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***Helicobacter cinaedi* Induced Typhlocolitis in Rag-2-Deficient Mice**

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Abstract

Background—*Helicobacter cinaedi*, an enterohepatic helicobacter species (EHS), is an important human pathogen and is associated with a wide range of diseases, especially in immunocompromised patients. It has been convincingly demonstrated that innate immune response to certain pathogenic enteric bacteria is sufficient to initiate colitis and colon carcinogenesis in recombina-activating gene (Rag)-2-deficient mice model. To better understand the mechanisms of human IBD and its association with development of colon cancer, we investigated whether *H. cinaedi* could induce pathological changes noted with murine enterohepatic helicobacter infections in the Rag2^{-/-} mouse model.

Materials and Methods—Sixty 129SvEv Rag2^{-/-} mice were experimentally or sham infected orally with *H. cinaedi* strain CCUG 18818. Gastrointestinal pathology and immune responses in infected and control mice were analyzed at 3, 6 and 9 months postinfection (MPI). *H. cinaedi* colonized the cecum, colon, and stomach in infected mice.

Results—*H. cinaedi* induced typhlocolitis in Rag2^{-/-} mice by 3 MPI and intestinal lesions became more severe by 9 MPI. *H. cinaedi* was also associated with the elevation of proinflammatory cytokines, interferon- γ , tumor-necrosis factor- α , IL-1 β , IL-10; iNOS mRNA levels were also upregulated in the cecum of infected mice. However, changes in IL-4, IL-6, Cox-2, and c-myc mRNA expressions were not detected.

Conclusions—Our results indicated that the Rag2^{-/-} mouse model will be useful to continue investigating the pathogenicity of *H. cinaedi*, and to study the association of host immune responses in IBD caused by EHS.

Keywords

Helicobacter cinaedi; typhlocolitis; mice

Inflammatory bowel diseases (IBD), classified as ulcerative colitis (UC) and Crohn's disease, affect millions of people worldwide and is characterized by chronic, uncontrolled inflammation of the intestinal mucosa. Epidemiological evidence indicates that humans with

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IBD have an increased risk of developing colon cancer [1]. The pathogenesis of IBD involves complex interactions including the genetic background of the host, the immunological responses of the host to intestinal microbiota, and environmental factors leading to dysbiosis of the intestinal microflora. Importantly, data from experimental models highlight that enteric flora play a crucial role in initiating and sustaining the inflammatory process [2].

Helicobacter species are microaerobic, gram-negative spiral bacteria that have been associated with gastric cancer in humans, as well as hepatitis, hepatocellular carcinoma, IBD, and colonic adenocarcinoma in mouse models [3–6], *H. cinaedi* (initially named *Campylobacter cinaedi*) is an enterohepatic *Helicobacter* species (EHS) first isolated from homosexual men suffering from enteritis, proctitis, or proctocolitis [7]. *Helicobacter cinaedi* was subsequently isolated from immunocompromised patients afflicted with meningitis, bacteremia, cellulitis, septic arthritis, and enteritis [8], as well as from immunocompetent patients with metabolic disease [9]. Recently, it has been associated with nosocomial transmission and systemic disease in hospitalized patients [10,11].

In our previous study, we reported that *H. cinaedi* induced typhlocolitis in IL-10 deficient mice; the disease was characterized by an elevated TH1 immune response. We also determined that cytolethal distending toxin plays a role in *H. cinaedi* induced intestinal inflammatory responses [12]. To further analyze the immune mechanisms of *H. cinaedi* induced IBD, we used recombina-activating gene (Rag)-2-deficient mice in the current study. In the Rag-deficient mouse model, which lack functional T and B lymphocytes, *Helicobacter hepaticus*-infected mice rapidly developed colitis and large bowel carcinoma, establishing the important relationship of microbial driven innate induced chronic inflammation and colon cancer development [5,6,13]. We investigated whether *H. cinaedi*, a human pathogen, colonized and induced pathological changes in the Rag2^{-/-} mouse model in a manner similar to *H. hepaticus*-induced colon adenocarcinoma [13,14].

Material and Methods

Animals

Sixty, 8-week old 129SvEv Rag2^{-/-} mice (30 male, 30 female), mice were used in this study. All mice were from a Rag2-deficient breeding colony housed in an Association for Assessment and Accreditation of Laboratory Care, International-accredited facility under barrier conditions for the duration of the 9-month experiment. Mice were maintained free of known murine viral pathogens, *Salmonella* spp, *Citrobacter rodentium*, ecto-and endoparasites, and *Helicobacter* spp. Animals were maintained in microisolator, solid-bottomed polycarbonate cages, fed a commercial pelleted diet (ProLab 3000; Purina Mills, St. Louis, MO, USA), and administered water *ad libitum*. The protocol was approved by the Committee on Animal Care of the Massachusetts Institute of Technology.

Experimental infection

Helicobacter cinaedi CCUG 18818 (ATCC type strain) was grown in Brucella broth containing 5% fetal calf serum under microaerobic conditions, screened for morphology and

motility, and resuspended in Brucella broth at approximately 10^9 organism/mL as determined by spectrophotometry at A_{660} . Mice received 0.2 mL of fresh inoculums by gastric gavage every other day for three doses or were sham dosed with broth only. Thirty mice were dosed with *H. cinaedi*, and 30 were sham dosed with broth. Equal numbers of male or female mice in each group were utilized in this study. Colonization with *H. cinaedi* was confirmed 1 month postinoculation (p.i.) by PCR analysis of fecal DNA using previously described methods [12]. Mice were necropsied at 12, 24, and 36 weeks postinfection (WPI). Ten control (five male and five female) and 10 infected mice were assayed at each time point.

Isolation of *Helicobacter cinaedi*

Fresh fecal samples and aseptically collected liver and stomach samples were placed in PBS and homogenized before passage through a 0.45- μ m filter onto blood agar for incubation at 37 °C in microaerobic conditions using vented jars containing N₂, H₂, and CO₂ (80:10: 10). The plates were assessed for growth after 3–5 days and were maintained for 2 weeks before a determination of no growth was made.

Histologic Evaluation

Mice were euthanized with CO₂, and stomach, liver, colon, cecum samples were collected for histologic analysis. Formalin-fixed tissues were routinely processed, embedded in paraffin, cut at 4 μ m, and stained with hematoxylin and eosin (H&E). Large bowel lesions were scored on a blinded basis; size and frequency of lesions for inflammatory, hyperplastic, edema, epithelial defects, and crypt atrophy were scored on a scale of 0–4 with ascending severity (0, none; 1, minimal; 2, mild; 3, moderate; and 4, severe). Epithelial dysplasia and neoplasia were graded using a scale of 0–4: 0, normal; 1, mild dysplastic changes; 2, moderate to severe dysplasia; 3, carcinoma *in situ*, or intramucosal carcinoma; and 4, invasive carcinoma; liver and stomach lesions were scored using criteria as previously described [6,16].

Mouse Tissue DNA Extraction

Tissue DNA was extracted from stomach, liver, colon, and cecum samples using a High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) following the manufacturer's protocol.

Real-time Quantitative PCR for *Helicobacter cinaedi* in Cecum and Colon Samples

Relative concentrations of *H. cinaedi* DNA in stomach, colon, and cecum samples were determined by use of real-time quantitative PCR analysis using the ABI Prism Taqman 7700 sequence detection system (PE Biosystems, Foster City, CA, USA), as previously described by Shen et al. in 2009 [12]. Samples were probed with *H. cinaedi* DNA primers generated from *H. cinaedi* *cdtB* gene using Primer Express software (Applied Biosystems Grand Island, NY, USA), with forward primer HcCDTF 5'-GAG CAA ATC GCG TGA ATC TTG CT-3'; and reverse primer HcCDTR 5'-TGA CAA TCG CAG GTG CAT CTC T-3'. The PCR mixture contained the following in duplicate 25 μ L volumes: 5 μ L of template DNA; 12.5 μ L SYBR Green Master mix; 500 nm of each primer. Thermocycling was performed at

50 °C for 2 minutes and 95 °C for 10 minutes and then 40 repeats of 95 °C for 15 seconds and 60 °C for 60 seconds. Samples were also probed with 18S rRNA-based primers for quantifying host DNA (Applied Biosystems) as described previously [17,18]).

Nested PCR for Detection of *Helicobacter cinaedi* in Liver Samples

Nested DNA PCR was performed using *Helicobacter* genus-specific primers in the first round that amplify a 1200 base-pair (bp) sequence in the 16S rRNA gene using a previously described protocol [19]. Ten percent of first-round product was amplified in a second round using another set of *Helicobacter* genus-specific primers to amplify a 383 bp product nested within the first-round amplicon (with forward primer C98F 5'-TGG TGT AGG GGT AAA ATC C-3' and reverse primer H3A-20 5'-GCC GTG CAG CAC CTG TTT C-3') [20]. The positive control was *H. cinaedi* genomic DNA and proven uninfected mouse tissue was used as a negative control.

Quantitative PCR for Cytokine mRNA Expression Profile in Cecum

Total RNA was extracted from approximately 25 mg of mouse cecum using Trizol reagent per the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Total RNA (2 µg) was converted into cDNA using a High Capacity cDNA Archive Kit following the manufacturer's protocol (Applied Biosystems). cDNA levels for TNF-α, IFN-γ, iNOS, IL-1β, IL-4, IL-6, IL-10, IL17, IL23, cox-2, and c-myc mRNA were measured by quantitative PCR using commercial primers and probes for each cytokine. Briefly, duplicate 25-µL reactions contained 5 µL of cDNA, 1.25 µL of a commercial 20× primer-probe solution, 12.5 µL of 2× master mix (all Applied Biosystems), and 6.25 µL of double-distilled H₂O. Relative expression of mRNA from the ceca of infected and control mice was calculated using the comparative C_T method with RNA input standardized between samples by expression levels of the endogenous reference gene, GAPDH. Results from duplicate samples of 10 mice in each group were plotted as fold changes noted between tissues from infected and uninfected control mice. (Applied Biosystems User Bulletin No. 2).

Immunofluorescence Staining for iNOS and Macrophages

Formalin-fixed cecal tissue sections were steam-treated for 20 minutes in citrate-buffered (pH 6) Target Retrieval Solution (DAKO Cytomation, Carpinteria, CA, USA) for epitope recovery. On an automated immunostainer (i6000, Biogenex; San Ramon CA, USA), deparaffinized and rehydrated tissue sections were sequentially overlaid with egg white avidin; biotin; primary antibody for Nos2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and F4/80 (Invitrogen); secondary biotinylated antibody; Streptavidin-Cy3 (Sigma, St. Louis, MO, USA) and fluorescein conjugated Streptavidin (Rockland Immunochemicals, Gilbertsville, PA, USA) as previously described [21]. Slides were mounted with antifade Vectashield plus DAPI (Vector Laboratories, Burlingame, CA, USA) and immunofluorescence visualized with a laser-scanning confocal imaging system (Carl Zeiss Microimaging, Thornwood, NY, USA).

Statistical Analysis

Cecal and colonic lesion scores were analyzed using the Mann–Whitney U nonparametric test for ordinal data; levels of *H. cinaedi* colonization and cytokine mRNA expression were compared by Student's *t*-test. Values of $p < .05$ were considered significant.

Results

***Helicobacter cinaedi* Colonize the Liver and Gastrointestinal Tract of Rag2^{-/-} Mice**

Helicobacter cinaedi was isolated by culture from the feces of all the infected mice at 12, 24, and 36 WPI. *H. cinaedi* was also isolated from the stomach samples of 20% of infected mice at 12 and 36 WPI (Table 1). Although only one of the liver samples was positive for *H. cinaedi* by culture at 36 WPI, 40% of the liver samples of mice infected with *H. cinaedi* were positive at 24 and 36 WPI by nested PCR analysis (Table 1). All the sham-dosed mice remained *H. cinaedi*-free as confirmed by bacterial culture and PCR throughout the 36-week experiment.

Quantitative PCR results of stomach, cecum, and colon samples indicated that *H. cinaedi* colonization reached the highest level at 12 WPI ($p < .05$) and then gradually decreased by 24 and 36 WPI. The *H. cinaedi* numbers were greater in the cecum compared to the colon at all time points (Fig. 1). *H. cinaedi* also consistently colonized Rag2^{-/-} mouse stomachs during the 9 months of infection. The bacterial numbers were relatively low compared to the colonization levels of the cecum and colon (1000 times less than cecum colonization) (Fig. 1, Table 1).

***Helicobacter cinaedi* Induced Typhlocolitis in Rag2^{-/-} Mice**

Helicobacter cinaedi induced significant typhlocolitis in Rag2^{-/-} mice at all the time points ($p < .001$). Infection with *H. cinaedi* resulted in mild to moderate typhlitis with hyperplasia, submucosal edema, epithelial defects, and dysplasia. Inflammation was limited to the mucosa and submucosa, consisting of few to moderate numbers of neutrophils in segmental areas. Hyperplastic crypts were elongated and lined by densely packed epithelial cells with diminished goblet cell differentiation. Occasional crypt abscesses with dilated crypt glands lined by attenuated epithelial cells and luminal cell debris were observed. Lower bowel inflammation usually was most severe at the cecocolic junction with limited numbers of focal lesions present in the mid-to distal colon. Colonic lesions contained mild mucosal and submucosal inflammation, hyperplasia, edema, and occasional crypt abscesses (Fig. 2). The intestinal lesions became more severe from 12 to 36 WPI. At 12 WPI, 20% of Rag2^{-/-} mice infected with *H. cinaedi* had developed moderate to severe inflammation that yielded scores ranging to >2 . At 36 WPI, 70% infected Rag2^{-/-} mice developed moderate to severe inflammation. Comparing the total lesion scores of ceca, which included inflammatory, hyperplastic, edema, epithelial defects, crypt atrophy, and dysplasia, the lesions of infected mice at 36 WPI were significantly more severe than earlier time points ($p < .01$) (Fig. 3). There was no difference in the pathology scores of infected male and female mice. There was no significant helicobacter induced gastric inflammation in infected mice. By 36 WPI, 50% of infected mice had mild liver inflammation, while only 10% had similar lesions in the

control group. ($p < .05$). No significant lesions were observed in the gastrointestinal tracts of the control mice at any time point (Fig. 4).

Pro-inflammatory Cytokine and iNOS mRNA Levels in Mouse Cecum were Increased in *Helicobacter cinaedi*-infected Rag2^{-/-} Mice

Inflammatory cytokines and iNOS expression were measured from the cecum RNA samples of both infected and non-infected control animals. *H. cinaedi* progressively increased mRNA levels of IL1 β , IL10, TNF α , IFN γ , and iNOS expression throughout the 36-week study. The mRNA intestinal levels of TNF α , IFN γ , and iNOS of *H. cinaedi*-infected mice were significantly increased at all time points compared to the noninfected control mice ($p < .001$). The TNF α mRNA level in the lower bowel of infected Rag2^{-/-} mice at 24 WPI was significantly higher than TNF α levels noted in lower bowel infected tissues at 36 WPI (Fig. 5). The mRNA level of IFN γ was significantly elevated as well at all three time points in infected mice compared to control groups ($p < .001$), with the highest level at 12 WPI (Fig. 5) All infected mice had elevated iNOS mRNA at 12, 24, and 36 WPI compared to control group ($p < .05$). IL10 and IL1 β expression levels began to increase at 12 WPI; reaching the highest level at 24 WPI, with no statistical differences noted with control mice at 36 WPI. No significant changes in mRNA expression of IL4, IL6, Cox-2, IL17, IL23, and c-myc mRNA were noted in cecal samples.

Immunofluorescence Staining for iNOS and Macrophages

iNOS was upregulated in focal areas in the cecum of infected mice when compared to control tissues, iNOS expression was highest in the surface epithelium. Although there were macrophages infiltrating between glands of the mucosa, macrophage infiltration was more noticeable in the submucosa (Fig. 4).

Discussion

Inflammatory bowel disease is a chronic inflammatory disorder of the gastrointestinal tract that affects millions of humans. The initiation and pathogenesis of IBD involve interactions among genetic, environmental, and immune factors. Results from clinical and experimental studies indicate that the normal intestinal mucosal immune response of the host develops tolerance to enteric flora [22,23]. This complex homeostasis is breached under conditions of chronic intestinal inflammation driven by an excessive host immune responses to intestinal microflora.

Helicobacter cinaedi causes bacteremia, cellulitis, arthritis, and osteomyelitis in humans [8,24–31]. Although *H. cinaedi* reportedly affects mostly immunocompromised patients [9,24,32–34], it has also been isolated from diarrheic and bacteremic immunocompetent adults and children [35,36]. Recently, *H. cinaedi* has been identified in immunocompetent patients suffering from bacteremia and cellulitis following orthopedic surgery [25]. Also, it has been frequently isolated from children with diarrhea residing in South Africa [37,38].

Helicobacter cinaedi has also been isolated from rhesus monkeys with colitis and hepatitis. The organism has also been cultured from the feces of hamsters, dogs, and cats, which raises the zoonotic potential of the organism [39–41].

Helicobacter species have been widely used to study bacteria induced IBD in animal models [42]. Rag2^{-/-} mice on a 129 genetic background lack functional T and B lymphocytes and have been used extensively in our laboratory and others to study IBD [6,16]. These studies have demonstrated that innate immune responses to certain pathogenic enteric *Helicobacter* spp. are sufficient to initiate colitis, which can progress to colon cancer [5,6,13,14].

In the current study, *H. cinaedi* colonized Rag2^{-/-} mice, resulting in chronic typhlocolitis. The immunocompromised model provides further evidence that innate immune responses elicited by select bacteria are sufficient to induce chronic inflammation in the lower bowel. In our previous study, *H. cinaedi*-induced typhlocolitis in B6.129P2 IL10 deficient mice caused dysplasia and intramucosal carcinoma with elevated mRNA expression of TNF α , IFN- γ , and iNOS in the cecum. In *H. cinaedi*-infected IL10^{-/-} mice, the severity of typhlitis reached the highest level at 12 WPI, and decreased at 24 and 36 WPI. This contrasts with this study where the pathology of *H. cinaedi*-induced in Rag2^{-/-} mice persisted and became more severe throughout the 36 weeks of the study. *H. cinaedi* persistently colonized gastrointestinal tracts of Rag2^{-/-} mice during the 9 months of infection, suggesting the lack of an adaptive immune system confers susceptibility to progressive *H. cinaedi*-associated colitis. Although the colonization levels decreased over the period of the study, *H. cinaedi* was consistently isolated from feces, stomach, and liver of infected mice at 9-MPI. The primary colonization niche was the cecocolic junction, which had the highest colonization levels. Even though *H. cinaedi*-induced pathology was primarily localized to the lower bowel, its isolation from the liver at 9-MPI indicated that *H. cinaedi* colonizing the gastrointestinal tract can translocate into the enterohepatic circulation and colonize the liver. This observation highlights the ability of *H. cinaedi* to induce bacteremia, cellulitis, arthritis, osteomyelitis, meningitis, and myopericarditis in human patients [9–11]. Recent data also documents that certain individuals can be intestinal carriers of *H. cinaedi*, and increase the risk of nosocomial infection with *H. cinaedi* in hospitalized patients [43–45]. Further, the ability of *H. cinaedi* to persistently colonize the stomach of mice emphasizes earlier studies where *H. cinaedi* was identified in the stomachs of humans [46,47].

The pathology scores did not directly correlate with bacterial colonization density; at 9 MPI, bacterial numbers were at the lowest level while pathology scores were elevated. The decrease of the bacteria colonization levels noted over the course of the experiment may be the result of the host's inflammatory responses which prompted reduction in the quantity of *H. cinaedi*. In *H. hepaticus*-induced colon cancer in Rag^{-/-} mice, *H. hepaticus* counts were not directly correlated with severity of colonic lesions, supporting the hypothesis that host inflammatory responses to EHS, rather than a direct quantitative effect of the pathogenic organisms, can sustain chronic inflammation, which progresses to premalignant lesions and in some cases, cancer [6]. The inflammation was more severe in the cecal colic junction, the primary site of *H. cinaedi* colonization, rather than in the colon during the early phase of infection. In Rag2^{-/-} mice, *H. hepaticus* also efficiently colonized the ileal–cecal colic junction. In this model, however, *H. hepaticus* resulted in the development of prominent tumors, including mucinous and poorly differentiated mucosal adenocarcinomas, which were more frequent in the transverse and descending colon and not the ileal–cecal junction mice [5,6]. Importantly, colonization of EHS is consistent with *Helicobacter pylori* infection

in mice; colonization levels of *H. pylori* are inversely correlated with severity of gastric pathology [48,49].

The genetic background of mice also influences the severity of lower bowel pathology. After 9 months of *H. hepaticus* or *Helicobacter bilis* infection in the C57BL/6J Rag2^{-/-} model, there was no clinical indication of IBD (diarrhea, or rectal prolapse); however, two of the five Rag2^{-/-} mice, in both the *H. hepaticus* and *H. bilis* group, developed mild focal mucosal inflammation characterized by minimal to mild epithelial hyperplasia and low numbers of a predominantly neutrophilic infiltration [50]. Interestingly, lesions in *H. bilis*-infected Rag2^{-/-} mice were found solely in the proximal colon, whereas the lesions in the *H. hepaticus*-infected Rag2^{-/-} mice were noted in the cecum [50]. In contrast, *Smad3*^{-/-} (129-*Smad3*^{tm/Par/J}) mice infected with *H. bilis* or coinfecting with *H. bilis* and *H. hepaticus* developed severe IBD; the mean survival time for these Rag2^{-/-} mice was only 19 weeks [51].

Helicobacter spp. infections in mice are known to induce the expression of proinflammatory cytokines [52–54]. In this study, the mRNA levels of IL1 β , TNF α , IFN γ and iNOS of *H. cinaedi*-infected mice were significantly increased compared to the noninfected control mice ($p < .001$), suggesting that these pro, inflammatory cytokines are primarily produced by nonlymphoid cells such as macrophages dendritic cells, and potentially colonic epithelial cells. TNF- α is believed to play a key role in initiating and amplifying the inflammatory reaction in the intestinal mucosa of IBD animal models and patients with IBD [55–57]. TNF- α stimulates the production of genotoxic molecules such as reactive oxygen species, leading to DNA damage and tumor initiation [58]. Studies have indicated that increased levels of TNF- α and IFN- γ increase intestinal epithelial paracellular permeability and thus may be an important mechanism contributing to intestinal inflammation [59,60]. A recent study identified *H. typhlonius* another EHS, as a key trigger in initiating excess TNF α production and promoting colitis in Tbx21^{-/-} Rag2^{-/-} ulcerative colitis (TRUC) mice [61]. IL-1 β is a prototypical proinflammatory cytokine; it is involved in both the initiation and amplification of the inflammatory response leading to intestinal injury. IL-1 β has been shown to play an important role in the pathogenesis of intestinal inflammation in IBD in humans and in animal models of intestinal inflammation. Patients with IBD have elevated levels of IL-1 β in their intestinal tissue [62]. IBD also requires the presence of phagocytic cells such as monocytes, macrophages, and neutrophils. When stimulated, these cells produce reactive nitrogen and oxygen species. These free radicals are responsible for killing phagocytosed pathogens. However, as a by-product of this protective effect, they also induce local tissue injury. Patients with both forms of IBD (Crohn's disease and ulcerative colitis) have increased inducible NO synthase (iNOS) activity and citrulline production in the mucosa of colonic biopsy specimens [63]. Infection of 129 Rag2^{-/-} mice with *H. hepaticus* led to infiltration of macrophages and neutrophils into the colon, which was temporally related to upregulation of iNOS expression at the site of infection and increased NO production evidenced by urinary excretion of nitrate [6]. Concurrent administration of an iNOS inhibitor prevented NO production, abrogated the epithelial pathology, and inhibited the onset of cancer [6]. Inflammation-mediated carcinogenesis involving infiltration of activated phagocytes at sites of infection, with subsequent generation of reactive oxygen,

nitrogen, and halogen species that cause molecular damage leads to cell dysfunction, mutation, and cell death [13,14]. In the current study, the iNOS expression was significantly increased in all *H. cinaedi*-infected mice, particularly surface epithelium and macrophages located in the submucosa and between glands. At all time points. In an in vitro model to study oxidative damage, macrophage and epithelial cells synthesized NO when co-infected with *H. cinaedi*. Interestingly, colonic epithelial cells were capable of synthesizing NO at rates comparable to macrophages [64]. When infected with *H. cinaedi*, the NO_2^- concentration in the cells was fourfold higher than in the uninfected control cells [64]. We also found that alkyl hydroperoxide reductase C (AhpC), which is responsible for detoxification of peroxides and protection of peroxide-induced stress played a role in *H. cinaedi*-induced pathogenicity as well. The *H. cinaedi* ahpC mutant had diminished resistance to organic hydroperoxide toxicity. The AhpC mutant was more susceptible to killing by macrophages than the WT strain and had reduced colonization ability in BALB/c IL-10^{-/-} mice [65].

Helicobacter cinaedi expresses the genotoxin cytolethal distending toxin (CDT) [66]. This toxin causes DNA strand breaks, inhibits ATM-dependent response pathways, and suppresses repair of DNA adducts [67]. CDT contributed to the severity of the typhlocolitis in the IL10 deficient mouse model [12]; whether *H. cinaedi* CDT functions in Rag2^{-/-} mice in the same manner requires further investigation.

In summary, we have documented that the human pathogen, *H. cinaedi*, persistently colonizes the lower bowel and induced typhlocolitis in Rag2^{-/-} mice. Although the roles of lymphoid cells in the induction of intestinal inflammation have received considerable attention, our results emphasized that innate immune mechanisms are also able to mediate significant and persistent intestinal inflammation upon stimulation with pathogenic organisms. The availability of this model will allow further in vivo investigations regarding the pathogenesis of this emerging human pathogen.

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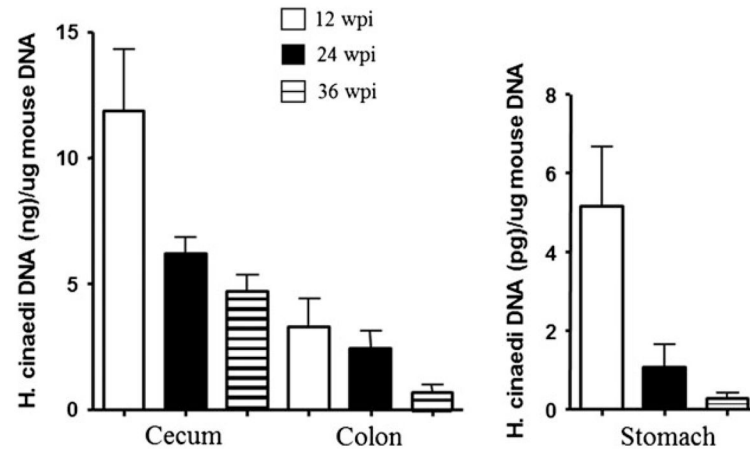


Figure 1. Quantitative PCR of *Helicobacter cinaedi* colonization levels in cecum, colon, and stomach samples of infected mice.

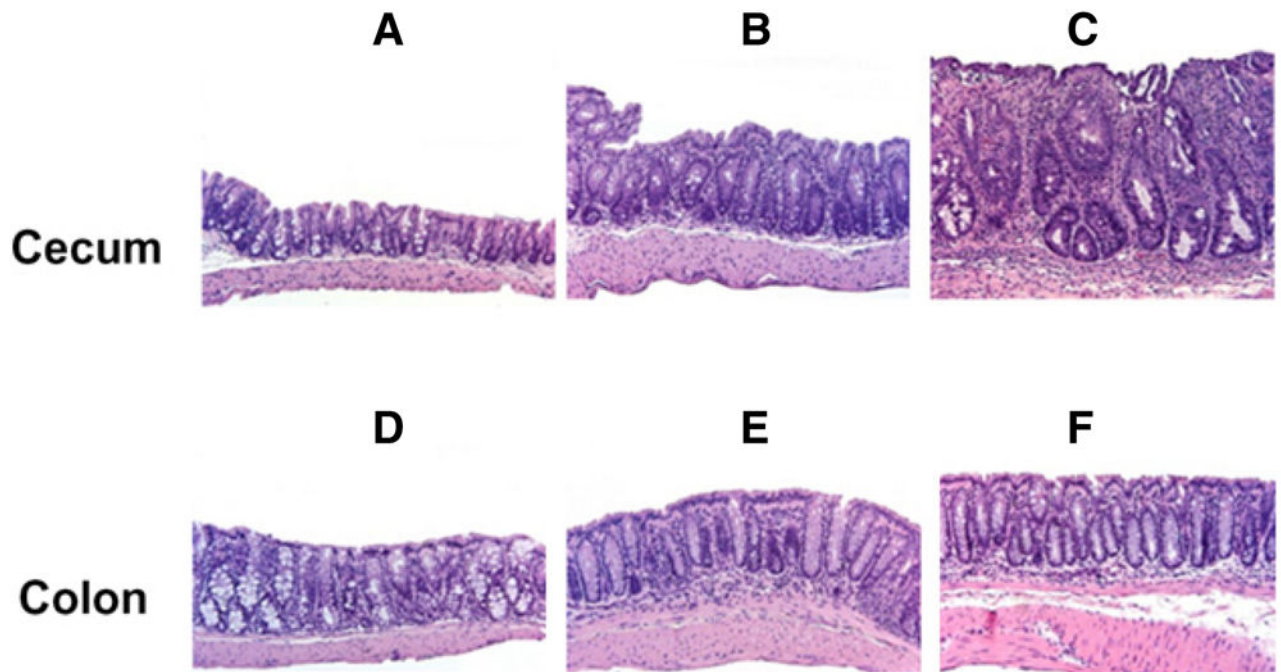


Figure 2. *Helicobacter cinaedi*-induced Typhlocolitis. (A) Cecum from a 12-WPI control mouse. (B) Cecum from a 12-WPI infected mouse with multifocal, mild lamina propria inflammation, and mild hyperplasia. (C) Cecum from a 36-week-infected RAG mouse with coalescing, moderate lamina propria, and submucosal inflammation, with moderate dysplasia, epithelial defects, crypt atrophy, and hyperplasia. (D) Colon from a 12-WPI control mouse. (E) Colon from a 12 week and a 36 week. (F) *H. cinaedi*-infected mouse with multifocal, mild lamina propria, and submucosal inflammation. Magnification $\times 200$.

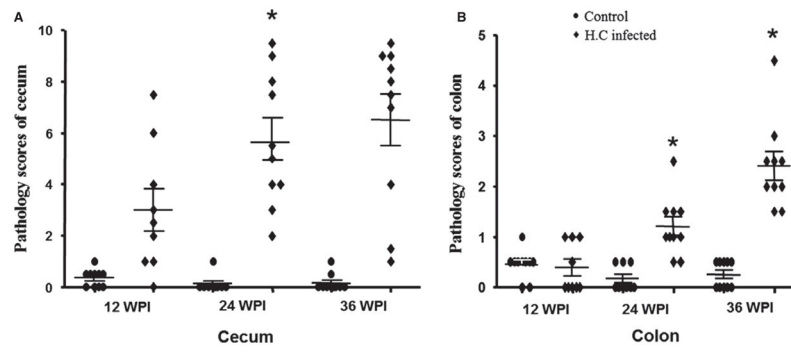


Figure 3. Total pathology scores of inflammation, hyperplasia, dysplasia, edema, epithelia defects, and crypt atrophy. (A) cecum; *p < 0.01 (B) colon.

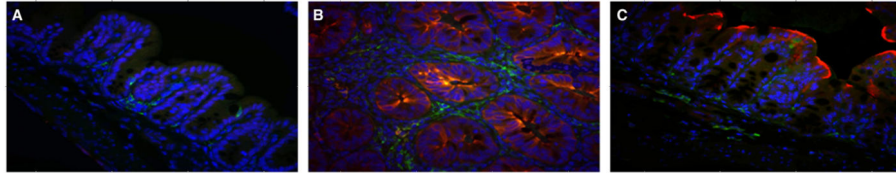


Figure 4. Immunofluorescence stains of *Helicobacter cinaedi*-infected and noninfected mouse cecum with antibodies against iNOS and macrophage marker F4/80. Green, F4/80; Red, iNOS; Blue nucleus. (A) noninfected control mouse; (B and C) *H. cinaedi*-infected mouse. Magnification $\times 200$.

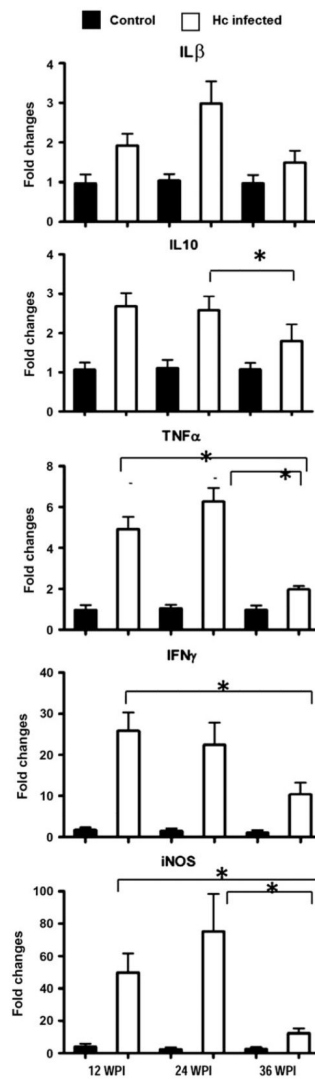


Figure 5. Relative expressions of select cytokine and iNOS mRNA levels in the cecum. Expression levels of IL10, IL1 β , TNF α , IFN γ , and iNOS were higher in infected mice compared to control mice at all the time points. Data represent mean and standard error of fold changes in mRNA expression from infected mice compared to uninfected controls. *p*-values when compared with sham control, * *p* < .05.

Table 1Culture and PCR results of *Helicobacter cinaedi*-infected Rag2^{-/-} mice

	<u>Rag2^{-/-} mice time points at postinfection^{a,b}</u>		
	<u>3 months</u>	<u>6 months</u>	<u>9 months</u>
Liver culture	0/10	0/10	1/10
Liver PCR	1/10	4/10	4/10
Stomach culture	2/10	0/10	2/10
Stomach PCR	8/10	9/10	7/10

^a All fecal samples from infected mice were culture positive for *H. cinaedi*.

^b All ceca and colon samples from infected mice were PCR positive for *H. cinaedi*.

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