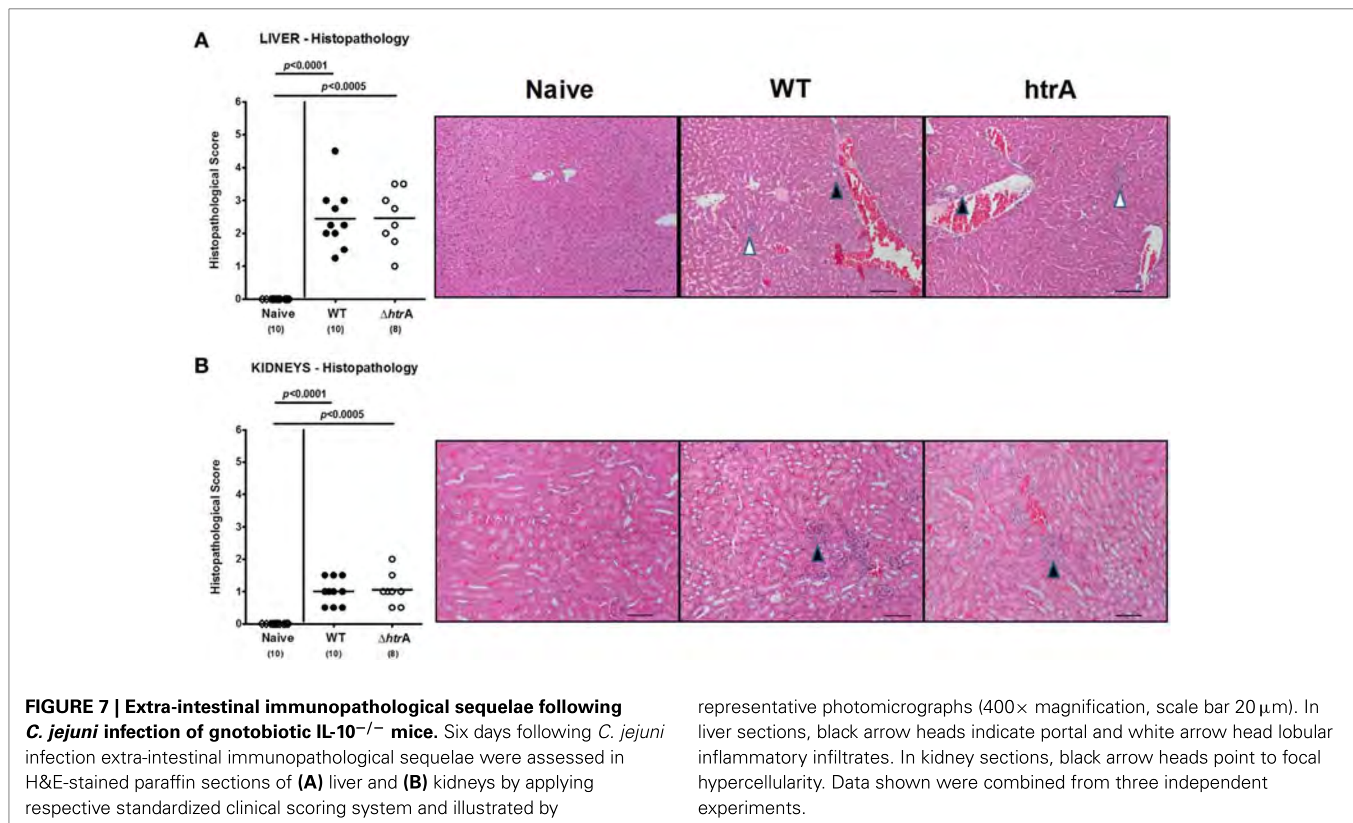

FIGURE 7 | Extra-intestinal immunopathological sequelae following *C. jejuni* infection of gnotobiotic IL-10$^{-/-}$ mice. Six days following *C. jejuni* infection extra-intestinal immunopathological sequelae were assessed in H&E-stained paraffin sections of **(A)** liver and **(B)** kidneys by applying respective standardized clinical scoring system and illustrated by representative photomicrographs (400× magnification, scale bar 20 μm). In liver sections, black arrow heads indicate portal and white arrow head lobular inflammatory infiltrates. In kidney sections, black arrow heads point to focal hypercellularity. Data shown were combined from three independent experiments.

present in the intestinal lumen. Previous infection studies in mice have revealed that mutation of the serine protease HtrA in a number of pathogenic bacteria results in attenuated virulence properties (Li et al., 1996; Humphreys et al., 1999; Wilson et al., 2006; Yuan et al., 2008; Lewis et al., 2009; De Stoppelaar et al., 2013). Bacterial HtrA may be involved in degradation of damaged bacterial proteins that accumulate during the hostile conditions in the macrophages and accordingly, *htrA* mutants of the facultative intracellular *Salmonella typhimurium*, *Listeria monocytogenes*, and *Yersinia enterocolitica* are all sensitive to oxidative agents and show reduced survival rates in macrophages (Bäumler et al., 1994; Yamamoto et al., 1996; Wilson et al., 2006). The individual contribution of chaperone and protease activity of HtrA to bacterial survival and multiplication in the host is difficult to dissect. However, for *S. typhimurium*, both activities appear to be required for proper systemic infection in mice (Lewis et al., 2009). Interestingly, after oral infection with an *htrA* mutant, the pathogen cannot translocate accurately from the Peyer's patches to other organs. In contrast, intravenous infection of

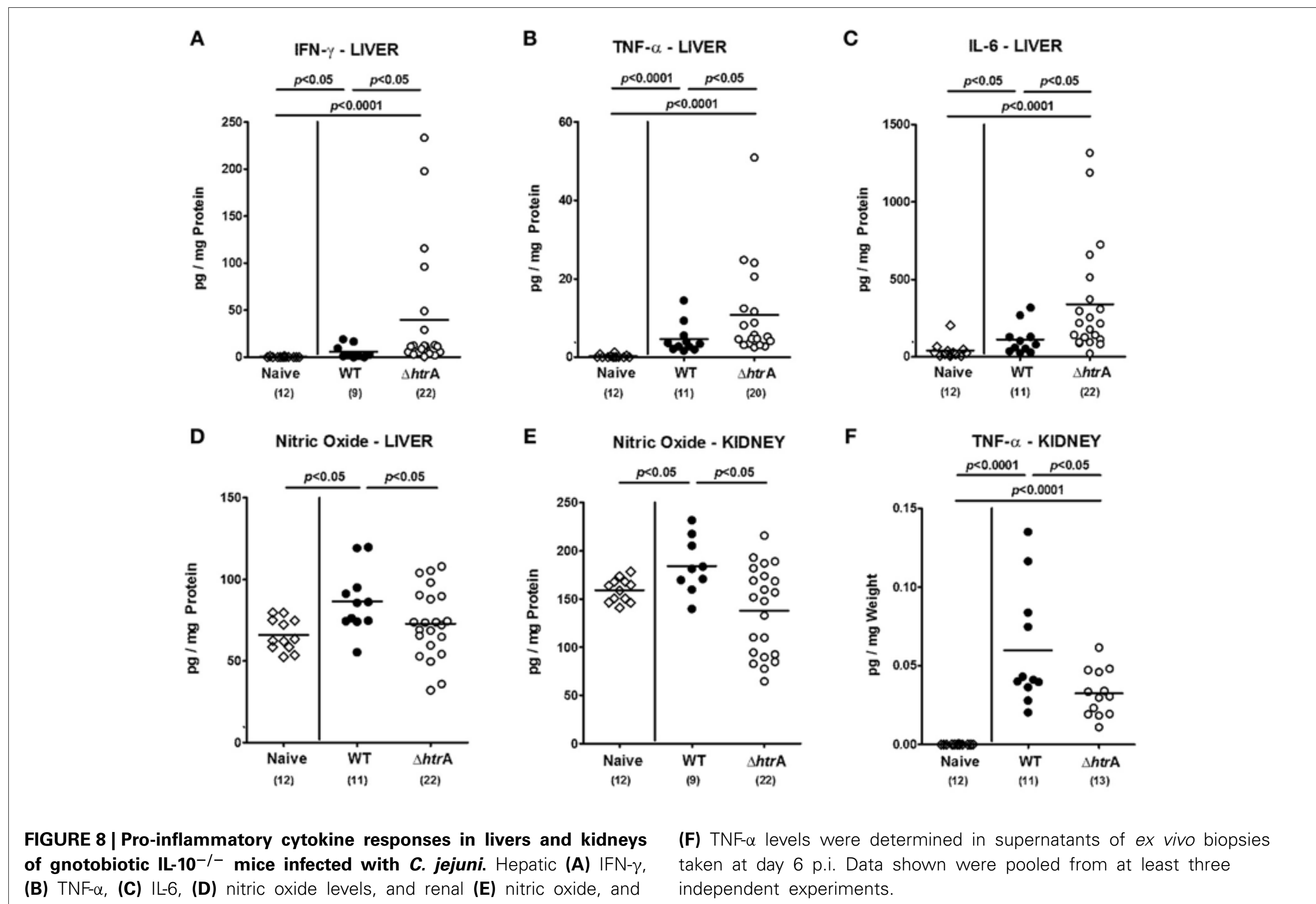

FIGURE 8 | Pro-inflammatory cytokine responses in livers and kidneys of gnotobiotic IL-10$^{-/-}$ mice infected with *C. jejuni*. Hepatic **(A)** IFN-γ, **(B)** TNF-α, **(C)** IL-6, **(D)** nitric oxide levels, and renal **(E)** nitric oxide, and **(F)** TNF-α levels were determined in supernatants of *ex vivo* biopsies taken at day 6 p.i. Data shown were pooled from at least three independent experiments.

mice resulted in full colonization of livers and spleens by the *S. typhimurium htrA* mutant, suggesting that HtrA is required to overcome the intestinal barrier (Humphreys et al., 1999). Expression of HtrA is also important for cell-to-cell spread of *Shigella flexneri* (Purdy et al., 2002, 2007). Previous *in vitro* infection studies with *C. jejuni* have indicated that inactivation of *htrA* reduced the bacterial adherence to cultured epithelial cells (Brøndsted et al., 2005). In addition, *C. jejuni htrA* mutants exhibited reduced invasion rates (Novik et al., 2010; Bæk et al., 2011a). Interestingly, HtrA chaperone activity appears to be important for efficient binding of *C. jejuni* to epithelial cells, while the HtrA protease activity seems required for maximal host cell entry after the bacteria have adhered to them (Bæk et al., 2011a). Under laboratory conditions *in vitro,* HtrA chaperone activity is necessary for growth of *C. jejuni* at high temperatures or under oxidative stress conditions, whereas HtrA protease activity is only essential during severe stress (Bæk et al., 2011b).

We have recently shown in a series of *in vitro* experiments that HtrA secreted by *C. jejuni* constitutes a novel bacterial virulence determinant, which opens cell-to-cell junctions through cleavage of E-cadherin and probably other host factors (Boehm et al., 2012, 2013; Hoy et al., 2012). In the present study, we investigated for the first time the impact of the *htrA* gene in *C. jejuni*-induced immunopathology *in vivo* and applied the acute *C. jejuni* infection model of gnotobiotic IL-10$^{-/-}$ mice. To prevent conventionally colonized IL-10$^{-/-}$ mice from chronic colitis exerted by antigenic stimuli through the conventional intestinal microbiota, mice were pre-treated for 4 months with a quintuple antibiotic regimen starting immediately after weaning (Haag et al., 2012a). Six days following peroral infection with *C. jejuni*, mice harbored high intestinal loads of the non-polar knockout mutant strain NCTC11168Δ*htrA*, which were comparable to those detected in mice upon infection with the parental WT strain NCTC11168. Hence, inactivation of the *htrA* gene did not down-regulate the overall high colonization capacity of *C. jejuni* in gnotobiotic mice. At first sight, these data seem to contradict a previous report where HtrA chaperone activity was shown to be required for efficient binding of *C. jejuni* to cultured INT-407 epithelial cells (Bæk et al., 2011a). However, INT-407 cells do not form polarized cell layers, while polarized epithelial cell layers are found in the intestine of live mice. Thus, the receptor availability in both systems is certainly different, which could be one reason to explain these findings.

Importantly, gnotobiotic IL-10$^{-/-}$ mice infected with the parental WT strain were severely compromised and developed ulcerative enterocolitis with bloody diarrhea and wasting symptoms, hence mimicking severe campylobacteriosis in immunocompromised patients (Haag et al., 2012a). Remarkably, at day 6 p.i. ulcerative enterocolitis was less distinct in mice infected

with the Δ*htrA* mutant, which displayed significantly less severe immunopathology in the intestinal tract as compared to mice infected with the *C. jejuni* WT strain. Interestingly, Δ*htrA* mutant infected mice exhibited higher $Ki67^+$ proliferating cell numbers in the colonic mucosa as compared to WT strain infected controls. This might be indicative for up-regulated compensatory properties in order to counteract cell destruction during immunopathology. Furthermore, not only local, but even systemic immune responses were less pronounced upon Δ*htrA* mutant infection as indicated by significant lower serum levels of pro-inflammatory cytokines such as TNF-α and IL-6 in Δ*htrA* mutant as compared to WT strain infected mice. The amelioration of devastating enteric and systemic disease, as in this hyper-acute model system, underlines the biological relevance of individual *C. jejuni* virulence factors *in vivo*. Furthermore, in the present and previous infection studies with conventional infant, germfree or with human microbiota re-associated germfree WT mice (Bereswill et al., 2011; Haag et al., 2012b), translocation of viable *C. jejuni* of either strain from the intestine to the MLNs could be observed in a subset of animals, whereas no bacterial CFUs could be cultured from extra-intestinal locations such as spleen, liver, kidney, or cardiac blood. This is good agreement with the observation of very rare cases of extra-intestinal *C. jejuni*-associated disease affecting the liver, lung, heart, or spleen that have been reported in immunocompromised patients suffering from *C. jejuni* bacteremia (Pigrau et al., 1997; Tee and Mijch, 1998; Crushell et al., 2004) and previous infection studies of isolator-raised germfree mice (Fauchère et al., 1985). Whereas viable *C. jejuni* could be cultured from MLNs of infected mice more than 3 weeks p.i., the pathogen was cleared from the circulation and extra-intestinal organs such as liver and spleen within 24 h. The authors proposed that non-specific bactericidal factors such as complement or phagocytic cells might have been responsible for rapid pathogenic clearance. However, the histopathological sequelae within the respective organs were not reported (Fauchère et al., 1985). Surprisingly, in the present study histopathological analysis revealed rather mild inflammatory changes in extra-intestinal organs such as liver and kidneys that did not differ 6 days p.i. with either *C. jejuni* strain. In gnotobiotic WT mice, however, which can be readily colonized by *C. jejuni*, we did not observe any overt extra-intestinal histopathological changes in respective H&E stained paraffin sections by day 42 p.i. (unpublished observations). It is therefore most likely that a significant intestinal pro-inflammatory scenario (as seen in the gnotobiotic $IL\text{-}10^{-/-}$ mouse model) is a prerequisite for subsequently induced extra-intestinal inflammatory responses as shown in the present study. In line with this, in another infection model, 3 weeks old infant mice (harboring a conventional microbiota) were infected with *C. jejuni* B2 strain immediately after weaning and exhibited histopathological sequelae in liver, kidneys, lungs, and cardiac muscle more than 100 days following *C. jejuni* infection (Haag et al., 2012b). Interestingly, mice were asymptomatic *C. jejuni* carriers until day 103 p.i. and the extra-intestinal organs were exclusively *C. jejuni* culture negative (Haag et al., 2012b). Furthermore, the vast majority of inflammatory cells that had accumulated in the respective extra-intestinal organs comprised $CD3^+$ T lymphocytes (Heimesaat et al., 2013). It is highly likely that pro-inflammatory immune cells have been attracted to the site of infection and cleared the pathogen early in the course of infection and reside further in the respective organ. This would explain the sterile inflammatory responses in liver and kidneys, both in less than 1 week (this study) and more than 3 months p.i. (Haag et al., 2012b; Heimesaat et al., 2013). Hence, the influx of pro-inflammatory immune cells upon even short-term *C. jejuni* infection might explain the increased levels of pro-inflammatory cytokines such as IFN-γ, IL-6, TNF-α, and NO detected in livers and the latter two in kidneys at day 6 p.i. Like in the colon, renal TNF-α and NO as well as hepatic NO levels were significantly lower in Δ*htrA* knockout mutant as compared to parental strain infected gnotobiotic $IL\text{-}10^{-/-}$ mice at day 6 p.i. Unexpectedly, IFN-γ, IL-6, and TNF-α concentrations in spleen and liver were even higher 6 days following Δ*htrA* knockout mutant as compared to parental WT strain infection. Notably, the standard deviations in the cohorts infected with the Δ*htrA* gene knockout mutant were rather high, which held true for individual experiments as well as for pooled data sets. Hence, the individual variabilities upon infection needs to take into account when judging for the biological relevance of the observed effects in the absence of *htrA*. In line with these unexpected data derived from livers of infected mice, splenic IFN-γ, IL-6, and TNF-α levels increased only upon Δ*htrA* knockout mutant, but not parental WT strain infection when compared to naive controls. It is therefore tempting to speculate whether in the course of the observed systemic immune responses more immune cells (such as dendritic cells and/or lymphocytes) might have been activated/imprinted in the spleen by the *C. jejuni* Δ*htrA* mutant present in the intestine as compared to parental WT strain infection or stimulated by distinct (so far unknown) circulating soluble bacterial factors derived from the pathogen which in turn could counteract intestinal disease. Further studies need to unravel this fascinating infection phenomenon.

Taken together, *C. jejuni* is one of the most important zoonotic pathogens causing food-borne gastroenteritis and potentially more severe diseases. Crossing the intestinal epithelial barrier and host cell invasion by *C. jejuni* are considered to constitute primary reasons of gut tissue damage in humans. However, the molecular mechanisms as well as major bacterial and host cell factors involved in these activities are poorly understood. Using the $IL\text{-}10^{-/-}$ knockout mouse infection model system, the results presented in this study demonstrate for the first time that the *C. jejuni* HtrA serine protease is a novel virulence factor which aggravates enterocolitis *in situ* by causing a substantial amount of cell damage, aggravation of intestinal apoptosis and inflammation upon *C. jejuni* infection *in vivo* accompanied by significant systemic pro-inflammatory immune responses. These observations are in line with our earlier *in vitro* studies showing that HtrA of *C. jejuni* targets epithelial E-cadherin-based cell-to-cell junctions (Boehm et al., 2012, 2013; Hoy et al., 2012). Future work should address important questions such as how *C. jejuni* can trigger HtrA secretion into the extracellular space, to identify E-cadherin cleavage sites by HtrA and to search for novel host cell targets involved in the above discovered activities *in vivo*. We further demonstrated non-polarity of

the Δ*htrA* knockout mutant strain by complementation of phenotypes *in vitro*; however, this does not warranty non-polarity of the mutation *in vivo*. The investigation of epithelium-bound or internalized *C. jejuni* in ongoing studies will further complete our understanding of the complex interactions of HtrA with the epithelium.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Markus M. Heimesaat, André Fischer, Marie Alutis, Steffen Backert, Stefan Bereswill. Performed the experiments: Markus M. Heimesaat, André Fischer, Marie Alutis, Ursula Grundmann. Analyzed the data: Markus M. Heimesaat, André Fischer, Marie Alutis, Anja A. Kühl, Manja Böhm, Nicole Tegtmeyer, Steffen Backert, Stefan Bereswill. Contributed reagents/materials/analysis tolls: Ulf B. Göbel, Manja Böhm, Nicole Tegtmeyer, Anja A. Kühl. Wrote the paper: Markus M. Heimesaat, Anja A. Kühl, André Fischer, Steffen Backert, Stefan Bereswill.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2014.00077/abstract

Figure S1 | Genetic complementation of HtrA reveals that the *C. jejuni* NCTC11168Δ*htrA* mutant is non-polar. (A) Expression of HtrA proteins by *C. jejuni* NCTC11168 wild-type (WT), NCTC11168Δ*htrA* mutant and complemented NCTC11168Δ*htrA/htrA* were investigated by Western blotting using an α-HtrA antibody. As control, equal amounts of protein per sample were confirmed by immunoblotting using the α-MOMP antibody. **(B)** Analysis of protease activities in all three indicated strains by casein zymography. The position of proteolytically active multimeric HtrA proteins is indicated with arrows.

Figure S2 | Effect of high temperature and oxygen concentration on growth of the *C. jejuni* Δ*htrA* mutant and genetically complemented *htrA* strain. Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) of the indicated *C. jejuni* strains [NCTC11168 wild-type (WT), NCTC11168Δ*htrA* and complemented NCTC11168Δ*htrA/htrA*] with an OD_{600} of 0.1 were spotted in 10 μl volumes onto Müller-Hinton agar plates. The plates were incubated for 3 days in jars under microaerobic conditions at **(A)** 42°C, **(B)** 44°C, or **(C)** 42°C in the presence of 18% O_2. Representative sections of the agar plates from three independent experiments are presented.

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Impact of *Campylobacter jejuni cj0268c* Knockout Mutation on Intestinal Colonization, Translocation, and Induction of Immunopathology in Gnotobiotic IL-10 Deficient Mice

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Abstract

Background: Although *Campylobacter jejuni* infections have a high prevalence worldwide and represent a significant socioeconomic burden, the underlying molecular mechanisms of induced intestinal immunopathology are still not well understood. We have recently generated a *C. jejuni* mutant strain NCTC11168::*cj0268c*, which has been shown to be involved in cellular adhesion and invasion. The immunopathological impact of this gene, however, has not been investigated *in vivo* so far.

Methodology/Principal Findings: Gnotobiotic IL-10 deficient mice were generated by quintuple antibiotic treatment and perorally infected with *C. jejuni* mutant strain NCTC11168::*cj0268c*, its complemented version (NCTC11168::*cj0268c*-comp-*cj0268c*), or the parental strain NCTC11168. Kinetic analyses of fecal pathogen loads until day 6 post infection (p.i.) revealed that knockout of *cj0268c* did not compromise intestinal *C. jejuni* colonization capacities. Whereas animals irrespective of the analysed *C. jejuni* strain developed similar clinical symptoms of campylobacteriosis (i.e. enteritis), mice infected with the NCTC11168::*cj0268c* mutant strain displayed significant longer small as well as large intestinal lengths indicative for less distinct *C. jejuni* induced pathology when compared to infected control groups at day 6 p.i. This was further supported by significantly lower apoptotic and T cell numbers in the colonic mucosa and lamina propria, which were paralleled by lower intestinal IFN-γ and IL-6 concentrations at day 6 following knockout mutant NCTC11168::*cj0268c* as compared to parental strain infection. Remarkably, less intestinal immunopathology was accompanied by lower IFN-γ secretion in *ex vivo* biopsies taken from mesenteric lymphnodes of NCTC11168::*cj0268c* infected mice versus controls.

Conclusion/Significance: We here for the first time show that the *cj0268c* gene is involved in mediating *C. jejuni* induced immunopathogenesis *in vivo*. Future studies will provide further deep insights into the immunological and molecular interplays between *C. jejuni* and innate immunity in human campylobacteriosis.

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Introduction

Campylobacter jejuni is the most important cause of bacterial diarrhea in developing as well as in industrialized countries. The characteristic features of the disease vary from watery to bloody diarrhea accompanied by abdominal cramps and fever. In rare cases complications such as the Guillain-Barré syndrome might arise post infection (p.i.) [1,2]. Although many virulence factors of *C. jejuni* have been described yet, the overall image of this bacterial infection is still incomplete [3,4].

A successful infection with *C. jejuni* requires adherence of the pathogen to host cells and several proteins of *C. jejuni* that contribute to this initial interaction have been characterized in the past. MOMP, CadF and FlpA, for instance, were shown to possess fibronectin-binding properties whereby specifically CadF and FlpA initiate the remodelling of the actin cytoskeleton *via* the activation of integrin receptors to allow internalization of *C. jejuni* into the host cell [5,6,7]. Furthermore, PEB1 as an element of an ABC transporter and CapA, representing an autotransporter protein, mediate adherence and are important for *C. jejuni*

colonization of mice and chicken, respectively [8,9]. Cj0091 and JlpA are additionally necessary for the adherence of *C. jejuni* to host cells whereby a JlpA-HSP 90alpha interaction is going along with the activation of NF-κB and the p38 MAP kinase [10,11,12]. Complementary to the proteins described above, lipooligosaccharides (LOS) contribute to the adherence properties since a *C. jejuni* strain deficient in LOS metabolism possesses a significantly reduced interaction with chicken embryo fibroblasts [13]. Furthermore, we characterized a *C. jejuni* mutant, which lacks a functional sulphite:cytochrome c oxidoreductase (SOR) leading to a diminished transcription of genes involved in legionaminic acid synthesis and a reduced adherence to Caco2 cell [14,15].

Recently, we investigated the *in vitro* properties of *C. jejuni* protein Cj0268c, which has been shown by our and other groups to be important for the invasion of Caco2 cells by the pathogen [15,16,17]. Thereby, we could show that the invasion-relevant phenotype of Cj0268c is due to its adherence mediating function, not only in *C. jejuni* but also when this protein is expressed heterologously in *E. coli* [17]. However, the functional relevance of Cj0268c for the interaction of *C. jejuni* with the host immune system has not been demonstrated so far.

To address this we here applied the gnotobiotic murine IL-10$^{-/-}$ infection model. In order to eradicate the colitogenic stimuli derived from the conventional intestinal microbiota, IL-10$^{-/-}$ mice were subjected to a broad-spectrum antibiotic treatment for at least 3 months starting immediately after weaning [18]. Upon *C. jejuni* infection gnotobiotic IL-10$^{-/-}$ mice get readily colonized by the pathogen and display acute enterocolitis within one week p.i.mimicking severe campylobacteriosis in humans, whereas gnotobiotic or with human microbiota reassociated wildtype mice display intestinal pro-inflammatory immune responses but no overt clinical symptoms such as bloody diarrhea upon *C. jejuni* infection [18]. We here for the first time investigated i) the colonization capacities and ii) clinical as well as iii) intestinal pro-inflammatory immune cell and cytokine responses upon infection of gnotobiotic IL-10$^{-/-}$ mice with the *C. jejuni* mutant strain NCTC11168::*cj0268c*, its complemented version NCTC11168::*cj0268c*-comp-*cj0268c* and the parental strain NCTC11168.

Results

Impact of Cj0268c on *C. jejuni* Colonization Capacity in Infected Gnotobiotic IL-10$^{-/-}$ Mice

Given that the murine commensal gut microbiota is essential for the physiological host resistance against *C. jejuni* infection [19], we generated gnotobiotic IL-10$^{-/-}$ mice by quintuple antibiotic treatment for at least 3 months (refer to [20,21]) to investigate the colonization capacity of *C. jejuni* mutant strain NCTC11168::*cj0268c*. Following peroral infection on two consecutive days with a comparable challenge of 10^9 viable mutant *C. jejuni* NCTC11168::*cj0268c*, its complemented version NCTC11168::*cj0268c*-comp-*cj0268c*, or the parental strain NCTC11168, each in the stationary phase (not shown), gnotobiotic mice were readily colonized with comparably high loads of 10^9 to 10^{10} colony forming units (CFU) of either strain per g feces over time until day 6 p.i. (**Fig. 1**). In addition, when luminal samples were taken from the entire gastrointestinal (GI) tract on the day of necropsy (day 6 p.i.), either *C. jejuni* strain could be cultured from the stomach, duodenum, ileum and colon, with the highest loads in the large intestine of approximately 10^9 to 10^{10} CFU per g luminal content (**Fig. 2**). Thus, deficiency of the *cj0268c* gene did not impact gastrointestinal colonization capacities of *C. jejuni* in gnotobiotic IL-10$^{-/-}$ mice upon peroral infection.

Impact of Cj0268c on Clinical Symptoms in *C. jejuni* Infected Gnotobiotic IL-10$^{-/-}$ Mice

We were next interested whether a knockout of the *cj0268c* gene impacts induction of immunopathology in gnotobiotic IL-10$^{-/-}$ mice. Daily survey of clinical conditions revealed that irrespectively whether mice had been infected with the *C. jejuni* parental strain NCTC11168, the mutant strain NCTC11168::*cj0268c* or its complemented version NCTC11168::*cj0268c*-comp-*cj0268c*, similar intestinal colonization densities were accompanied by comparable disease symptoms of enterocolitis as indicated by similar clinical scores over time (**Fig. 3**). Overt clinical symptoms started to occur around day 3 p.i. with either strain and progressed further over time reaching maximum scores at day 5 and 6 p.i. (**Fig. 3**). Notably, clinical scores of mice infected with respective *C. jejuni* strains at defined time points did not differ.

Given that acute intestinal inflammation is accompanied by a significant shortening of the intestinal tract [18,20,22], we determined the absolute lengths of the small as well as large intestines at day 6 p.i. Interestingly, gnotobiotic IL-10$^{-/-}$ mice infected with the *C. jejuni* mutant strain NCTC11168::*cj0268c* displayed longer small intestines (approximately 10% mean difference; **Fig. 4A**) and colons (approximately 20% mean difference; **Fig. 4B**) as compared to mice infected with the parental strain NCTC11168 ($p<0.05$) or complemented strain NCTC11168::*cj0268c*-comp-*cj0268c* ($p<0.05$ and $p<0.01$, respectively; **Fig. 4AB**) indicative for significantly less distinct intestinal pathology. Furthermore, viable bacteria of the *C. jejuni* parental strain NCTC11168 and the complemented strain NCTC11168::*cj0268c*-comp-*cj0268c* could be cultured from mesenteric lymphnodes (MLNs) in 20.00% (2 out of 10) and 8.33% (1 out of 12) of infected animals at day 6 p.i., respectively, whereas the mutant strain NCTC11168::*cj0268c* did not translocate into MLNs at all (not shown). Furthermore, virtually no pathogenic translocation to extra-intestinal compartments could be detected given that spleen, liver, kidney and cardiac blood were exclusively *C. jejuni* culture-negative (not shown). Taken together, uncompromised colonization capacities of *C. jejuni* lacking *cj0268c* were accompanied by comparable induction of gross disease (clinical symptoms of ulcerative enterocolitis). Longer small and large intestines as well as lower translocation frequencies in *C. jejuni* mutant strain NCTC11168::*cj0268c* infected gnotobiotic IL-10$^{-/-}$ mice, however, hint towards less pronounced intestinal immunopathology caused by absence of the *cj0268c* gene.

Impact of Cj0268c on Induction of Intestinal Pro-inflammatory Immune Responses in *C. jejuni* Infected Gnotobiotic IL-10$^{-/-}$ Mice

We further assessed the immunopathological responses of mice upon infection with the *C. jejuni* knockout mutant NCTC11168::*cj0268c*. Irrespective of the strain, gnotobiotic mice displayed comparable histopathological changes in hematoxylin and eosin (H&E) stained colonic paraffin sections at day 6 p.i. (not shown). Given that apoptosis is a commonly used diagnostic marker in the histopathological evaluation and grading of intestinal disease [21] and a key feature of *C. jejuni* induced ulcerative enterocolitis in gnotobiotic IL-10$^{-/-}$ mice [18], we quantitatively assessed caspase-3$^+$ cells within the colonic mucosa following infection with the respective *C. jejuni* strains. Six days upon peroral challenge, mice infected with the mutant strain NCTC11168::*cj0268c* displayed significantly less distinct colonic epithelial apoptosis when compared to wildtype and complemented controls as indicated by approximately 35% lower caspase-3$^+$

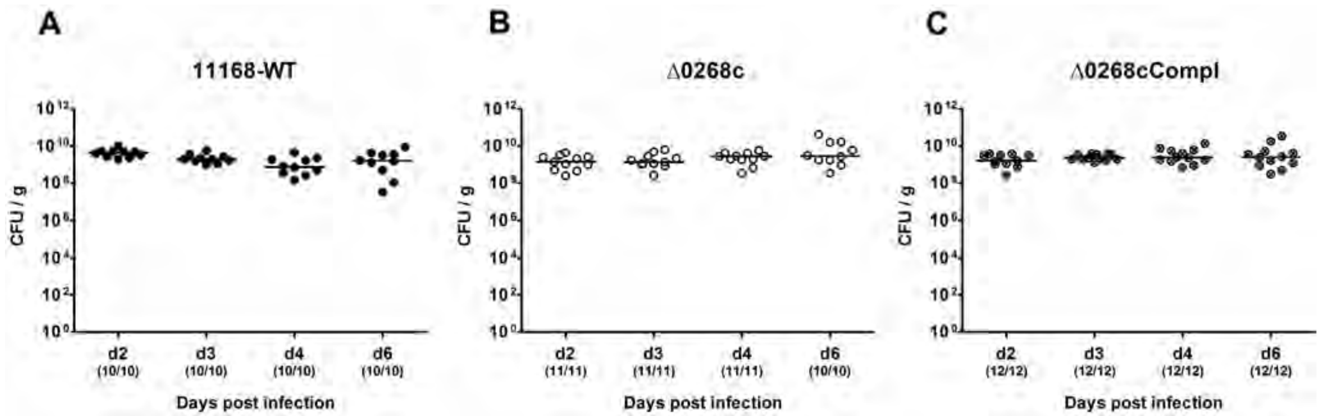

Figure 1. Kinetic survey of *C. jejuni* knockout mutant NCTC11168::*cj0268c* colonization in gnotobiotic IL-10$^{-/-}$ mice. Gnotobiotic IL-10$^{-/-}$ mice were generated by antibiotic gut decontamination and perorally infected with *C. jejuni* NCTC11168 (11168-WT, closed circles; **A**), mutant strain NCTC11168::*cj0268c* (Δ0268c, open circles; **B**), or the complemented strain NCTC11168::*cj0268c*-comp-*cj0268c* (Δ0268cCompl, crossed circles; **C**) as described (see methods). The intestinal colonization capacities over time were determined by quantification of live *C. jejuni* in fecal samples applying cultural analysis (CFU, colony forming units) starting two days until six days post infection as indicated on the x-axis. Medians (black bars) are indicated and numbers of animals harbouring the respective *C. jejuni* strain out of the total number of analyzed animals given in parentheses. Data shown were pooled from three independent experiments.
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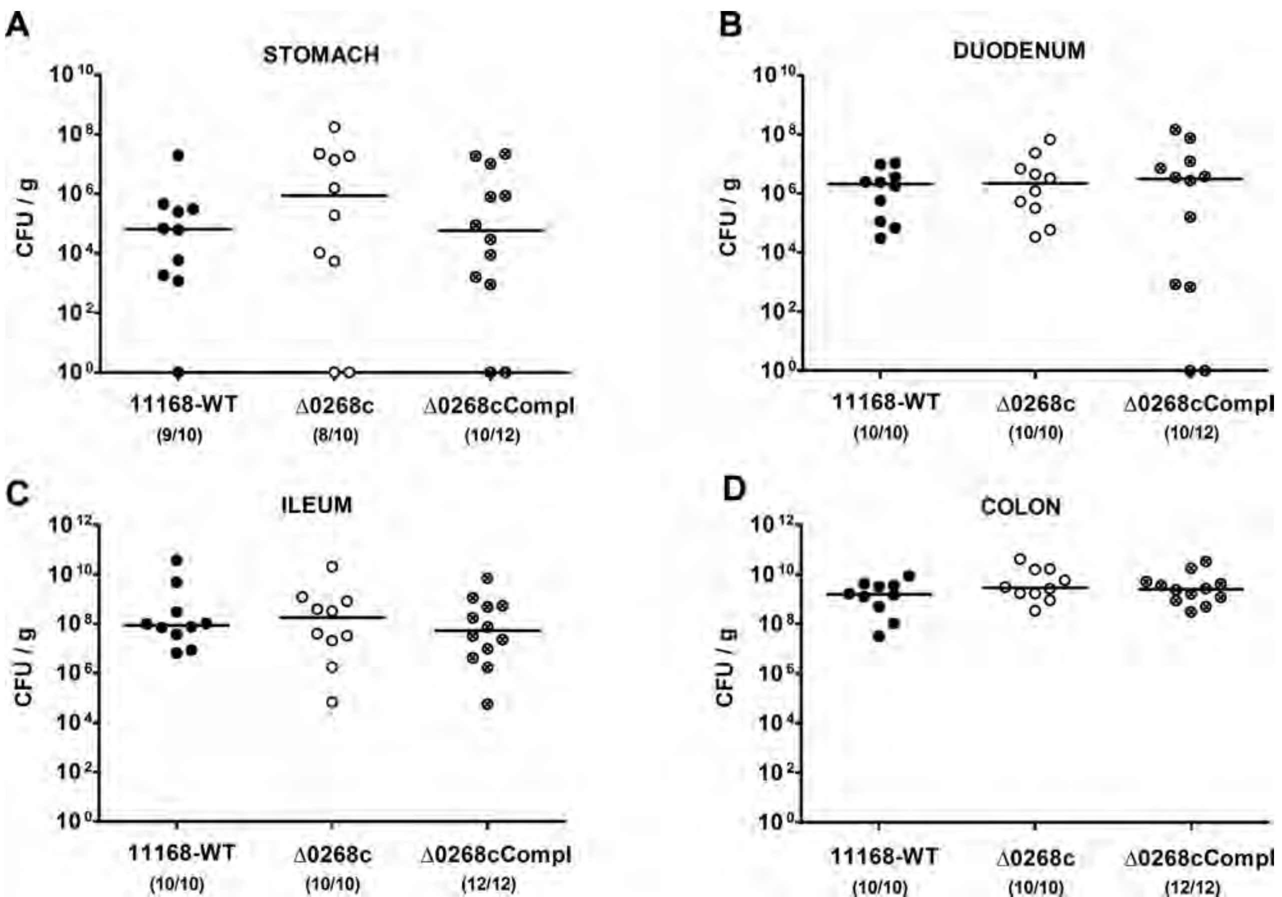

Figure 2. *C. jejuni* knockout mutant NCTC11168::*cj0268c* colonization along the gastrointestinal tract of gnotobiotic IL-10$^{-/-}$ mice. Gnotobiotic IL-10$^{-/-}$ mice were generated by antibiotic gut decontamination and perorally infected with *C. jejuni* NCTC11168 (11168-WT, closed circles), mutant strain NCTC11168::*cj0268c* (Δ0268c, open circles), or the complemented strain NCTC11168::*cj0268c*-comp-*cj0268c* (Δ0268cCompl, crossed circles) as described (see methods). The pathogen densities in distinct compartments of the gastrointestinal tract were determined by quantification of live *C. jejuni* in luminal samples taken from stomach, duodenum, ileum, and colon at day 6 p.i. by cultural analysis (CFU, colony forming units). Medians (black bars) are indicated and numbers of animals harbouring the respective *C. jejuni* strain out of the total number of analyzed animals given in parentheses. Data shown were pooled from three independent experiments.
doi:10.1371/journal.pone.0090148.g002

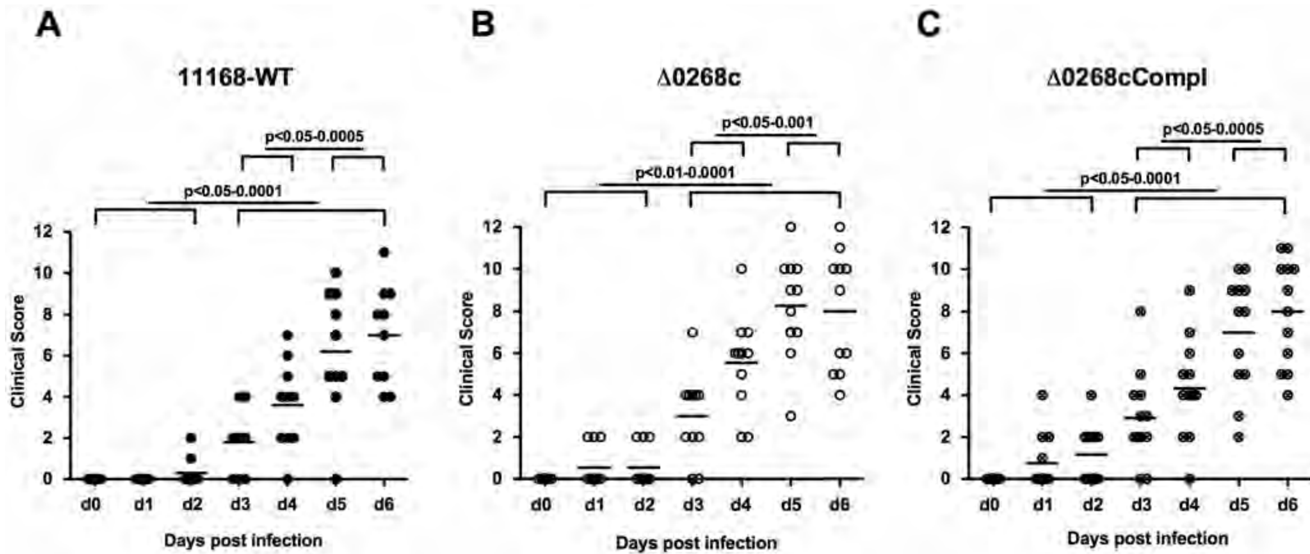

Figure 3. Kinetic survey of clinical symptoms following *C. jejuni* knockout mutant NCTC11168::*cj0268c* infection of gnotobiotic IL-10$^{-/-}$ mice. Gnotobiotic IL-10$^{-/-}$ mice were generated by antibiotic gut decontamination and perorally infected with *C. jejuni* NCTC11168 (11168-WT, closed circles, n = 10; A), mutant strain NCTC11168::*cj0268c* (Δ0268c, open circles, n = 11; B), or the complemented strain NCTC11168::*cj0268c*-comp-*cj0268c* (Δ0268cCompl, crossed circles; n = 12; C) as described (see methods). Disease activity before and following *C. jejuni* infection was assessed daily by applying a standardized clinical scoring system. Means (black bars) and levels of significance (*P*-values) determined by the Mann-Whitney-U test are indicated. Data shown were pooled from three independent experiments.
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positive cell numbers in the colonic mucosa of the former ($p<0.05$; **Fig. 5A**).

Given that recruitment of pro-inflammatory immune cell populations to sites of inflammation is a hallmark of human campylobacteriosis [21], we next quantitatively assessed the influx of innate and adaptive immune as well as effector cell populations into the large intestine by applying *in situ* immunohistochemical staining of colonic paraffin sections. Following *C. jejuni* infection, a marked influx of $CD3^+$ cells (i.e. T lymphocytes) into the colonic mucosa and lamina propria could be detected until day 6 p.i. (**Fig. 5B**). This increase, however, was significantly less pronounced in mice infected with the knockout mutant NCTC11168::*cj0268c* as compared to parental strain NCTC11168 and complemented strain NCTC11168::*cj0268c*-comp-*cj0268c* infected control animals ($p<0.01$ and $p<0.05$, respectively; **Fig. 5B**). Irrespective of the *C. jejuni* strain, infected mice displayed comparable increases of $Foxp3^+$ regulatory T cells, $B220^+$ B lymphocytes, $MPO7^+$ neutrophils, and $F4/80^+$ macrophages and monocytes in the colonic mucosa at day 6 p.i. as compared to naïve animals ($p<0.0005$–0.0001; **Fig. 5C–F**).

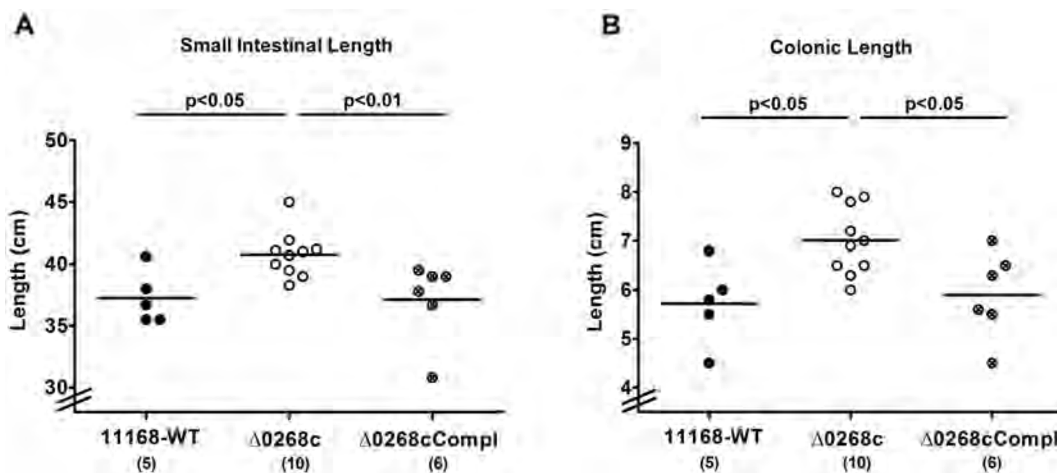

Figure 4. Intestinal lengths following *C. jejuni* knockout mutant NCTC11168::*cj0268c* infection of gnotobiotic IL-10$^{-/-}$ mice. Gnotobiotic IL-10$^{-/-}$ mice were generated by antibiotic gut decontamination and perorally infected with *C. jejuni* NCTC11168 (11168-WT, closed circles), mutant strain NCTC11168::*cj0268c* (Δ0268c, open circles), or the complemented strain NCTC11168::*cj0268c*-comp-*cj0268c* (Δ0268cCompl, crossed circles) as described (see methods). Six days following *C. jejuni* strain infections, (C) small as well as (D) large intestinal lengths (in cm) were measured at necropsy. Means (black bars), levels of significance (*P*-values) as compared to the respective control group (determined by the Mann-Whitney-U test), and numbers of analyzed animals (in parentheses) are indicated. Data shown were pooled from three independent experiments.
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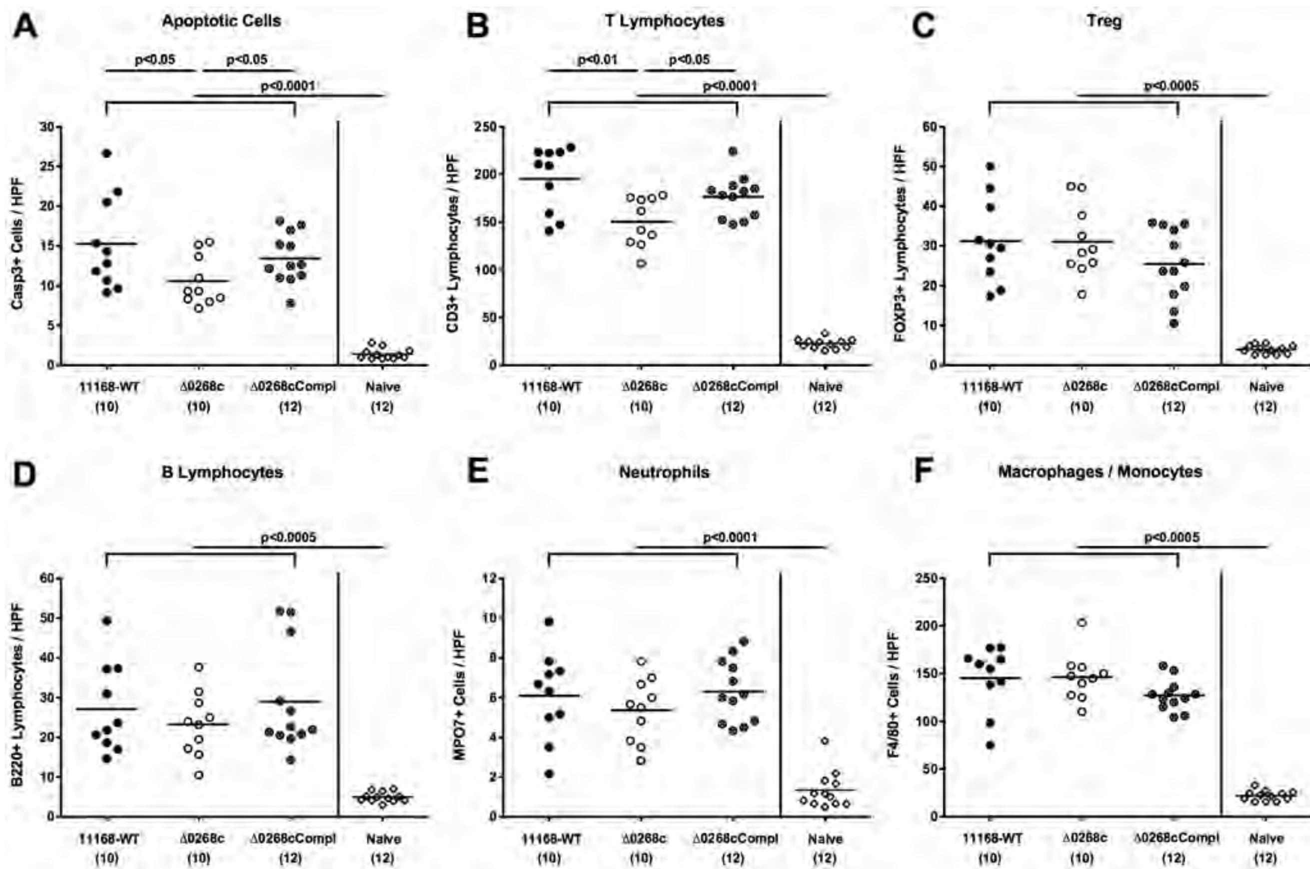

Figure 5. Impact of the *cj0268c* gene on colonic inflammatory and immune cell responses following infection of gnotobiotic IL-10$^{-/-}$ mice. Gnotobiotic IL-10$^{-/-}$ mice were generated by antibiotic gut decontamination and perorally infected with *C. jejuni* NCTC11168 (11168-WT, closed circles), mutant strain NCTC11168::*cj0268c* (Δ0268c, open circles), or the complemented strain NCTC11168::*cj0268c*-comp-*cj0268c* (Δ0268cCompl, crossed circles) as described (see methods). The average numbers of apoptotic cells (positive for caspase-3, panel A), T lymphocytes (positive for CD3, panel B), regulatory T cells (Treg, positive for Foxp3, panel C), B lymphocytes (positive for B220, panel D), neutrophils (positive for MPO7, panel E), and macrophages/monocytes (positive for F4/80, panel F) from at least six high power fields (HPF, 400x magnification) per animal were determined microscopically in immunohistochemically stained colon sections at day 6 p.i. uninfected animals (Naïve; open diamonds) served as negative controls. Numbers of analyzed animals are given in parentheses. Means (black bars) and levels of significance (*P*-values) determined by the Mann-Whitney-U test are indicated. Data shown were pooled from three independent experiments.
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We next determined intestinal pro-inflammatory cytokine expression levels upon *C. jejuni* infection. Lower colonic apoptotic cell and T lymphocyte counts were accompanied by lower IL-6 and IFN-γ protein concentrations in *ex vivo* colonic biopsies obtained from gnotobiotic IL-10$^{-/-}$ mice six days following infection with the knockout mutant NCTC11168::*cj0268c* as compared to the parental strain NCTC11168 (**Fig. 6A, B**). The impact of *cj0268c* in mediating *C. jejuni* induced immunopathology was further underlined by lower IFN-γ levels in *ex vivo* biopsies of draining mesenteric lymphnodes in mutant strain NCTC11168::*cj0268c* as compared to parental strain NCTC11168 infected mice ($p<0.05$; **Fig. 6C**). Whereas intestinal pro-inflammatory cytokine levels in complemented and wildtype strain infected mice did not differ, a trend towards higher intestinal IL-6 and IFN-γ concentrations six days following complemented as compared to knock-out mutant strain infection could be observed. Given high standard deviations in the respective groups, however, the differences did not reach statistical significance (**Fig. 6**).

Taken together, *cj0268c* gene deficiency does not alter *C. jejuni* NCTC11168 colonization capacities *in vivo*. In addition, the Cj0268*c* protein is involved in mediating *C. jejuni* induced acute enteritis as indicated by i.) less shrinkage of the small as well as large intestines, ii.) less abundance of colonic epithelial apoptotic cells, iii.) less distinct T lymphocyte infiltrations in the colonic mucosa and iv.) less pro-inflammatory cytokine secretion at intestinal tissue sites including mesenteric lymphnodes of gnotobiotic IL-10$^{-/-}$ mice infected with the knockout mutant strain NCTC11168::*cj0268c* when compared to control animals.

Discussion

We have recently shown that the *C. jejuni* protein Cj0268c is an important prerequisite for pathogen adhesion and invasion of host cells *in vitro* [17]. In the present study we investigated the impact of *cj0268c* in *C. jejuni* induced immunopathology *in vivo*. To prevent conventionally colonized IL-10$^{-/-}$ mice from spontaneous chronic colitis due to antigenic stimuli derived from the intestinal microbiota, mice were subjected to at least 3 months broad-spectrum antibiotic treatment starting immediately after weaning [18]. Upon peroral *C. jejuni* infection gnotobiotic IL-10$^{-/-}$ mice develop non-selflimiting ulcerative enterocolitis within one week p.i. mimicking severe campylobacteriosis in immuno-compromized patients [18]. Here, kinetic analyses revealed that until day

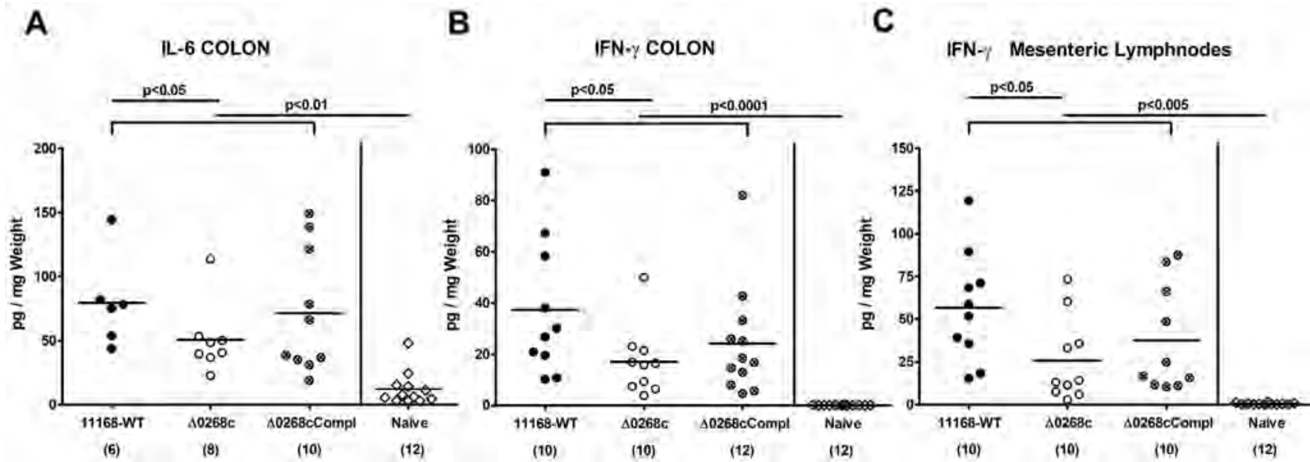

Figure 6. Impact of the *cj0268c* gene on intestinal pro-inflammatory cytokine responses following infection of gnotobiotic IL-10$^{-/-}$ mice. Gnotobiotic IL-10$^{-/-}$ mice were generated by antibiotic gut decontamination and perorally infected with *C. jejuni* NCTC11168 (11168-WT, closed circles), mutant strain NCTC11168::*cj0268c* (Δ0268c, open circles), or the complemented strain NCTC11168::*cj0268c*-comp-*cj0268c* (Δ0268cCompl, crossed circles) as described (see methods). Colonic (A) IL-6 and (B) IFN-γ levels as well as IFN-γ secretion in (C) mesenteric lymphnodes (MLNs) were determined in culture supernatants of *ex vivo* biopsies taken from the respective organs at day 6 p.i. Uninfected animals (naïve; open diamonds) served as negative controls. Numbers of analyzed animals are given in parentheses. Means (black bars) and levels of significance (*P*-values) determined by the Mann-Whitney-U test are indicated. Data shown were pooled from three independent experiments.
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6 following peroral infection, mice harboured high intestinal loads of the knockout mutant strain NCTC11168::*cj0268c*, which were comparable to those detected in mice upon infection with the parental strain NCTC11168 or the complemented version NCTC11168::*cj0268c*-comp-*cj0268c*. Hence, knockout of the *cj0268c* gene did neither compromise infection capacities *in vitro* [17] nor *in vivo*. Of note, genetic complementation clearly demonstrates that the generated NCTC11168::*cj0268c* knock-out mutant strain is not polar. Remarkably, mice infected with the mutant strain NCTC11168::*cj0268c* displayed significantly less severe immunopathology in the intestinal tract as compared to mice infected with the parental strain NCTC11168 or the complemented *C. jejuni* strain NCTC11168::*cj0268c*-comp-*cj0268c* as indicated by a plethora of results. First, knockout mutant strain NCTC11168::*cj0268c* infected gnotobiotic IL-10$^{-/-}$ mice displayed less shrinkage of the small as well as large intestines which is a rather rough, but reliable indicator for less pronounced intestinal pathology [20,22,23,24]. Second, this was further supported by less abundance of caspase-3^{+} cells in the colonic mucosa given that apoptosis is a commonly used diagnostic marker in the histopathological evaluation and grading of intestinal disease [21] and a key feature of *C. jejuni* induced ulcerative enterocolitis in gnotobiotic IL-10$^{-/-}$ mice [18]. Third, T lymphocytes well known to play a pivotal role in induction and perpetuation of *C. jejuni* induced immunopathology in mice [18,21,25,26,27] were infiltrating the intestinal mucosa and lamina propria of gnotobiotic IL-10$^{-/-}$ mice following infection with knockout mutant NCTC11168::*cj0268c* to a far lesser extent as compared to the applied control strains. Fourth, virtually no translocation of viable *C. jejuni* from the intestinal tract to MLNs was observed upon infection with mutant strain NCTC11168::*cj0268c*. Fifth, expression of pro-inflammatory cytokines such as IL-6 and IFN-γ was more than 50% lower in *ex vivo* biopsies derived from colon and MLNs upon infection with the mutant versus the parental strain. In our previous work where we independently studied the *C. jejuni*-induced immunopathological sequelae in two other murine *C. jejuni* infection models we could unequivocally demonstrate that severity of campylobacteriosis was paralleled by up-regulated expression levels of IFN-γ and IL-6 in both, the colon and MLNs [18,21,25], further supporting significance of the results presented here. It is tempting to speculate that the decreased intestinal IFN-γ and IL-6 levels following mutant as compared to parental strain infection might be indicative for shifted intestinal T cell populations in the absence of *cj0268c* which needs to further unraveled.

Irrespective of the *C. jejuni* strain, however, infected gnotobiotic IL-10$^{-/-}$ mice developed comparable clinical symptoms of enteritis over time in the presented study, which was contrasting the less pronounced immunopathological outcome in the intestinal tract. Despite the observation of comparable clinical symptoms upon infection with the knockout mutant strain NCTC11168::*cj0268c*, one needs to take into account that the clinical picture of a disease is rather the sum of different effects resulting from several levels of immunopathological mechanisms. Furthermore, the *cj0268c* gene is by far not the only factor involved in adhesion and invasion and subsequent induction of immunopathology [28]. Nevertheless, severity of *C. jejuni* induced enteritis can vary considerably between infected human individuals and range from very mild, sublatent and self-limiting complaints to severe symptoms such as abdominal cramps, fever, and bloody diarrhea depending on the dysbalance between the immune status of the host and the respective pathogenicity factors of the pathogen expressed in parallel [29].

One needs to take further into account, that *C. jejuni* infection in the *in vivo* infection model applied here results in a devastating outcome, namely non-selflimiting acute ulcerative enterocolitis leading to death within 10 days [18]. Hence, if any beneficial effect is observed in such a hyper-acute model system, the biological relevance gets more plausible. Furthermore, our *in vitro* results revealed that adhesive properties of the mutant strain were not 100%, but reached rather 60% [17]. Moreover, we have recently shown in different murine infection models that Toll-like-receptor (TLR)-4 dependent signalling of *C. jejuni* lipooligosaccharide is a key factor in *C. jejuni* induced immunopathology as indicated by ameliorated clinical and intestinal immunopathology in *C. jejuni*

infected gnotobiotic TLR-4 deficient as well as IL-10 deficient mice lacking TLR-4 [18,21,30].

Taken together, our previous and actual results have shown that *cj0268c* is involved in *C. jejuni* adhesion and invasion of vertebrate cells subsequently inducing significant immunopathology in the host with varying clinical features. Due to the lack of appropriate animal models in the past, the impact of most of the so far identified pathogenicity factors of *C. jejuni* involved in pathogen-host-interaction and thus their biological relevance in inducing campylobacteriosis have not been investigated *in vivo* yet.

In conclusion, future *in vivo* studies should further unravel the distinct molecular mechanisms and orchestration of different pathogenicity factors contributing to *C. jejuni* induced disease.

Materials and 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, Germany; registration numbers G0123/12). Animal welfare was monitored twice daily by assessment of clinical conditions.

Mice

IL-$10^{-/-}$ 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.

To eradicate the commensal gut flora, mice were transferred to sterile cages and treated by adding ampicillin (1 g/L; Ratio-pharm), vancomycin (500 mg/L; Cell Pharm), ciprofloxacin (200 mg/L; Bayer Vital), imipenem (250 mg/L; MSD), and metronidazole (1 g/L; Fresenius) to the drinking water *ad libitum* as described earlier [20] starting at 3 weeks of age right after weaning. Age matched female mice were subjected to the quintuple antibiotic treatment for 3–4 months before the infection experiment.

C. jejuni Infection of Mice

Mice were infected with approximately 10^9 viable CFU of *C. jejuni* strains NCTC11168 (parental strain), the *C. jejuni* mutant strain NCTC11168::*cj0268c* (lacking the *cj0268c* gene [17]), or its complemented version NCTC11168::*cj0268c*-comp-*cj0268c* [17], respectively, by gavage in a total volume of 0.3 mL PBS on two consecutive days (day 0 and day 1).

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 points: no blood; 2 points: microscopic detection of blood by the Guajac method using Haemoccult, Beckman Coulter/PCD, Krefeld, Germany; 4 points: overt 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 [18].

Sampling Procedures

Mice were sacrificed by isofluran treatment (Abbott, Germany). Cardiac blood and tissue samples from mesenteric lymphnodes, spleen, liver, kidney and GI tract (stomach, duodenum, ileum, colon) were removed under sterile conditions. Absolute small and large intestinal lengths were determined by measuring the distances from the transition of the stomach to the duodenum to the very distal terminal ileum and from the ascending colon leaving the caecum to the rectum, respectively, by a ruler and expressed in cm. GI samples from each mouse were collected in parallel for immunohistochemical, microbiological, and immunological analyses. Immunohistopathological changes were determined in colonic samples immediately fixed in 5% formalin and embedded in paraffin. Sections (5 µm) were stained with H&E or respective antibodies for *in situ* immunohistochemistry.

Immunohistochemistry

In situ immunohistochemical analysis of colonic paraffine sections was performed as described previously [18,21,25,31]. Primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, USA, 1:200), CD3 (#N1580, Dako, Denmark, dilution 1:10), myeloperoxidase-7 (MPO-7, # A0398, Dako, 1:10000), F4/80 (# 14-4801, clone BM8, eBioscience, 1:50), Foxp3 (FJK-16s, eBioscience, 1:100), and B220 (eBioscience, San Diego, CA, USA, 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 three independent investigators.

Quantitative Analysis of *C. jejuni* (Translocation)

Live *C. jejuni* were detected in feces or at time of necropsy (day 6 p.i.) in luminal samples taken from the stomach, duodenum, ileum, or colon dissolved in sterile PBS by culture as described earlier [18,21]. To quantify bacterial translocation, MLNs, spleen, liver ($\approx$1 cm^2) and kidney were homogenized in sterile PBS and analyzed by cultivating on karmali agar (Oxoid, Wesel, Germany) in a microaerophilic atmosphere at 37°C for at least 48 hours. Cardiac blood ($\approx$200 µL) was directly streaked onto karmali agar 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 $\approx$100 CFU per g.

Cytokine Detection in Culture Supernatants of *ex vivo* Biopsies taken from Colon and Mesenteric Lymphnodes

Colon biopsies were cut longitudinally, and washed in PBS. Mesenteric lymphnodes or strips of approximately 1 cm^2 colon tissue were placed in 24-flat-bottom well culture plates (Nunc, Wiesbaden, Germany) containing 500 µL serum-free RPMI 1640 medium supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL; PAA Laboratories). After 18 h at 37°C, culture supernatants were tested for IL-6 and IFN-γ by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences) on a BD FACSCanto II flow cytometer (BD Biosciences).

Statistical Analysis

Mean values, medians, and levels of significance were determined using Mann-Whitney-U test. Two-sided probability (*P*) values $\leq$0.05 were considered significant. All experiments were repeated at least twice.

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Author Contributions

Conceived and designed the experiments: MMH AF MA SB. Performed the experiments: MMH AF MA. Analyzed the data: MMH AF MA AAK SB. Contributed reagents/materials/analysis tools: RL AAK AEZ UBG. Wrote the paper: MMH RL AF AAK UBG AMT SB.

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Original article

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SELECTIVE GELATINASE INHIBITION REDUCES APOPTOSIS AND PRO-INFLAMMATORY IMMUNE CELL RESPONSES IN *CAMPYLOBACTER JEJUNI*-INFECTED GNOTOBIOTIC IL-10 DEFICIENT MICE

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Increased levels of the matrix metalloproteinases-2 and -9 (also referred to gelatinase-A and -B, respectively) can be detected in intestinal inflammation. We have recently shown that selective gelatinase blockage by the synthetic compound RO28-2653 ameliorates acute murine ileitis and colitis. We here investigated whether RO28-2653 exerts anti-inflammatory effects in acute *Campylobacter jejuni*-induced enterocolitis of gnotobiotic IL-10$^{-/-}$ mice generated following antibiotic treatment. Mice were perorally infected with *C. jejuni* (day 0) and either treated with RO28-2653 (75 mg/kg body weight/day) or placebo from day 1 until day 6 post infection (p.i.) by gavage. Irrespective of the treatment, infected mice displayed comparable pathogen loads within the gastrointestinal tract. Following RO28-2653 administration, however, infected mice exhibited less severe symptoms such as bloody diarrhea as compared to placebo controls. Furthermore, less distinct apoptosis but higher numbers of proliferating cells could be detected in the colon of RO28-2653-treated as compared to placebo-treated mice at day 7 p.i. Remarkably, gelatinase blockage resulted in lower numbers of T- and B-lymphocytes as well as macrophages and monocytes in the colonic mucosa of *C. jejuni*-infected gnotobiotic IL-10$^{-/-}$ mice. Taken together, synthetic gelatinase inhibition exerts anti-inflammatory effects in experimental campylobacteriosis.

Keywords: matrix metalloproteinases, gelatinases, RO28-2653, synthetic gelatinase blockage, *Campylobacter jejuni*, gnotobiotic IL-10 deficient mice, acute ulcerative enterocolitis, apoptosis, pro-inflammatory immune cell responses, proliferating cells

Introduction

Campylobacter (*C.*) *jejuni* infections comprise a significant health and socioeconomic burden in humans with rising prevalences worldwide especially in industrialized countries [1, 2]. The highly motile Gram-negative bacteria are part of the commensal gut microbiota in a multitude of wild and domestic animals. Zoonotic transmission from livestock animals takes place via consumption of contaminated meat products or water [3, 4]. Infected humans present a broad range of clinical manifestations. Symptoms vary from mild malaise to severe ulcerative enterocolitis requiring hospitalization especially in severely immune-compromized patients [5]. In most cases, however, human campylobacteriosis is self-limiting [6]. In the acute stage of *C. jejuni*-induced enterocolitis, patients suffer from abdominal cramps, fever, watery or bloody diarrhea [5, 7]. Histological examination of inflamed intestinal tissues reveals apoptosis, crypt abscesses, ulcerations, and infiltration of the intestinal mucosa and lamina propria with pro-inflammatory immune cell populations such as lymphocytes, macrophages, and neutrophils [8, 9]. We have recently shown that gnotobiotic IL-10$^{-/-}$ mice generated by broad-spectrum antibiotic treatment are excellently suited as *C. jejuni* infection model to study host–pathogen interactions *in vivo* [10–12]. It is well known that rodents are 1000 times more resistant to Toll-like receptor-4 agonists such as lipopolysaccharide (LPS) and lipooligosacharide (LOS) than humans [13]. In addition, IL-10$^{-/-}$ mice are much more sensitive to LPS and LOS as compared to wildtype mice [10]. Given the key role of LOS in mediating *C. jejuni*-induced disease, gnotobiotic IL-10$^{-/-}$ mice develop acute ulcerative enterocolitis within 1 week following *C. jejuni* infection mimicking key fea-

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tures of severe human campylobacteriosis [10, 14]. Matrix metalloproteinases (MMPs) comprise a tightly controlled heterogenous family of zinc- and calcium-dependent matrix-degrading endopeptidases [15, 16]. With respect to their substrate specificity, MMPs are categorized into collagenases (MMP-1, -8, -13, and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -7, -10, and -11), elastase (MMP-12), and membrane-type matrix metalloproteinases (MT-MMP-1 to -5) [17]. MMPs are physiologically involved in embryonic development and differentiation, tissue proliferation, and regeneration [16, 18]. A dysbalance between activators and inhibitors of MMP expression, however, results in diseases such as arthritis, atherosclerosis, or cancer [19, 20]. In experimental models of intestinal inflammation [21–23] and in patients suffering from human inflammatory bowel diseases such as Crohn's disease or ulcerative colitis [24–26], expression levels of the gelatinases A and B (MMP-2 and MMP-9, respectively) were shown to be upregulated. We have recently demonstrated that selective gelatinase blockage by the synthetic compound RO28-2653 ameliorated acute small intestinal inflammation [22] and acute colitis in mice [23]. The synthetic compound exerts its antigelatinase effects via direct binding to MMP-2 and MMP-9 in a manner that saturates all possible interactions with the pyrimidine core moiety to the protein [27]. Since RO28-2653 lacks anti-MMP-1 and -MMP-7 properties [27] – the major reasons for serious side effects exerted by nonselective MMP-blocking agent in clinical studies – the likelihood of unwanted adverse effects following gelatinase inhibition can be considered as rather low [28]. We were, therefore, interested in potential beneficial effects of the gelatinase-blocking compound RO28-2653 in acute *C. jejuni*-induced ulcerative enterocolitis. To address this, we here investigated 1) the gastrointestinal colonization properties of *C. jejuni*, 2) the clinical course of infection, and 3) the abundances of apoptotic, regenerating, and pro-inflammatory immune cell populations in the colonic mucosa and lamina propria of *C. jejuni*-infected gnotobiotic IL-10$^{-/-}$ mice following synthetic selective gelatinase blockage.

Materials and 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, Germany). Animal welfare was monitored twice daily by assessment of clinical conditions.

Mice

IL10$^{-/-}$ mice (in C57BL/10 background) were bred and maintained under specific pathogen-free (SPF) conditions in the Forschungseinrichtung für Experimentelle Medizin (FEM, Charité, Berlin, Germany). Gnotobiotic IL-10$^{-/-}$ mice were generated by broad-spectrum antibiotic treatment as described previously [10, 29]. Briefly, to eradicate the commensal gut microbiota, mice were transferred to sterile cages and treated by adding a mix of ampicillin (1 g/l; Ratiopharm), vancomycin (500 mg/l; Cell Pharm), ciprofloxacin (200 mg/l; Bayer Vital), imipenem (250 mg/l; MSD), and metronidazole (1 g/l; Fresenius) to the drinking water *ad libitum* starting at 3 weeks of age immediately after weaning. Age-matched female mice were subjected to the quintuple antibiotic treatment for approximately 4 months before the infection experiments.

C. jejuni infection

Three days prior to infection, the antibiotic cocktail was withdrawn and replaced by sterile tap water. Immediately before infection, sterility of mice was confirmed by transferring individual fecal samples to thioglycollate enrichment broths (Oxoid, Wesel, Germany) and applying subsequent cultural analyses as described earlier [29]. Mice were then 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) by gavage on day 0.

Treatment with RO28-2653

Gnotobiotic IL-10$^{-/-}$ mice were treated perorally with RO28-2653 (75 mg/kg body weight/day; kindly provided by Dr. H.-W. Krell, Roche, Penzberg, Germany) in 0.3 ml PBS once daily by gavage starting at day 1 post infection (p.i.) following *C. jejuni* infection for 6 days until necropsy (day 7 p.i.). PBS-treated animals (0.3 ml perorally once daily) served as negative controls. A potential antibacterial effect of the compound was excluded as described previously [30].

Clinical scoring

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 points: no blood; 2 points: microscopic detection of blood using Haemoccult, Beckman Coulter/PCD, Krefeld, Germany; 4 points: overt 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 [10, 31].

Sampling procedures and histologic scoring

Mice were sacrificed by isofluran treatment (Abbott, Germany) 7 days following *C. jejuni* infection. Colon samples from each mouse were removed under sterile conditions and collected in parallel for histopathological, immunohistochemical, and microbiological analyses.

For immunohistochemical stainings, colon samples were immediately fixed in 5% formalin and embedded in paraffin, and sections (5 μm) were stained with the respective antibodies as described below. Histopathology was investigated in paraffin-embedded hematoxylin and eosin (H&E) stained tissue sections. A published standardized histologic score ranging from 0 to 6 was used for blinded evaluation of the inflammatory processes in the colon [12].

Immunohistochemistry

In situ immunohistochemical analysis of 5 μm thin colonic paraffin sections was performed as described previously [10, 31–34]. Primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, USA, 1:200), Ki67 (TEC3, Dako, Denmark, 1:100), F4/80 (# 14-4801, clone BM8, eBioscience, 1:50), myeloperoxidase-7 (MPO-7, # A0398, Dako, 1:500), CD3 (M-20, Santa Cruz, dilution 1:1000), FOXP3 (FJK-16s, eBioscience, 1:100), and B220 (eBioscience, San Diego, CA, USA, 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) was determined microscopically by two independent double-blinded investigators and subjected to statistical analysis as indicated below.

Statistical analysis

Mean values, medians, and levels of significance were determined using Mann–Whitney *U* test. Two-sided probability (*P*) values ≤ 0.05 were considered significant. All experiments were performed twice.

Results

Uncompromized C. jejuni *infection of gnotobiotic IL-10$^{-/-}$ mice following selective gelatinase blockage*

Given that selective gelatinase blockage by the synthetic compound RO28-2653 was shown effective in preventing and treating acute small intestinal as well as colonic inflammation [22, 23], we were interested whether RO28-2653 could affect murine acute ulcerative enterocolitis following *C. jejuni* infection. To address this, we applied our gnotobiotic IL-10$^{-/-}$ mouse infection model [10]. In order to eradicate the chronic colitogenic stimuli derived from the commensal intestinal microbiota, IL-10$^{-/-}$ mice were subjected to a quintuple antibiotic regimen for 4 months starting immediately after weaning [10]. The resulting gnotobiotic mice were then perorally infected with 10^9 CFU *C. jejuni* strain 81-176 on day 0. Starting at day 1, infected IL-10$^{-/-}$ mice were then perorally treated with RO28-2653 or placebo for 6 days on a daily basis. Notably, neither the synthetic compound nor placebo exerted any antimicrobial effects in respective disk diffusion assays (not shown) that might interfere with establishment of *C. jejuni* in the murine gastrointestinal tract. This was further confirmed by daily assessment of the *C. jejuni* loads in fecal samples demonstrating comparable pathogen burdens in the intestines of RO28-2653- and placebo-treated mice over time *(Fig. 1)*. On day 7 post

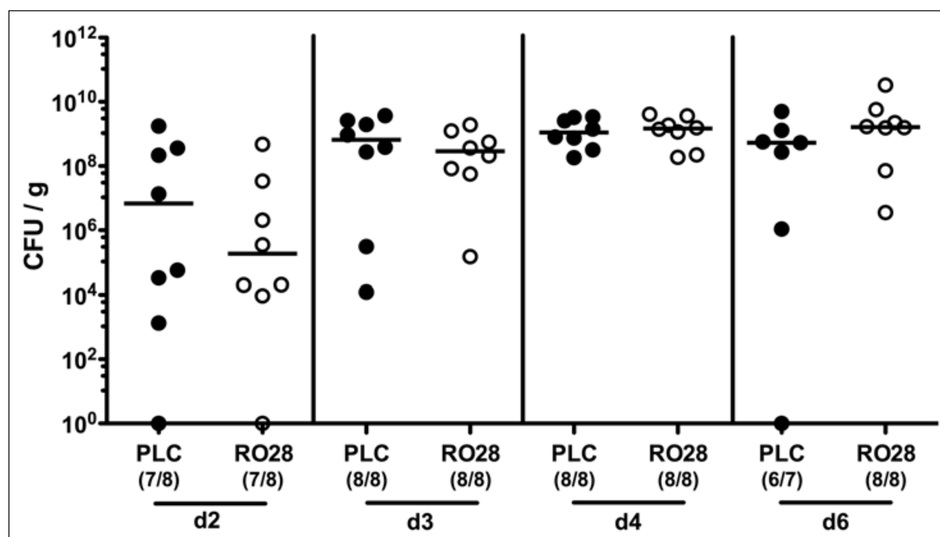

Fig. 1. Kinetic analysis of fecal *C. jejuni* loads following selective gelatinase blockage. Gnotobiotic IL-10$^{-/-}$ mice were generated by antibiotic treatment and orally infected with *C. jejuni* strain 81-176 on day 0. Starting at day 1 post infection, infected mice were either treated with RO28-2653 (RO28, open circles) or placebo (PLC, solid circles) once daily. *C. jejuni* loads were determined in fecal samples from day (d) 2 until d6 post infection by culture (CFU, colony forming units). Numbers of mice harboring the pathogen out of the total number of analyzed animals are given in parentheses. Medians (black bars) and significance levels (*p* values) determined by Mann–Whitney *U* test are indicated. Data are representative for two independent experiments

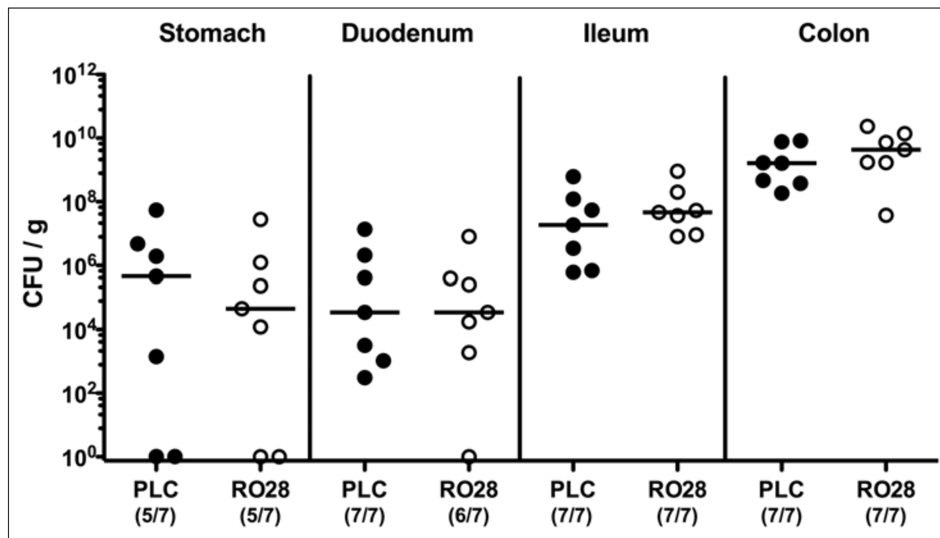

Fig. 2. *C. jejuni* colonization of the gastrointestinal tract following selective gelatinase blockage. Gnotobiotic IL-$10^{-/-}$ mice were generated by antibiotic treatment and orally infected with *C. jejuni* strain 81-176 on day 0. Starting at day 1 post infection, infected mice were either treated with RO28-2653 (RO28, open circles) or placebo (PLC, solid circles) once daily. *C. jejuni* loads were determined in luminal samples of the gastrointestinal tract at day 7 post infection by culture (CFU, colony forming units). Numbers of mice harboring the pathogen out of the total number of analyzed animals are given in parentheses. Medians (black bars) and significance levels (p values) determined by Mann–Whitney U test are indicated. Data shown are representative for two independent experiments

infection (p.i.), when gnotobiotic IL-$10^{-/-}$ mice suffered from severe *C. jejuni*-induced ulcerative enterocolitis, all animals displayed comparable pathogen loads throughout the entire gastrointestinal tract irrespective whether treated with RO28-2653 or placebo *(Fig. 2)*. Hence, synthetic gelatinase blockage did not interfere with *C. jejuni* infection of gnotobiotic IL-$10^{-/-}$ mice.

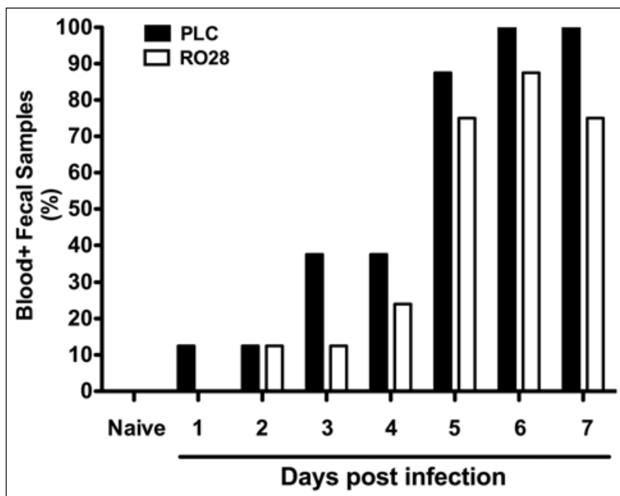

Fig. 3. Bloody diarrhea. Kinetic analysis of blood-positive fecal samples in *C. jejuni*-infected mice following selective gelatinase blockage. Gnotobiotic IL-$10^{-/-}$ mice were generated by antibiotic treatment and orally infected with *C. jejuni* strain 81-176 on day 0. Starting at day 1 post infection, infected mice were either treated with RO28-2653 (RO28, white bars) or placebo (PLC, black bars) once daily. Indicated relative rates of blood-positive fecal samples of uninfected (naive) and *C. jejuni*-infected IL-$10^{-/-}$ mice were determined by haemoccult test on a daily basis. Data shown are representative for two independent experiments

Better clinical outcome of C. jejuni-*infected mice following selective gelatinase blockage*

We next assessed clinical symptoms such as bloody diarrhea, a hallmark of severe campylobacteriosis [5, 7], in *C. jejuni*-infected mice upon selective gelatinase blockage over time. Remarkably, kinetic analyses of the occurrence of blood in fecal samples following *C. jejuni* infection revealed that RO28-2653-treated mice exhibited less frequently bloody diarrhea from day 3 until day 7 p.i. as compared to placebo control animals (*Fig. 3* and *Fig. 4A*, $p < 0.05$). The beneficial effect of selective gelatinase blockage was further substantiated by assessing a more differential cumulative clinical score. At day 7 p.i., placebo control mice were severely compromised, whereas RO28-2653-treated mice exhibited approximately 50% lower clinical scores as compared to placebo animals ($p < 0.05$; *Fig. 4B*). Thus, selective gelatinase blockage resulted in better clinical outcomes of *C. jejuni*-infected gnotobiotic IL-$10^{-/-}$ mice.

Macroscopic and histopathological colonic changes in C. jejuni-*infected mice following selective gelatinase blockage*

Given that intestinal inflammation results in a significant shortening of the intestines [10, 29, 33], we next deter-

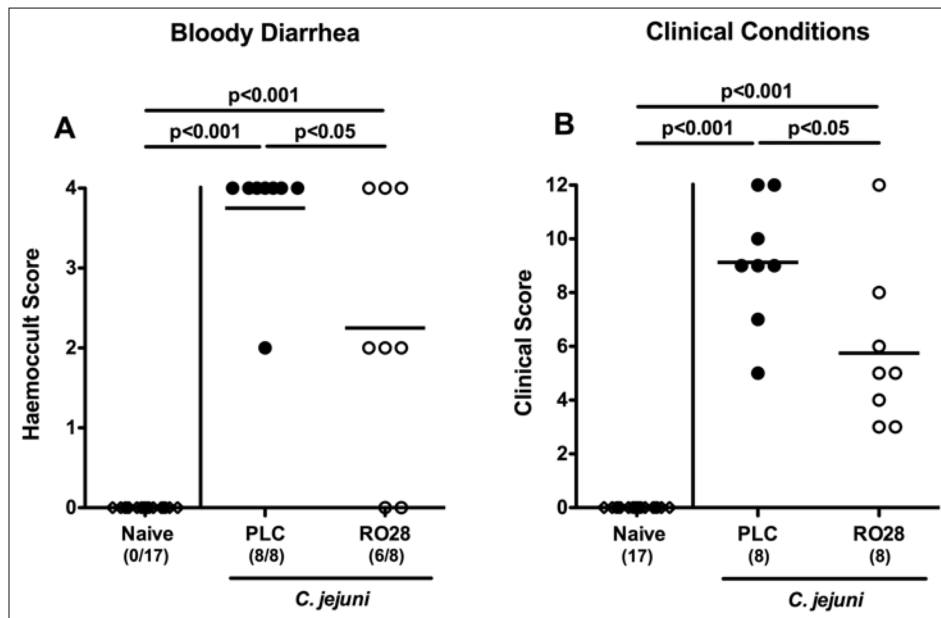

Fig. 4. Clinical symptoms of *C. jejuni*-infected mice following selective gelatinase blockage. Gnotobiotic IL-$10^{-/-}$ mice were generated by antibiotic treatment and orally infected with *C. jejuni* strain 81-176 at day 0. Naive uninfected mice served as negative controls (open diamonds). Starting on day 1 post infection, infected mice were either treated with RO28-2653 (RO28, open circles) or placebo (PLC, solid circles) once daily. (A) The occurrence of blood in fecal samples and (B) clinical symptoms were assessed at day 7 post infection applying respective standardized scores as described in methods. Numbers of haemoccult-positive animals (A) out of the total numbers of analyzed mice (A, B) are given in parentheses. Means (black bars) and significance levels (*p* values) determined by Mann–Whitney *U* test are indicated. Data shown are representative for two independent experiments

mined the absolute colonic lengths in *C. jejuni*-infected mice following the respective treatment regimen. At day 7 p.i., placebo- and RO28-2653-treated animals exhibited significantly shorter large intestines as compared

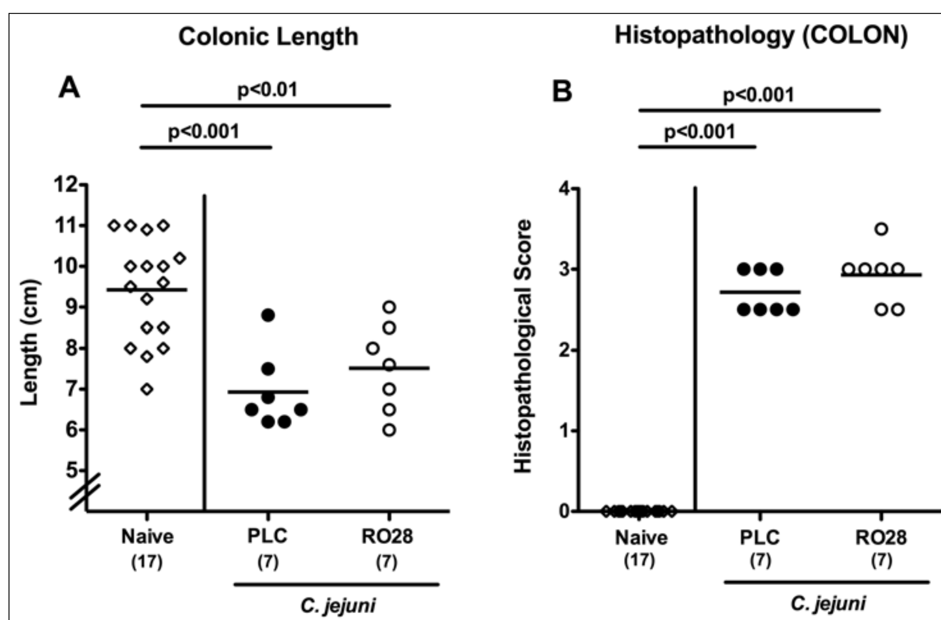

Fig. 5. Macroscopic and histopathological colonic changes in *C. jejuni*-infected mice following selective gelatinase blockage. Gnotobiotic IL-$10^{-/-}$ mice were generated by antibiotic treatment and orally infected with *C. jejuni* strain 81-176 on day 0. Naive uninfected mice served as negative controls (open diamonds). Starting at day 1 post infection, infected mice were either treated with RO28-2653 (RO28, open circles) or placebo (PLC, solid circles) once daily. (A) Absolute colonic lengths and (B) histopathological changes applying a standardized histopathological score in H&E-stained colonic paraffin sections were determined at day 7 post infection. Numbers of analyzed mice are given in parentheses. Means (black bars) and significance levels (*p* values) determined by Mann–Whitney *U* test are indicated. Data shown are representative for two independent experiments

to naive controls ($p < 0.001$ and $p < 0.01$, respectively; *Fig. 5A*). Although not significantly different, infected mice subjected to selective gelatinase blockage displayed a trend towards higher large intestinal lengths as compared to the placebo group *(Fig. 5A)*. We further determined the *C. jejuni*-induced colonic histopathological sequelae following gelatinase blockage. Irrespective of the treatment regimen, *C. jejuni*-infected gnotobiotic IL-10$^{-/-}$ mice displayed comparable histopathological scores determined in colonic H&E stained paraffin sections at day 7 p.i. *(Fig. 5B)*.

Colonic apoptotic and proliferating cells in C. jejuni*-infected mice following selective gelatinase blockage*

Given that apoptosis is a commonly used diagnostic marker in the histopathological evaluation and grading of intestinal diseases [33] and furthermore a key feature of *C. jejuni*-induced ulcerative enterocolitis in gnotobiotic IL-10$^{-/-}$ mice and infected humans [10], we next quantitatively assessed caspase-3^{+} cells within the colonic mucosa in *C. jejuni*-infected mice following selectice gelatinase inhibition. Seven days following *C. jejuni* infection numbers of apoptotic cells increased multifold ($p < 0.001$; *Fig. 6A*), but to a significantly lesser extent in RO28-2653-treated as compared to placebo-treated mice ($p < 0.01$; *Fig. 6A*). Conversely, numbers of colonic Ki67^{+} proliferating cells increased in *C. jejuni*-infected mice following selective gelatinase inhibition only as compared to naive or placebo treated animals at day 7 p.i. ($p < 0.001$ and $p < 0.05$, respectively; *Fig. 6B*). Hence, selective gelatinase blockage resulted in less apoptosis and more cell proliferation thereby counteracting mucosal inflammation in *C. jejuni*-infected gnotobiotic IL-10$^{-/-}$ mice.

Less pronounced immune cell responses in the colonic mucosa of C. jejuni*-infected mice following selective gelatinase blockage*

Given that human colitis is accompanied by the recruitment of pro-inflammatory immune cell populations to sites of inflammation in the large intestine [23], we next quantitated inflammatory immune cells *in situ* by immunohistochemical staining of colonic paraffin sections of mice with antibodies against F4/80 (macrophages and monocytes), MPO7 (neutrophils), CD3 (T-lymphocytes), FOXP3 (regulatory T-cells, Treg), and B220 (B-lymphocytes). At day 7, following *C. jejuni* infection, placebo control mice displayed multifold increases in colonic macrophage, monocyte, and neutrophil numbers exerting oxidative stress to the epithelium ($p < 0.001$; *Fig. 7A* and *B*). The increase of F4/80^{+} macrophages and monocytes, however, was less pronounced in RO28-2653 treated mice ($p < 0.05$; *Fig. 7A*). Even though not statistically significant due to a relatively high standard deviation in the placebo group, a trend towards a less pronounced in-

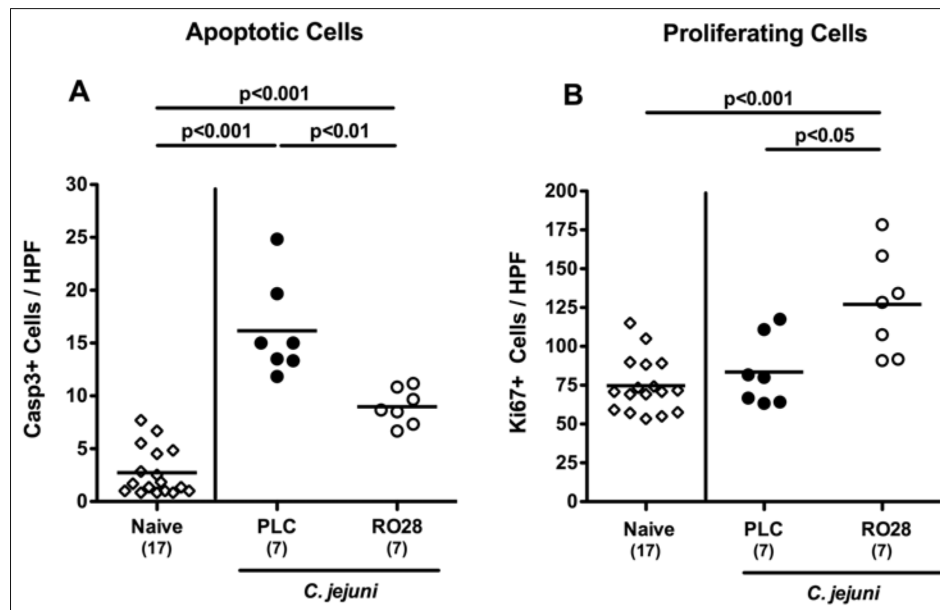

Fig. 6. Colonic apoptotic and proliferating cells in *C. jejuni*-infected mice following selective gelatinase blockage. Gnotobiotic IL-10$^{-/-}$ mice were generated by antibiotic treatment and orally infected with *C. jejuni* strain 81-176 on day 0. Naive, uninfected mice served as negative controls (open diamonds). Starting at day 1 post infection, infected mice were either treated with RO28-2653 (RO28, open circles) or placebo (PLC, solid circles) once daily. The average number of (A) apoptotic (positive for caspase-3, Casp3) and (B) proliferating cells (positive for Ki67) from at least six high power fields (HPF, 400× magnification) per animal was determined microscopically in immunohistochemically stained colonic paraffin sections at day 7 post infection. Means (black bars) and significance levels (p values) determined by Mann–Whitney U test are indicated. Data shown are representative for two independent experiments.

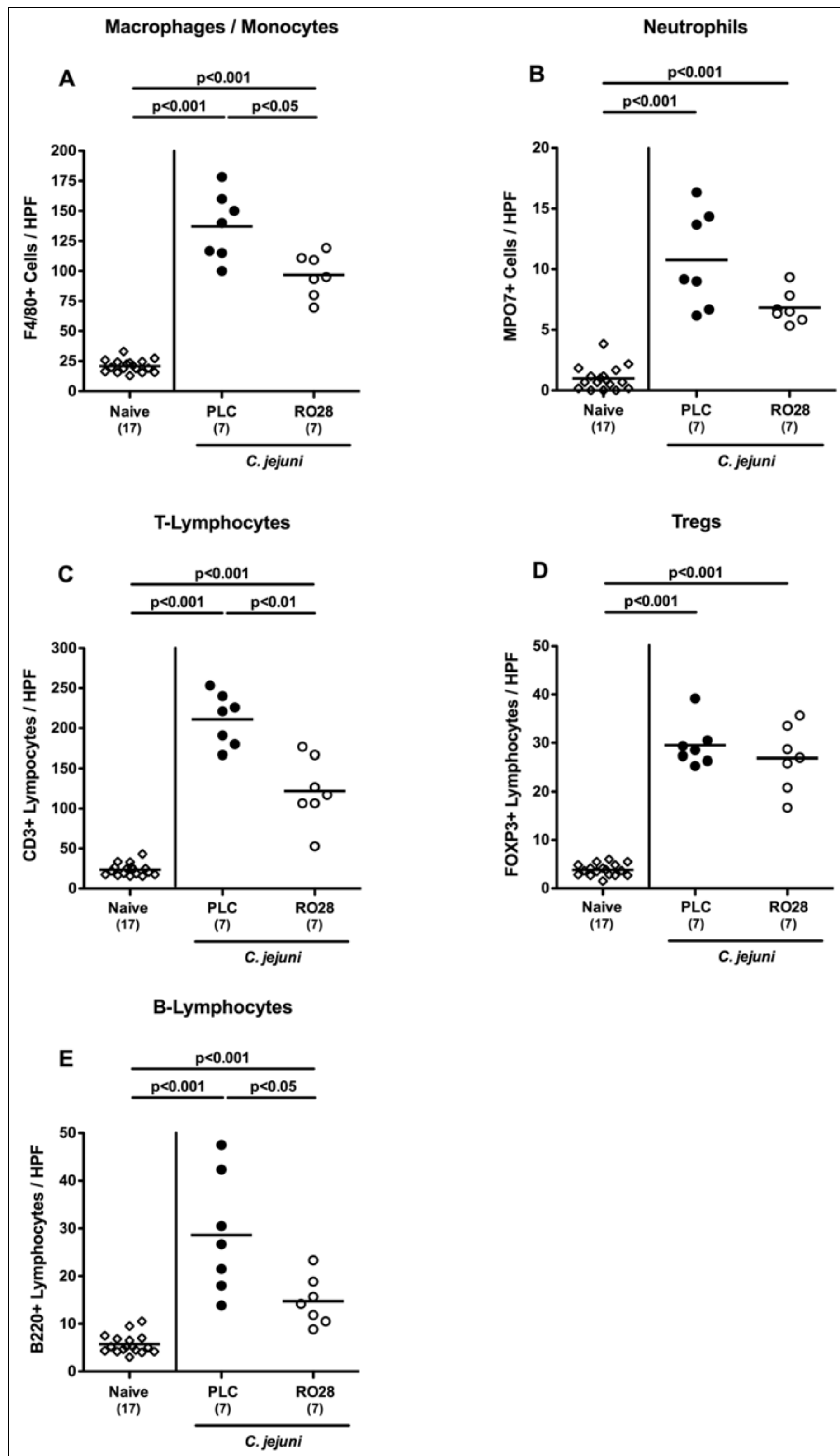

Fig. 7. Colonic immune cell responses in *C. jejuni*-infected mice following selective gelatinase blockage. Gnotobiotic IL-$10^{-/-}$ mice were generated by antibiotic treatment and orally infected with *C. jejuni* strain 81-176 on day 0. Naive uninfected mice served as negative controls (open diamonds). Starting at day 1 post infection, infected mice were either treated with RO28-2653 (RO28, open circles) or placebo (PLC, solid circles) once daily. The average number of (A) macrophages and monocytes (positive for F4/80), (B) neutrophils (positive for MPO7), (C) T-lymphocytes (positive for CD3), (D) regulatory T-cells (Tregs, positive for FOXP3), and (E) B-lymphocytes (positive for B220) from at least six high power fields (HPF, 400× magnification) per animal was determined microscopically in immunohistochemically stained colonic paraffin sections at day 7 post infection. Means (black bars) and significance levels (*p* values) determined by Mann–Whitney *U* test are indicated. Data shown are representative for two independent experiments.

flux of MPO7$^+$ neutrophils into the colonic lamina propria could be observed following RO28-2653 as compared to placebo treatment *(Fig. 7B)*. We have recently shown that mucosal infiltration with CD3$^+$ T-cells is a major characteristic feature of acute and chronic *C. jejuni* infection of mice [10, 31, 33–35]. Accordingly, a multifold increase of T-lymphoctes into the colonic mucosa and lamina propria could be observed 7 days following *C. jejuni* infection of gnotobiotic IL-10$^{-/-}$ mice ($p < 0.001$; *Fig. 7C*). This increase, however, was significantly less distinct following selective gelatinase blockage as compared to placebo treatment ($p < 0.01$; *Fig. 7C*). Interestingly, the *C. jejuni*-induced increase of FOXP3$^+$ Tregs was similar in RO28-2653- and placebo-treated mice at day 7 p.i. ($p < 0.001$; *Fig. 7D*). Furthermore, numbers of colonic B220$^+$ B-lymphocytes were lower in RO28-2653-treated as compared to placebo-treated mice seven day following *C. jejuni* infection ($p < 0.05$; *Fig. 7E*). Hence, selective gelatinase blockage resulted in reduced immune cell numbers within the colonic mucosa and lamina propria of *C. jejuni*-infected gnotobiotic IL-10$^{-/-}$ mice.

Discussion

The gelatinases A and B play pivotal roles in intestinal inflammation in mice and men [21–26]. Given that MMPs are also essentially involved in physiological homeostasis of tissues including the cartilage and the gut [36, 37], compounds for nonselectively blocking MMPs (including the collagenases) can exert serious adverse effects such as muscle pain, arthralgia, and compromised wound healing limiting clinical application. Hence, selective and effective MMP blocking agents with limited side effects would be utmost desirable. Furthermore, MMPs are known to shed biologically active pro-inflammatory molecules such as TNF and IL-6 from surfaces of macrophages subsequently inducing MMP expression in epithelial, parenchymal, as well as immune cells [36, 38]. Therefore, from the therapeutic perspective, an effective MMP-blocking agent should cut this vicious pro-inflammatory cycle due to this positive MMP-feedback loop by downregulating expression and function of MMPs, immune cells such as macrophages, and pro-inflammatory cytokines all at once without compromising health-promoting MMP properties such as cell regeneration [23].

To date, no data exist regarding the roles of MMPs and particularly the gelatinases in *C. jejuni* infection. To address this, we applied the gnotobiotic IL-10$^{-/-}$ mouse infection model. Within 1 week, following *C. jejuni* infection, mice suffer from non-self-limiting acute ulcerative enterocolitis [10]. In the present study, selective gelatinase blockage by peroral treatment with the synthetic gelatinase blocking compound RO28-2653 effectively ameliorated *C. jejuni*-induced enterocolitis. We could show here that RO28-2653 treatment resulted in 1) better clinical conditions of infected mice, 2) less frequent bloody diarrhea (a clinical hallmark of human campylobacteriosis), 3) less colonic apoptosis, but 4) more cell proliferation counteracting the inflammatory process, and finally, 5) less distinct influx of pro-inflammatory immune cell populations such as T- and B-cells as well as macrophages and monocytes in *C. jejuni*-infected gnotobiotic IL-10$^{-/-}$ mice. Notably, RO28-2653 did not affect *C. jejuni* replication either *in vitro* or *in vivo* as indicated by comparable pathogen loads in the gastrointestinal tract of RO28-2653 as compared to placebo treated mice. These results are well in line with our two previous murine studies of acute intestinal inflammation. We could recently show that the selective gelatinase blocking compound RO28-2653 was effective in preventing and treating murine acute ileitis following oral *Toxoplasma gondii* infection [22]. In this hyper-acute inflammatory scenario, RO28-2653 could dampen the Th1-type immune responses and ameliorate small intestinal disease. In addition, RO28-2653 treatment could also ameliorate acute DSS-induced colitis [23]. Like in our *C. jejuni* infection study, influx of immune cells such as T- and B-lymphocytes, macrophages, and monocytes as well as neutrophils into the colonic mucosa and lamina propria (perpetuating the inflammatory process) was diminished upon selective gelatinase blockage. Notably, RO28-2653 did neither show significant adverse effects in rat and monkey toxological studies [27] nor any antimicrobial effects that might affect the commensal microbiota compositon upon treatment [23]. Notably, regenerative properties of the colonic epithelium were not compromised by gelatinase inhibition in *C. jejuni*-mediated enterocolitis. Instead, numbers of Ki67$^+$ proliferating cells in the colonic mucosa were even higher in RO28-2653-treated and *C. jejuni*-infected mice thereby counteracting the inflammatory process.

Conclusion

In conclusion, we propose the selective gelatinase blocker RO28-2653 as a promising future option for prophylaxis and treatment of intestinal inflammation in humans including campylobacteriosis.

Outlook

We have recently shown that MMP-2, but not MMP-9, is essentially involved in mediating acute *T. gondii*-induced ileitis [22] and acute DSS-induced colitis in mice [23]. In an ongoing study, we currently unravel whether MMP-2, MMP-9, or both are essentially involved in *C. jejuni*-induced inflammation.

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Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

5. Publikationsliste

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