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Helicobacter hepaticus infection in mice: models for understanding lower bowel inflammation and cancer

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Pioneering work in the 1990s first linked a novel microaerobic bacterium, *Helicobacter hepaticus*, with chronic active hepatitis and inflammatory bowel disease in several murine models. Targeted *H. hepaticus* infection experiments subsequently demonstrated its ability to induce colitis, colorectal cancer, and extraintestinal diseases in a number of mouse strains with defects in immune function and/or regulation. *H. hepaticus* is now widely utilized as a model system to dissect how intestinal microbiota interact with the host to produce both inflammatory and tolerogenic responses. This model has been used to make important advances in understanding factors that regulate both acquired and innate immune response within the intestine. Further, it has been an effective tool to help define the function of regulatory T cells, including their ability to directly inhibit the innate inflammatory response to gut microbiota. The complete genomic sequence of *H. hepaticus* has advanced the identification of several virulence factors and aided in the elucidation of *H. hepaticus* pathogenesis. Delineating targets of *H. hepaticus* virulence factors could facilitate novel approaches to treating microbially induced lower bowel inflammatory diseases.

INITIAL ISOLATION AND CHARACTERIZATION OF *H. HEPATICUS* AND *H. BILIS*

Since the isolation and characterization of *Helicobacter pylori*, the etiological agent of peptic ulcer disease, in 1982 a number of other *Helicobacter* species have been isolated, many of which can produce serious nongastric disease in their respective hosts. *Helicobacter* species that colonize the lower bowel and biliary tract of mice have been associated with the development of colitis resembling human inflammatory bowel disease (IBD) in susceptible hosts. Further, *H. hepaticus* infection has been demonstrated to exacerbate the development of cancer at both intestinal and extraintestinal sites. The most well studied member of this group of enterohepatic *Helicobacter* species (EHS) is *H. hepaticus*. In this review, we will focus on the initial identification of *H. hepaticus*, the basis for *H. hepaticus*-induced lower bowel inflammation, and the characterization of bacterial virulence factors.

In the early 1990s, a chronic active hepatitis was detected in several inbred strains of mice including A/JCr, originating from a barrier-maintained facility. A distinctive morphological pattern of liver damage was noted, which had not been previously

identified in mice. Using a special silver stain, a spiral-like organism was observed in the liver. Infected A/JCr mice were then shipped to our laboratory for characterization and diagnostic evaluation. A microaerobic bacterium was consistently isolated from the liver and colon of these mice between 3 and 18 months of age. By biochemical and molecular methodologies these bacteria were confirmed to represent a new *Helicobacter* sp., which we named *H. hepaticus*.^{1–3} Shortly after the isolation of *H. hepaticus*, we isolated a fusiform bacterium from the liver, bile, and lower intestine of aged, inbred mice. On the basis of 16S rRNA gene sequence analysis, the organism was classified as a novel *Helicobacter* sp., *H. bilis*.

Currently there are nine formally named EHS isolated from mice as well as several other novel *Helicobacter* spp. awaiting formal naming. Most recently, *Helicobacter pullorum*, a human pathogen, has been isolated from commercial, barrier maintained mice.⁴ Surveys conducted in 1995 and 2007 indicated that *H. hepaticus* and other EHS have a worldwide distribution in academic and commercial mice.^{5,6} Contrary to the earlier survey, no *Helicobacter* spp., however, were found in mice in 2007 from advertised *Helicobacter*-free production areas from

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two US vendors. These results demonstrated that infection with EHS is widespread in most, if not all, mouse colonies used for experimental investigations.

SPONTANEOUS IBD IN IMMUNODEFICIENT AND GENETICALLY ENGINEERED MICE: ASSOCIATION WITH *HELICOBACTER* SPP.

Before the isolation of EHS, it had been speculated that microflora might have an important role in the etiology of spontaneous colitis that developed in a number of immunodeficient laboratory mouse strains, including interleukin (IL)-10-deficient, *scid*, and Rag. Berg *et al.*⁷ also demonstrated that inbred mice on different strain backgrounds with undefined intestinal flora influenced the degree of colitis and lower bowel adenocarcinoma noted in mice; C57BL IL10^{-/-} mice were less prone to develop colitis and carcinoma, BALBc IL10^{-/-} mice were intermediate in susceptibility, and 129 IL10^{-/-} mice developed the most severe disease. Once identified, we found that although *H. hepaticus* infection could be demonstrated in a number of immunologically normal mice without colitis, it was strongly associated with chronic proliferative typhlitis, colitis, and rectal prolapse in several immunodeficient mouse strains housed at the National Cancer Institute and Massachusetts Institute of Technology.^{8,9} The specific pathogen-free health status of these colonies was verified to include lack of serum antibodies to murine viruses and negative cultures for respiratory pathogens, *Salmonella* sp., and *Citrobacter rodentium*. These findings suggested infection with *H. hepaticus* was strongly associated with the development of colitis in a number of laboratory mouse strains, and raised the question of whether *H. hepaticus* might be an etiological agent of murine colitis.

ESTABLISHING THE LINK BETWEEN *HELICOBACTER* SPP. AND IBD

To establish the link between *H. hepaticus* and the development of colitis, we demonstrated that *H. hepaticus* inoculated into *Helicobacter* spp. free defined flora C.B. 17 *scid* mice reconstituted with CD45RB^{high} T cells resulted in severe IBD, similar to that noted with human disease.¹⁰ The expression of typhlitis and colitis was statistically less severe in defined flora *scid* mice that had been reconstituted with T cells but not infected with *H. hepaticus* when compared to reconstituted *scid* mice infected with *H. hepaticus*,¹⁰ demonstrating that infection with this single species could significantly exacerbate colitis in a murine disease model.^{11,12} Similar results were obtained following targeted infection of C57BL IL-10-deficient mice with *H. hepaticus*.^{13,14} Targeted infection experiments have now demonstrated that *H. hepaticus* is able to induce colitis and in some cases lower bowel carcinoma in a number of mouse strains with defects in immune function and/or regulation (Table 1). Interestingly, the same strain susceptibility noted by Berg *et al.* in IL10^{-/-} mice was also documented in Rag^{-/-} mice developing colitis-associated lower bowel carcinoma when experimentally infected with *H. hepaticus*.^{7,15}

Table 1 *H. hepaticus*- and *H. bilis*-associated IBD in mice^a

Genetic status of mice	Type of defect	Pathology	Reference
CD45RB (high)-reconstituted Institute Cancer Research-defined flora <i>scids</i>	Reconstitution with naïve CD4 ⁺ T cell	Typhlocolitis	Cahill <i>et al.</i> ¹⁰
TCR- α , β mutants	Abnormal T-cell receptors	Typhlocolitis	Chin <i>et al.</i> ⁸⁸
Scid ICR-defined flora ^b	Lack T and B cells	Typhlocolitis	Shomer <i>et al.</i> ^{89,90}
C57BL/IL-10 ^{-/-c}	Knockout	Typhlocolitis	Burich <i>et al.</i> ¹⁴ and Kullberg <i>et al.</i> ^{18,19,36}
129SvEv/Rag2 ^{-/-}	Knockout	Typhlocolitis, colon cancer	Erdman <i>et al.</i> ^{28,56}
C57BLRag2 ^{-/-}	Knockout	Typhlocolitis	Burich <i>et al.</i> ¹⁴
IL-7 ^{-/-} Rag-2 ^{-/-}	Double knockout	None	von Freeden-Jeffry <i>et al.</i> ²⁷
A/JCr	Normal	Typhlitis	Fox <i>et al.</i> ³
Swiss Webster gnotobiotic	Normal	Enterocolitis	Fox <i>et al.</i> ⁹¹
129SvEv/NF- κ β (p50 ^{-/-} p65 ^{+/-})	Double knockout	Typhlocolitis	Erdman <i>et al.</i> ⁹²
<i>mdra</i> ^{-/-d}	Lack P-glycoprotein	Typhlocolitis	Maggio-Price <i>et al.</i> ⁹³
SMAD3 ^{-/-d}	Knockout	Typhlocolitis, colon cancer	Maggio-Price <i>et al.</i> ⁹⁴

Abbreviations: IBD, inflammatory bowel disease; IL, interleukin; TCR- α , T-cell receptor alpha.

^aIn mice of the same genetic status, which had *H. hepaticus* (or other *Helicobacter* spp.)-negative microflora, no intestinal disease was noted.

^bMice infected with *H. bilis* also produced IBD.⁸⁹

^cIBD also produced in C57Bl/IL-10^{-/-} mice experimentally infected with a novel urease-negative *Helicobacter* sp.;⁹⁵ now named *H. typhlonius*;⁹⁶ also IBD produced with *H. trogonum*⁹⁷ and *H. cinaedi*.⁷⁴

^d*H. bilis* produces IBD^{93,94} and colon cancer.⁹⁴

BASIS OF THE GASTROINTESTINAL INFLAMMATORY RESPONSE TO *H. HEPATICUS*

The experiments described indicate that *H. hepaticus* infection can induce colitis in certain susceptible mouse strains, but the nature of the inflammatory response and why certain strains were susceptible required further investigation. Seminal experiments from the early 1990s demonstrated that mice lacking IL-10 developed spontaneous colitis, which was significantly less severe when mice were housed in SPF conditions, suggesting that enhanced susceptibility to microbiota induced inflammation.¹⁶ In contrast to *H. hepaticus* monoassociated C57BL IL10^{-/-} mice, which did not develop colitis, Kullberg and co-workers directly demonstrated that targeted infection of SPF-housed C57BL IL-10 mice with *H. hepaticus* significantly exacerbated the development of colitis.^{13,17} The inflammatory response in the *H. hepaticus*-infected C57BL IL-10-deficient mice was characterized by a Th1-like cytokine profile.^{13,18} More recently, these results have been replicated by demonstrating that wild-type (WT) C57BL

mice treated with an antibody directed at the IL-10 receptor (anti-IL-10R), but not those treated with a control antibody, develop colitis following infection with *H. hepaticus*.¹⁹ Colitis in anti-IL-10R-treated mice is characterized by increased expression of mRNA coding for interferon (IFN)- γ and IL-17 within the colon. Mesenteric lymph node cells (MLNs) derived from *H. hepaticus*-infected mice treated with anti-IL-10R secreted higher levels of IL-17 and IFN- γ compared with control MLNs following exposure to soluble helicobacter antigen.¹⁹ Mice deficient in IL-12p40 but not those deficient in IL-12p35 were resistant to *H. hepaticus*-induced colitis following anti-IL-10R treatment, which was associated with decreased expression of IFN- γ and IL-17, suggesting that IL-23 rather than IL-12p70 was necessary for the generation of both Th1 and Th17 responses observed following *H. hepaticus* infection. These experiments indicated that *H. hepaticus* can induce antigen-specific Th1 and Th17 responses in mice with impaired IL-10 signaling.

Although *H. hepaticus* infection of mice with impaired IL-10 signaling induces CD4-mediated Th1 and Th17 responses, the exact role of both IFN- γ and IL-17 in *H. hepaticus*-mediated colitis has been more difficult to discern. Initial experiments with an IFN- γ -depleting antibody suggested that IFN- γ was necessary for *H. hepaticus* to induce colitis in IL-10-deficient mice.¹³ However, the severity of *H. hepaticus*-induced colitis in mice doubly deficient for IL-10 and IFN- γ was very similar to that in mice lacking IL-10 alone.¹⁸ Further, although *H. hepaticus*-induced inflammation within the colon of IFN- γ -deficient mice treated with anti-IL-10R was decreased compared with similarly treated WT mice, there was no difference in the level of inflammation in the cecum between genotypes.¹⁹ Although, to our knowledge, the role of IL-17 in regulating *H. hepaticus*-induced inflammation in mice with impaired IL-10 signaling has not been directly tested, there are conflicting results from experiments testing the role of IL-17 in the development of colitis that develops following the adoptive transfer of CD4⁺CD45RB^{high} cells into lymphocyte-deficient hosts.^{20,21} Further, as it has been suggested that other Th17 cell-derived cytokines such as IL-17F²⁰ and IL-22^{22,23} can modulate disease severity in T cell-dependent models of colitis, the role of these cytokines in *H. hepaticus*-dependent colitis requires additional study.

Although the exact role of T-helper subsets in the development of *H. hepaticus*-induced colitis in susceptible hosts remains to be determined, increasing evidence has focused attention on the innate inflammatory response to *H. hepaticus*. Early studies demonstrated that *H. hepaticus* could induce colitis in certain strains of lymphocyte-deficient mice including *scid* and 129SvEv/Rag^{-/-},^{10,24–27} and progressed to colonic carcinoma with neoplastic peritoneal invasion in some murine models.^{24,28} *H. hepaticus*-induced innate colitis appeared to be dependent upon IL-23, as antibody depletion of both IL-12 p40 and IL-12 p19 (the components of IL-23) inhibited *H. hepaticus*-induced colitis in 129SvEv/Rag^{-/-} mice.²⁹ Antibody depletion assays have also demonstrated an important role of tumor necrosis factor (TNF) in *H. hepaticus*-induced innate colitis.²⁵

Surprisingly, even in the innate colitis model, *H. hepaticus* infection induced robust levels of the T-helper-associated

cytokines IL-17 and IFN- γ within the colon of 129SvEv/Rag^{-/-} mice,²⁹ and treatment of *H. hepaticus*-infected 129SvEv/Rag^{-/-} mice with either IL-17 or IFN- γ -depleting antibody ameliorated colitis.³⁰ This suggested that cells of the innate immune system express IL-17 and IFN- γ within the colon following *H. hepaticus* infection. Indeed, in 129SvEv/Rag^{-/-} mice infected with *H. hepaticus*, there was a marked increase in the percentage of colonic lamina propria (LP) cells that stain positively for IL-17 alone or both IL-17 and IFN- γ following overnight stimulation with IL-23.³⁰ These IL-17⁺ cells lacked lineage markers (CD11b, GR1, B220), but expressed high levels of Thy1,³⁰ and thus bear similarities to a previously described rare population of LP cells termed lymphoid tissue inducer-like (LTi-like) cells.^{31,32} Some LTi-like cells express CD4 on their cell surface,³¹ whereas these newly described IL-17⁺ innate lymphoid cells do not.³⁰ However they share a number of other cell surface markers including IL-7R α .^{30,31} Interestingly, there was a marked increase in the frequency of Thy1^{high} innate lymphoid cells within the colonic lamina propria of 129SvEv/Rag^{-/-} following *H. hepaticus* infection, and when isolated by sorting, these cells produced IFN- γ , IL-17, and IL-22 after overnight culture, which was enhanced by treatment with IL-23.³⁰

It has previously been shown that the development of LTi and LTi-like cells, as well as classic Th17 cells require the transcription factor, retinoic acid related receptor γ t (ROR γ t).^{31–33} Interestingly, *H. hepaticus*-induced innate-Thy1^{high} LP cells expressed higher levels of ROR γ t than remaining Thy1⁻ LP cells,³⁰ and both C57BL/6Rag^{-/-} mice treated with anti-Thy1 antibody, or C57BL/6Rag^{-/-} mice that lacked the gene for ROR γ t, were resistant to anti-CD40-induced innate colitis.³⁰ These data strongly suggest that Thy1⁺ ROR γ t-expressing innate lymphoid cells have a critical role in driving intestinal inflammation. Further, if these innate lymphoid cells require the IL-7 receptor for their survival and/or expansion in similar manner to LTi cells,³⁴ this could explain the earlier observation that *H. hepaticus* is unable to induce colitis in 129SvEv/Rag^{-/-} mice that also lack IL-7.²⁷

These studies argue that innate immune mechanisms have a critical role in the ability of *H. hepaticus* to induce colitis in susceptible hosts. However, it is important to recognize that *H. hepaticus* infection does not induce colitis in most WT mouse strains despite persistent infection, suggesting that a lymphocyte-mediated mechanism inhibits *H. hepaticus*-induced innate inflammation. Consistent with this, several groups have shown that the adoptive transfer of CD4⁺CD25⁺ T-regulatory cells into 129SvEv/Rag^{-/-} mice can inhibit the development of colitis following *H. hepaticus* infection.^{25,28} Inhibition depends upon the ability of transferred cells to express IL-10, as IL-10-deficient CD4⁺CD25⁺ T cells are unable to inhibit *H. hepaticus*-induced innate inflammation, and in fact appear to exacerbate disease.^{24,25} Remarkably, transfer of CD4⁺CD25⁺ T-regulatory cells into *H. hepaticus*-infected 129SvEv/Rag^{-/-} mice with colitis leads to rapid resolution of disease.^{24,35} Several studies revealed that potency of CD4⁺CD25⁺ cells to protect against or treat IBD can be enhanced by *H. hepaticus* infection of donor mice.^{36,37} Thus, CD4⁺CD25⁺ T-regulatory cells can both inhibit and treat *H. hepaticus*-induced innate inflammation in the lower bowel,

based on an IL-10-dependent mechanism. Interestingly, CD25⁺ cells are not the only CD4⁺ subtype with regulatory activity, as it has been demonstrated that CD4⁺CD45RB^{low}CD25⁻ cells harvested from *H. hepaticus*-infected C57BL/10 mice are potent inhibitors of colitis induced by adoptive transfer of IL-10-deficient CD4⁺ T cells into *H. hepaticus*-infected C57BL/10Rag^{-/-} mice.³⁶

Experiments described above, indicating that the recognition of *H. hepaticus* by the innate immune system has a key role in regulating the ensuing inflammatory response in the lower bowel, suggest that understanding the innate inflammatory signaling pathways and receptors utilized by *H. hepaticus* could lead to important insights into the basis of microbiota-driven colonic inflammation. *H. hepaticus* challenge of *ex vivo*-cultured bone marrow-derived macrophages induces activation of both the nuclear factor (NF)- κ B and ERK pathways.^{26,38} Evidence for both pro- and anti-inflammatory roles of individual NF- κ B subunits in the response to *H. hepaticus* come from experiments demonstrating that 129SvEv/Rag^{-/-} mice that also lack the NF- κ B subunit c-Rel are resistant to *H. hepaticus*-induced colonic inflammation,³⁹ whereas 129SvEv mice that lack the p50/p105 subunit of NF- κ B are sensitized to *H. hepaticus*-induced colitis, based on a defect within the innate immune system.^{26,40}

Identifying the innate immune receptors responsible for inducing inflammatory signaling in response to *Helicobacter spp.* infection is an active area of research. Whole *H. hepaticus* bacteria are able to induce NF- κ B activation in HEK293 cells transfected with toll-like receptor 2 (TLR2), but not those transfected with TLR4.⁴¹ However, a recent study demonstrated that the absence of TLR2 in 129SvEv/Rag^{-/-} mice does not influence the severity of the *H. hepaticus*-induced colitis.⁴² Nonetheless, there is strong evidence suggesting the involvement of TLRs in mediating the *in vivo* inflammatory response to *H. hepaticus* as irradiated 129SvEv/Rag^{-/-} reconstituted with Myd88-deficient bone marrow cells are resistant to *H. hepaticus*-induced innate colitis.⁴³

Intracellular receptors of the Nod family plan an important role in recognizing bacterial products such as peptidoglycan that are injected by bacterial secretion systems.⁴⁴ It has recently been reported that the genome of *H. hepaticus* codes for a type VI secretion system that may function to inject substrates into host epithelial cells.⁴⁵ Indeed, it has been suggested that the ability of *H. muridarum*, a murine EHS, to induce NF- κ B reported activity in HEK293 cells depends upon Nod1.⁴⁶ Although, of note, *H. hepaticus* is a very poor inducer of NF- κ B activation in HEK293 cells,⁴⁶ and a weak inducer of inflammatory gene expression in the murine intestinal crypt epithelial cells (m-IC_{cl2} cells),⁴⁷ suggesting that *H. hepaticus* is unlikely to engage Nod1 in these epithelial cell lines. In contrast, it has been found that *H. hepaticus*, as well as LPS derived from *H. hepaticus*, can inhibit TLR4-induced inflammatory gene expression in m-IC_{cl2} cells,⁴⁷ raising the possibility that *H. hepaticus* may have prominent anti-inflammatory properties on epithelial cells. An anti-inflammatory role of an *H. hepaticus*-derived product is supported by studies showing that a *H. hepaticus* mutant lacking the type VI secretion

system colonized the intestine of C57BL/6Rag^{-/-} recipients of CD4⁺CD45RB^{hi} T cells to higher levels than the WT *H. hepaticus* strain, and induced elevated levels of inflammatory gene expression.⁴⁵ Finally, a role of Nod2 in recognition of *H. hepaticus* has been suggested by studies demonstrating higher levels of *H. hepaticus* in infected Nod2-deficient C57BL/6 mice than in infected WT mice, although intestinal pathology was not detected following infection of either WT or Nod2-deficient mice.⁴⁸

H. HEPATICUS INFECTION MODULATES TUMORIGENESIS AT INTESTINAL AND EXTRAINTestinal SITES

At the time of initial characterization in A/JCr mice, it was recognized that in addition to chronic active hepatitis, infection with *H. hepaticus* was also associated with the development of hepatocellular carcinoma in A/JCr as well as liver tumor development in AXB, B6C3F₁, and a substrain of C57BL mice.^{1,3,49-52} Previous studies using germ-free mice link chronic bacterial infection with chronic inflammatory diseases in the lower bowel.⁵³ Targeted bacterial infection with *H. hepaticus* in 129SvEv/Rag2^{-/-} mice lead to colitis-associated colorectal cancer (CRC) with malignancy invading the peritoneal cavity.^{24,28} Gut bacteria-induced lesions in 129SvEv/Rag2^{-/-} deficient mice resembled neoplasia in human patients⁵⁴ with IBD, in that carcinoma clearly arises from colitis-associated dysplastic epithelial foci that become locally invasive.⁵⁵ The finding that 129SvEv/Rag2^{-/-} mice lacking lymphocytes exhibited CRC demonstrated that innate immunity is sufficient for development of carcinoma.²⁸ However, immunologically intact 129 WT mice infected with *H. hepaticus* did not develop carcinoma, showing that IL-10-dependent activities of adaptive immune cells normally serve to reinforce homeostasis and prevent cancer in the lower bowel.^{28,37} Adoptive transfer studies using CD4⁺ cell subsets demonstrated that an imbalance between IL-6, IL-10, and tumor growth factor- β increased risk of neoplastic epithelial invasion.³⁷ Interestingly, *H. hepaticus*-induced malignancy in the 129SvEv/Rag2^{-/-} mice was readily reversible by blocking underlying bacteria-driven inflammation with antibodies directed at TNF α and IL-6.^{37,56}

Pathogenic gut microbial infections have also been shown to trigger development of intestinal adenomatous polyps in mice with a mutated Apc gene (*Apc*^{Min/+} or *Min*).⁵⁷ Mice heterozygous for Apc gene (*Apc*^{Min/+}) develop a large number of intestinal polyps by 3 months of age.⁵⁸ This preneoplastic process has been shown to be inhibited by adoptive transfer of IL-10-dependent T_{Reg} cells, and depends on their ability to produce IL-10.^{59,60} Targeted infection with *H. hepaticus* increases multiplicity and malignancy of intestinal polyps in the *Apc*^{Min/+} mouse model by an inflammation-associated TNF- α -dependent mechanism.⁵⁷ Likewise, infection with other pathogenic enteric microbiota including *C. rodentium*⁶¹ and an enterotoxigenic *Bacteroides fragilis*⁶² have been shown to promote colon tumorigenesis in *Apc*^{Min/+} mice. Recent evidence points to key roles of IL-17 as well as IL-23 in colonic hyperplasia and tumor formation.^{62,63} Taken together, these models may provide novel mechanistic insights in colon carcinogenesis in humans.

More recently, experiments examining the ability of gastrointestinal (GI) tract bacteria and subpopulations of immune cells to induce or inhibit IBD have revealed surprising insights into extraintestinal carcinogenesis. For example, *H. hepaticus* infection was found to trigger mammary tumorigenesis in *Apc^{Min/+}* in the absence of IBD.^{57,64} Mammary tumors also developed rapidly in susceptible mice after transfer of CD4⁺ CD45RB^{high} T cells.^{57,63} Although WT Treg completely inhibited *H. hepaticus*-induced diseases in these models, IL-10-deficient Treg cells exacerbated rather than inhibited *H. hepaticus*-induced extraintestinal pathology.^{24,37} Interestingly, previous exposures of Treg donor mice to *H. hepaticus* enhanced their ability to inhibit extraintestinal pathology,^{57,63,64} raising the possibility that therapies aimed toward intestinal homeostasis may offer sustained protection from systemic diseases.

VIRULENCE FACTORS OF ENTEROHEPATIC HELICOBACTER

Human IBD as well as IBD in mouse models is multifactorial, with genetic and environmental factors contributing to pathogenesis. The complexity of the system necessitates parallel investigations into bacterial virulence factors, gene expression changes in intestinal tissue, host innate and adaptive immune responses, changes in the gut microbial flora, and of course, lesion development in the intestine. Using the *H. hepaticus* genome, we have begun studying in detail, with the use of isogenic mutants, how different virulence genes have a role in chronic intestinal inflammation. The pathogenic potential of several virulence factors from *H. hepaticus* have been well characterized.

Of the known EHS, *H. hepaticus* strain 3B1 (ATCC 51449), the prototype of EHS, has been the most extensively investigated. This murine pathogen contains a circular genome of ~1.8 Mb with 1875 predicted open reading frames.⁶⁵ The G + C content of the *H. hepaticus* genome is 35.9%, which places it between the G + C content of *H. pylori* (39%) and *Campylobacter jejuni* (30.5%). In addition, ~50% of predicted *H. hepaticus* genes are orthologous to those in the genome of either *H. pylori* or *C. jejuni*, both of which cause significant GI diseases in humans.⁶⁵

Cytolethal distending toxin

Bacterial cytolethal distending toxins (CDTs) are present in multiple Gram-negative pathogens including strains of *Salmonella* spp., *Shigella* spp., *C. jejuni*, and *Escherichia coli*, and belong to the AB₂-type of toxins. In general these toxins are comprised of subunits CdtA and CdtC, which together bind to subunit CdtB and deliver CdtB, the active subunit of the toxin, intracellularly.⁶⁶ Several biological functions of the CdtB subunit have been reported, including a DNase I-like function, cell-cycle arrest, phosphatase activity, and eventual cell death.⁶⁷ The *cdtB* ortholog and CDT activity are found in several enterohepatic helicobacters, including *H. hepaticus*, *H. bilis*, *H. canis*, and *H. marmotae*, as well as in *H. cinaedi* and *H. pullorum* isolated from humans.^{68–70}

H. hepaticus 3B1 and an isogenic mutant of the CDT gene (*cdtB*-deficient HhcdtBm7) were evaluated in outbred Swiss Webster mice. Although inoculation with WT 3B1 leads to

persistent colonization, colonization by the mutant failed in female mice and was transient in males.⁷¹ WT 3B1 infection caused significantly increased expression of ileal and cecal IFN- γ mRNA, higher Th1-induced IgG2a responses, and increased mucosal IgA secretion compared with the isogenic mutant.^{13,71} CDT-deficient isogenic *H. hepaticus* mutants also lose their ability to persistently colonize the large intestine of B6.129-IL10^{tm1Cgn} (IL10^{-/-}) mice. Importantly, the mutant-infected mice developed significantly less severe typhlocolitis when compared with WT 3B1-infected mice.^{71–73} Mutation of the *CDT* gene of *H. cinaedi*, a human pathogen, also reduced its ability to induce lower bowel pathology in IL-10-deficient mice.⁷⁴

Molecular mechanisms operable in *H. hepaticus* CDT-induced cell death are also being actively investigated. CDT activated the ATM-dependent DNA damage check point response in a Myc-dependent manner.⁷⁵ In addition, exposure of INT407 cells to *H. hepaticus* CDT resulted in upregulation or pro-apoptotic Bax, release of Cyt *c*, and activation of caspases 3 and 9.⁷⁶ Events operable in *H. hepaticus* CDT-induced cell arrest and apoptosis are summarized in **Figure 1**.

H. hepaticus pathogenicity island (PAI)

PAIs are distinct genetic elements on bacterial chromosomes that encode one or more virulence-associated factors and often have a G + C content distinct from the rest of the core genome.⁶⁵ Several PAIs, such as *LEE* in enteropathogenic *E. coli*, *cag* in

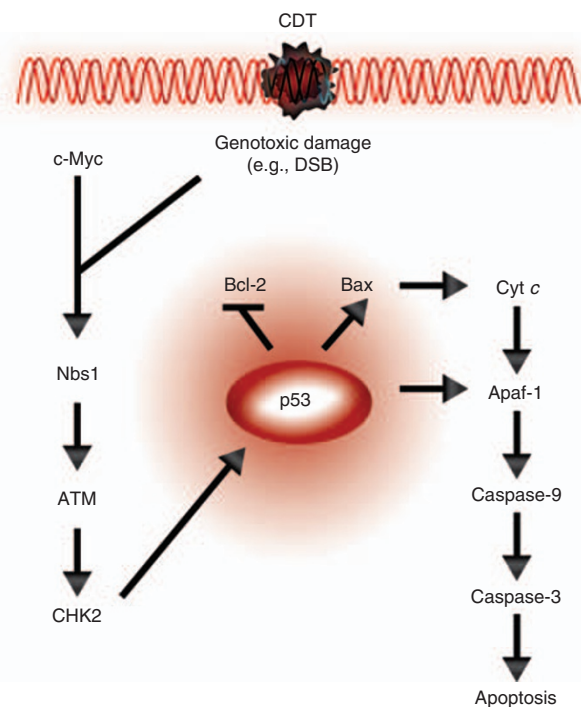


Figure 1 ATM-dependent checkpoint responses and apoptosis triggered by *H. hepaticus* cytolethal distending toxin (CDT)-induced DNA damage.^{75,76} Apaf-1, apoptotic peptidase-activating factor 1; ATM, ataxia telangiectasia mutated; Bax, Bcl-2-associated X protein; Bcl-2, B-cell leukemia/lymphoma 2; CHK2, checkpoint kinase 2; Cyt *c*, cytochrome *c*; DSB, double-strand breakage; Nbs 1, Nijmegen breakage syndrome 1 protein.

H. pylori, and *SaPI1* in *Staphylococcus aureus*, significantly contribute to pathogenicity of these infectious agents.⁷⁷ In the genome of *H. hepaticus* 3B1, a 71-kb region, *HHG11*, displays genetic features characteristic of PAIs.⁶⁵ *HHG11* is highly variable among certain *H. hepaticus* isolates. Male A/JCr mice infected with *H. hepaticus* strain MIT 96-1809 lacking the entire *HHG11* or strain MIT 96-284 missing ~62 out of 71 kb of *HHG11* developed less severe hepatitis than those infected with *H. hepaticus* 3B1, which has an intact *HHG11*.⁷⁸ An isogenic mutant (*HhPAId1*) of *H. hepaticus* 3B1 containing a deletion of 19 predicted genes (*HH0250* to *HH0268*) within *HHG11* colonized the intestine of B6.129-IL10^{tm1Cgn} (IL10^{-/-}) mice at higher levels but provoked less severe typhlocolitis and hyperplasia when compared with *H. hepaticus* 3B1.⁷⁹ The magnitude of the Th1-associated IgG2c response against this isogenic mutant (*HhPAId1*) was less than against WT *H. hepaticus* and was accompanied by suppressed cecal and colonic mRNA expression levels for proinflammatory IFN- γ , TNF- α , and IL17a. These results demonstrate that *HHG11* contributes to the pathogenicity of *H. hepaticus* at least in part via upregulation of proinflammatory mediators.

Recently, a study indicated that *HHG11* encodes for a set of 12 genes (*HH0237*, *HH0242* to *HH0245*, *HH0247* to *HH0252*, *HH0291*), which are homologous to known bacterial type VI secretion systems (T6SS).⁴⁵ T cell-reconstituted Rag1^{-/-} mice infected with a T6SS-deficient Δ *IcmF* *H. hepaticus* mutant (encoded by *HH0252*) developed higher colonization levels of the mutant compared with WT *H. hepaticus*-infected mice, consistent with higher levels of mutant *HhPAId1* cecal colonization.⁷⁹ In addition, infection with Δ *IcmF* *H. hepaticus* induced higher expression in the colon of proinflammatory mediators, including IL-17, IL-23, IL-1 β , TNF α , and IFN γ than infection with WT *H. hepaticus* 3B1.⁴⁵ However, unlike the *HhPAId1* mutant, the T6SS mutant Δ *IcmF*, and *H. hepaticus* 3B1 induced a similar degree of colitis by 2–4 weeks postinoculation.^{45,79} Therefore, further investigations will be required to define the role(s) of T6SS and additional components in *HHG11* in the pathogenesis of *H. hepaticus*.

Urease

Gastric helicobacters produce urease, which is essential for establishing persistent colonization in the acidic milieu of the stomach.⁸⁰ Approximately 40% of known EHS species, including *H. hepaticus*, are urease positive.⁸¹ The *H. hepaticus* urease gene cluster is similar to that of *H. pylori*; however, regulatory mechanisms of urease activity in *H. hepaticus* are distinct from those in *H. pylori*. For example, *H. hepaticus* urease does not confer acid resistance, transcription of *ure* genes is not induced by acid, and urease activity is suppressed by the transcriptional regulator Fur.^{82,83}

By infecting male A/JCr mice with a urease-deficient, isogenic mutant (*HhureNT9*) of *H. hepaticus* 3B1, we demonstrated that the lack of urease activity did not affect the level of cecal colonization but its presence was essential for hepatic colonization.⁸¹ Consequently, the *HhureNT9*-infected mice developed less-severe hepatitis and produced significantly lower hepatic

mRNA levels of proinflammatory cytokines IFN- γ and TNF- α when compared with the *H. hepaticus* 3B1-infected mice.⁸¹

Enzymes in oxidative stress resistance

To survive and establish colonization in the host, bacteria have evolved various mechanisms to combat oxidative stress that confers bactericidal effects via reactive oxygen species. The *H. hepaticus* catalase enzyme KatA, which converts hydrogen peroxide into molecular oxygen and water, has a key role in this process. The amino-acid sequence of *H. hepaticus* KatA contains three motifs (R-F-Y-D, RERIPER, and VVHAKG) in the heme ligand domain and three surface-predicted motifs that are shared among bacterial and mammalian catalases.⁸⁴ KatA-deficient *H. hepaticus* are more sensitive to exposure of oxygen and H₂O₂ and also suffer severe DNA fragmentation by H₂O₂ treatment compared with *H. hepaticus* 3B1.⁸⁵

Bacterial hydrogenase, a hydrogen uptake hydrogenase, oxidizes molecular hydrogen to yield protons and electrons, which have a key role in energy conservation in bacteria. *H. hepaticus* genes or *HH0056* to *HH0059* encode subunits of hydrogenase HyaA to HyaD. Mutation in the *H. hepaticus* *hyaB* (coding for the large subunit) does not affect cecal and hepatic colonization levels when compared with *H. hepaticus* 3B1 in male A/JCr mice; however, liver pathology is diminished in the *hyaB* mutant-infected mice.⁸⁶ It is unknown how hydrogenase contributes to the pathogenesis of *H. hepaticus*, but it is speculated that energy released by hydrogen oxidation may be utilized to promote synthesis of virulence-related proteins or enzymes that result in hepatic or intestinal inflammation and necrosis.⁸⁶

Flagellar genes

All *Helicobacter* species possess flagella for motility; *H. hepaticus* is a spiral rod with bipolar sheathed flagella. In other related bacteria, flagellar genes are mainly regulated by the sigma factor FliA (σ 28). *H. hepaticus* contains two identical copies of the gene encoding the major flagellin subunit FlaA (open reading frames HH1364 (*flaA1*) and HH1653 (*flaA2*)),⁶⁵ which are regulated by the sigma factor FliA (σ 28). Mutations in *fliA* or both copies of the *flaA* gene resulted in no detectable synthesis of FlaA as well as severely truncated flagella, and thus both mutants were non-motile and unable to colonize mice.⁸⁷ The *flaA_1H. hepaticus* mutant, which has flagella but decreased motility, also does not colonize mice, indicating that full motility is a prerequisite for intestinal colonization by *H. hepaticus*, and that the presence of flagella alone is not sufficient.

SUMMARY

As described in this review, *H. hepaticus* has had a central role in shaping our understanding of how intestinal microbiota interact with the host to produce both inflammatory and tolerant responses. The study of immunopathology that develops following *H. hepaticus* infection of susceptible mice has helped to define the importance of key inflammatory mediators, including IL-23 and IL-17, and highlighted the importance of the innate immune response in the development of chronic intestinal inflammation. The use of *H. hepaticus* infection models has

provided definitive evidence that regulatory T cells directly inhibit the innate inflammatory response to lower bowel microbiota and delineated the critical role of IL-10 in this process. Remarkably, *H. hepaticus* infection has powerful effects on carcinogenesis both within the intestine and at extraintestinal sites, and many of the same immunoregulatory pathways that influence the GI inflammatory response to *H. hepaticus* also appear to influence the development of cancer. The complete genomic sequence of *H. hepaticus* has advanced the identification of potential virulence factors, and evaluating the function of these factors is ongoing. In addition, genome sequencing of five additional EHS isolated from humans with GI disease (*H. bilis*, *H. canadensis*, *H. cinaedi*, *H. pullorum*, and *H. winghamensis*) has been completed and these results can be found at http://www.broadinstitute.org/annotation/genome/Helicobacter_group/. Comparative genome analysis of these five genomes plus *H. hepaticus* will provide insight into the etiopathogenesis of EHS infection in humans. For example, preliminary data indicate that both *H. cinaedi* and *H. pullorum* have PAIs with similar features to the HHGI1 found in *H. hepaticus*. Studies are needed to ascertain whether these PAIs impart similar *in vivo* characteristics in humans. Additional areas for future investigation include defining the cellular and molecular pathways that contribute to host recognition of *H. hepaticus*, and determining how initial recognition events lead to activation of innate and acquired inflammatory effector mechanisms. Further, clarifying how responses to *H. hepaticus* infection influences carcinogenesis at local and distant sites could lead to important new insights into the relationship between specific microbiota and the development of cancer. Finally, delineating the targets of *H. hepaticus*-associated virulence factors could lead to novel approaches to treating microbial induced inflammation in the lower bowel.

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DISCLOSURE

The authors declared no conflict of interest.

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