HOW IMMUNOLOGICAL STUDIES ARE BEING INFLUENCED BY
HELICOBACTER HEPATICUS STATUS IN THE MOUSE COLONIES

A Thesis in
Laboratory Animal Medicine

by
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ABSTRACT

Contamination with *Helicobacter hepaticus* is very common in research mouse colonies yet little is known about how this persistent infection affects immunological research conducted in infected mice. The goal of this study was to determine if herpes simplex virus type-1 (HSV-1)-specific immune responses and the characteristics of dendritic cells (DCs) can be altered by the *H. hepaticus* infection status of mouse colonies. We measured and compared virus-specific antibody and T cell-mediated immune responses in *H. hepaticus*-infected and non-infected mice that were inoculated intranasally with HSV-1. The effect of *H. hepaticus* on the HSV-1-specific antibody and T cell-mediated immune responses in superficial cervical and tracheobronchial lymph nodes (LNs) did not reach the level of statistical significance. In separate experiments, we compared the expression of maturation-associated surface markers CD40, CD80, CD86, and MHC II and proinflammatory cytokines IL-12p40 and TNF-α by DCs from the spleen and colic LNs of *H. hepaticus*-infected and non-infected mice. There was decreased surface expression of CD40, CD86, and MHC II and a decreased percentage of IL-12p40 and TNF-α-producing DCs from colic LNs of *H. hepaticus*-infected mice. In contrast, *H. hepaticus* infection did not inhibit the splenic-derived DCs. In fact, there was increased expression of CD40, CD80, CD86 and MHC II on splenic-derived DCs from *H. hepaticus*-infected mice following *in vitro* lipopolysaccharide (LPS) stimulation. These results indicate that *H. hepaticus* infection can influence the results of immunological assays in mice and support an argument for the use of *H. hepaticus*-free mice in immunological research.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
</tr>
<tr>
<td>ASF</td>
<td>altered Schaedler flora</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CDT</td>
<td>cytolethal distending toxin</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>DCs</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence in situ hybridization</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GMFI</td>
<td>geometric mean fluorescence intensity</td>
</tr>
<tr>
<td>HBSS</td>
<td>hank's-buffered salt solution</td>
</tr>
<tr>
<td>HSV-1</td>
<td>herpes simplex virus type 1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL-10</td>
<td>interleukin-10</td>
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IMDM  iscove’s-modified Dulbecco’s medium  
LNs  lymph nodes  
LPS  lipopolysaccharide  
MCP-1  macrophage chemo-attractant protein-1  
MHC  major histocompatibility complex  
MHV  mouse hepatitis virus  
MIG  monokine induced by gamma interferon  
MIP-1α  macrophage inflammatory protein-1 alpha  
ml  milliliter  
mM  millimolar  
NF-κβ  nuclear factor-κβ  
NK cells  natural killer cells  
PBS  phosphate-buffered saline  
PCR  polymerase chain reaction  
PE  phycoerythrin  
PE-Cy7  phycoerythrin-Cy7  
PFU  plaque forming unit  
Rag-2−/−  recombinant activating gene-2 knockout  
RFLP  restriction fragment-length polymorphism  
SCID  severe combined immunodeficiency  
SE  standard error  
Th  helper T cells  
TNF-α  tumor necrosis factor-α
units

μM micromolar
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CHAPTER 1

The effect of Helicobacter hepaticus infection on herpes simplex virus type 1-specific immune responses and characteristics of dendritic cells.

Contamination with Helicobacter hepaticus is very common in research mouse colonies yet little is known about how this persistent infection affects immunological research conducted in infected mice. The goal of this study was to determine if herpes simplex virus type-1 (HSV-1)-specific immune responses and the characteristics of dendritic cells (DCs) can be altered by the H. hepaticus infection status of mouse colonies. We measured and compared virus-specific antibody and T cell-mediated immune responses in H. hepaticus-infected and non-infected mice that were inoculated intranasally with HSV-1. The effect of H. hepaticus on the HSV-1-specific antibody and T cell-mediated immune responses in superficial cervical and tracheobronchial lymph nodes (LNs) did not reach the level of statistical significance. In separate experiments, we compared the expression of maturation-associated surface markers CD40, CD80, CD86, and MHC II and proinflammatory cytokines IL-12p40 and TNF-α by DCs from the spleen and colic LNs of H. hepaticus-infected and non-infected mice. There was decreased surface expression of CD40, CD86, and MHC II and a decreased percentage of IL-12p40 and TNF-α-producing DCs from colic LNs of H. hepaticus-infected mice. In contrast, H. hepaticus infection did not inhibit the splenic-derived DCs. In fact, there was increased expression of CD40, CD80, CD86 and MHC II on splenic-derived DCs from
*H. hepaticus*-infected mice following *in vitro* lipopolysaccharide (LPS) stimulation. These results indicate that *H. hepaticus* infection can influence the results of immunological assays in mice and support an argument for the use of *H. hepaticus*-free mice in immunological research.
Introduction

*Helicobacter hepaticus* is a Gram-negative, microaerophilic, curved to spiral-shaped bacterium with bipolar, sheathed flagella. *H. hepaticus* was described for the first time in 1994 as the cause of chronic active hepatitis associated with a high incidence of hepatocellular neoplasms in mice on a long-term toxicology study (31). Since then, *H. hepaticus* has been found to be a common contaminant of mouse colonies at research institutions. Although commercial breeders produce *H. hepaticus*-free animals, there are still many mouse colonies at public and private research institutions that harbor *H. hepaticus*. A recent survey found *H. hepaticus*-infected mice in 59% of commercial and academic institutions in Canada, Europe, Asia, Australia, and the United States (27).

*H. hepaticus* persistently colonizes the hepatic bile canaliculi and the cecal and colonic mucosa of mice (9, 31). Infection can cause chronic active hepatitis, hepatocellular neoplasms and typhlocolitis which vary in severity depending on the strain, age, gender, and immune status of the mouse (5, 9, 11, 31). In adult immunocompetent mice, *H. hepaticus* infection is usually asymptomatic. However, immune-dysregulated mice can develop inflammatory bowel disease, which may present as rectal prolapse and/or diarrhea (14).

Mice initiate immune responses against *H. hepaticus* primarily via its interaction with Toll-like receptor 2 present on the antigen presenting cells (18). Both systemic as well as local (at the site of infection) *H. hepaticus*-specific Th1-type immune responses are induced in immunocompetent mice (22, 32). Systemic antibody and cell-mediated immunity against the bacteria persist for at least 46 weeks following experimental
inoculation (32). Gene expression profiles of cecal tissue of *H. hepaticus*-infected mice have shown that inflammatory responses differ depending upon the mouse strain. For example, A/JCr mice had significant and prolonged expression of Th1-type cytokines IFN-γ and IL-12p40 in cecal mucosa, which persisted for at least 3 months after *H. hepaticus* infection. However, C57BL/6 mice had a lesser elevation of IFN-γ gene expression without an effect on IL-12p40. IFN-γ expression waned by 1 month post-inoculation in C57BL/6 mice (21). *H. hepaticus*-specific secretory IgA antibodies are also persistently detected in the feces of mice (32). It is unknown how these immune responses in *H. hepaticus*-infected mice might impact immunological research.

The goal of this study was to determine if herpes simplex virus type 1 (HSV-1)-specific immune responses and characteristics of dendritic cells (DCs) are altered in *H. hepaticus*-infected mice. Intranasal HSV-1 infection is a viral infection model widely used to study immune mechanisms in mice. Immunity to HSV-1 consists of virus neutralizing antibodies in the serum and virus-specific T cells in the draining LNs. Superficial cervical and mediastinal LNs have been described as draining LNs for intranasal HSV-1 infection (2). The peak response to HSV-1 infection is present at 7 days post infection, which leads to clearance of the viral load (2). In this study, we compared the levels of HSV-1-specific antibody and T cell-mediated immune responses in *H. hepaticus*-infected and non-infected mice.

DCs are important components of the immune system that play a role in antigen processing and presentation. Upon exposure to foreign antigen, DCs mature and express higher levels of MHC II, CD40, CD80, and CD86 on their surface. These maturation-associated surface markers interact with naive T and B cells to initiate antibody and cell-
mediated immune responses against the foreign antigen (23). Mature DCs also secrete proinflammatory cytokines, including TNF-α and IL-12p40. These cytokines lead to increased vascular permeability, complement activity, lymphocyte activation, lymphocyte proliferation, and increased antibody production (23). To determine if infection with *H. hepaticus* affects characteristics of DC, we measured the expression of maturation-associated surface markers CD40, CD80, CD86, and MHC II and proinflammatory cytokines IL-12p40 and TNF-α by DCs derived from the spleen and colic lymph nodes of *H. hepaticus*-infected and non-infected mice. Our findings indicate that *H. hepaticus* infection can influence the results of immunological research.
**Materials and Methods**

**Animals** – 3-4 week-old male, specific pathogen-free, C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in individually ventilated microisolation cages with sterile food, bedding, and water in an AAALAC International-accredited animal facility. All use of animals was approved by the Penn State Hershey Institutional Animal Care and Use Committee according to standards put forth in the *Guide for the Care and Use of Laboratory Animals*. All cage changes and animal manipulations were conducted in a class II biosafety cabinet following strict precautions to prevent cross contamination. All of the mice were maintained in a specific pathogen-free environment and were confirmed to be serologically negative for mouse hepatitis virus, minute virus of mice, mouse parvovirus, mouse norovirus, Sendai virus, *Mycoplasma pulmonis*, Theiler's murine encephalomyelitis virus, mouse rotavirus, pneumonia virus of mice, reovirus 3, lymphocytic choriomeningitis virus and ectrromelia virus at the conclusion of the experiments.

**H. hepaticus infection** – *H. hepaticus* ATCC 51449 was grown on chocolate agar or in brain heart infusion broth with 10% (v/v) fetal bovine serum (FBS) under microaerobic conditions obtained by flushing sealed jars with anaerobic gas (90% N₂, 5% CO₂ and 5% H₂). Mice were gavaged with 10⁹ colony forming units (CFUs) of *H. hepaticus* in 0.2 ml of phosphate-buffered saline (PBS). Control mice received 0.2 ml of PBS alone. Feces was collected one week post-inoculation and at the time of euthanasia to confirm the infection status of the mice using *Helicobacter* fecal PCR as previously described (3)
with primers 5’-CTATGACGGGTATCCGGC-3’ and 5’-ATTCCACCTACCTCTCCCA-3’ (25).

**HSV-1 infection** – HSV-1 strain McIntyre was used in this study. The stocks of HSV-1 were prepared by infection of Vero cells as previously described (1) and stored at -70°C. Viral titer of the stocks was assessed by plaque assay using Vero cells. Four weeks after *H. hepaticus* infection, mice were infected intranasally with 1x10^7 plaque forming units (PFUs) of HSV-1 in 20 µl of PBS containing 1% (v/v) FBS. For intranasal infection, mice were sedated briefly using isoflurane.

**Isolation of cells from LNs** – One week after the HSV-1 infection, mice were euthanized by exsanguination after sedation with isoflurane. Superficial cervical LNs were collected from the upper poles of the submandibular salivary glands (20, 28) and tracheobronchial LNs were collected from the tracheal bifurcation (30). The collected LNs were placed in Iscove’s-modified Dulbecco’s media (Gibco, Carlsbad, California) supplemented with 10% (v/v) FBS, 50 µM 2-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate (supplemented IMDM). A single cell suspension of the collected LNs was made as described previously (2) by mechanical dissociation and passage through a 70 µm nylon cell strainer (BD Biosciences, San Jose, CA). Trypan blue exclusion assay was used to determine the number of viable cells.

**Tetramer assay** – This assay was performed for the detection and quantification of the HSV-1-specific CD8^+ T cells in the LNs of HSV-1-infected mice (*H. hepaticus*-infected
and non-infected mice). Mice that were non-infected with either agent were used as negative controls. Cells isolated from LNs were incubated with a phycoerythrin (PE)-labeled tetramer as described previously (2). This tetramer is a complex of four gB_{498-505} peptides and four H-2K^{b}-restricted T cell receptors, linked to each other by streptavidin-biotin. gB_{498-505} peptide is an HSV-1-specific immunodominant epitope in C57BL/6 mice (4). Briefly, CD16/CD32 Fcγ receptors on isolated mononuclear cells were blocked with antibody from a 2.4G2 hybridoma cell culture supernatant supplemented with 20% (v/v) mouse serum (Sigma-Aldrich Inc. Saint Louis, MO). Cell surface expression of CD8 was detected using Alexa Fluor® 647-conjugated anti-CD8 antibody (clone 53-6.7; eBioscience, San Diego, CA). Following washes with FACS buffer (PBS supplemented with 1% [v/v] FBS, 0.02% [w/v] sodium azide), cells were resuspended in 2% (w/v) paraformaldehyde (prepared in PBS) prior to analysis by flow cytometry. Flow cytometry was performed using a FACS Calibur (Becton Dickinson, San Diego, CA) and data were analysed using Cell Quest software (Becton Dickinson, San Diego, CA).

**Intracellular cytokine staining for detection of IFN-γ** – This assay was performed to detect the IFN-γ-producing, HSV-1-specific CD8^{+} T cells in the LNs of HSV-1-infected mice (H. hepaticus-infected and non-infected mice) as described previously (2). LN-derived cells were re-suspended in supplemented IMDM and incubated with 1 µM gB_{498-505} peptide for 2 hours at 37°C. As a control, cells were stimulated with ovalbumin amino acid residues 257-264 (OVA_{257-264}; SIINFEKL) peptide. Cells were treated with Golgi plug™ (BD Biosciences, San Jose, CA) (final concentration 1 µg/ml) to prevent the secretion of cytokines and incubated for an additional 4 hours at 37°C. Cells were then
washed twice with FACS buffer and the CD16/CD32 Fcγ receptors blocked with 2.4G2 cell culture supernatant supplemented with mouse serum. To identify CD8+ T lymphocytes, cells were incubated with Alexa Fluor® 647-conjugated anti-CD8 antibody. Following staining for CD8, cells were permeabilized in BD cytofix/cytoperm solution (BD Biosciences, San Jose, CA) and incubated on ice for 20 minutes in the dark. Cells were then washed twice with 1X BD perm/wash buffer (BD Biosciences, San Jose, CA) and incubated with anti-IFN-γ-fluorescein isothiocyanate (FITC)-conjugated antibody (clone XMG1.2; eBioscience, San Diego, CA) diluted in 1X BD perm/wash buffer. Subsequently, cells were washed with 1X BD perm/wash buffer and then with FACS buffer. Finally, cells were fixed in 2% paraformaldehyde, and analyzed by flow cytometry. Flow cytometry was performed using a FACS Calibur and data were analysed using Cell Quest software.

**CD107a degranulation assay** – This assay was performed to detect and quantify HSV-1-specific cytotoxic CD107+/CD8+ T cells in HSV-1-infected mice (*H. hepaticus*-infected and non-infected mice) as described previously (2). Briefly, cells from the LNs were incubated in 96-well plates (5 x 10^5 cells/well) in the presence of 10 μM gB<sub>498−505</sub> or OVA<sub>257−264</sub> peptide at 37°C. Wells also contained FITC-conjugated anti-CD107a (1D4B; BD Pharmingen, San Jose, CA). After 1 hr, 10 mM NH₄Cl was added to cells to prevent endosome acidification. Three hours later, the cells were stained with PE-conjugated anti-I-A<sup>b</sup> (AF6-120-1; BD Pharmingen, San Jose, CA) and Alexa Fluor® 647-conjugated anti-CD8 antibody. Cells were stained for I-A<sup>b</sup> to exclude CD8α<sup>+</sup> DCs that constitutively undergo endocytosis and take up anti-CD107a. Flow cytometry was performed using a
FACS Calibur and data were analysed using Cell Quest software. Analyses were performed on the CD8⁺, I-Aᵇdim population of cells.

**Virus neutralization assay** – Blood samples were collected via cardiac puncture from anesthetized mice one week after HSV-1 infection. The amount of serum neutralizing antibody against HSV-1 was determined by plaque reduction assay as described previously with minor modifications (6, 10). Two-fold serial dilutions of serum from HSV-1-infected mice (*H. hepaticus*-infected and non-infected mice) were prepared in PBS containing 1% FBS, and incubated with an equal volume of HSV-1 suspension (112 PFU) for 30 minutes at 37 °C. Sera from mice that were not infected with either HSV-1 or *H. hepaticus* were used as the negative controls. The mixture was then used to infect monolayers of Vero cells. The cells were incubated with the mixture at 37°C for 1 hour and then overlaid with 1% (w/v) methylcellulose in 1X Eagles medium without phenol red supplemented with 5% (v/v) heat-inactivated FBS, 2mM L-glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin sulfate. The plates were incubated at 37°C for 4-5 days. At the end of the incubation period, cells were stained and fixed with 5% formaldehyde (v/v) and 0.5% (w/v) crystal violet solution. The number of viral plaques was counted.

**Isolation of cells from spleens and colic LNs** – Four weeks after *H. hepaticus* infection or PBS inoculation, mice were sedated with isoflurane for blood collection and euthanized by cervical dislocation for collection of spleens and colic LNs. Colic LNs, a subset of the mesenteric LNs, were collected from the mesocolon at the transition
between the ascending colon and the transverse colon (30). Spleen and colic LNs were enriched for DCs as described previously with minor modifications (12). Spleen and colic LNs were placed in Hank's-buffered salt solution without calcium and magnesium (HBSS) (Invitrogen, Carlsbad, CA) supplemented with 0.1% (w/v) bovine serum albumin (BSA) (Roche, Indianapolis, IN) at room temperature. Colic LNs from three mice were pooled together and treated as one sample whereas spleen from each mouse was used as a single sample. Spleen and colic LNs were treated with 1mg/ml of collagenase D (Roche, Indianapolis, IN) in HBSS for 30 minutes at 37°C in a 5% CO₂ incubator. To stop the collagenase D digestion, organs were suspended in IMDM supplemented with 10% (v/v) FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 ug/ml streptomycin sulfate. Single-cell suspensions were obtained by mechanical dissociation and passage through a 40 μm nylon cell strainer (BD Biosciences, San Jose, CA). Splenocytes were then treated with ACK lysis buffer (Lonza BioWhittaker, Walkersville, MD) for RBC lysis. The number of viable cells was determined by trypan blue dye exclusion assay.

**Lipopolysaccharide (LPS) treatment of cells from spleen and colic LNs for DC assay**

– 1 x 10⁶ splenocytes and colic LN cells from *H. hepaticus*-infected and non-infected mice were stimulated with 1μg/ml *Escherichia coli* 055:B5 LPS (Sigma, Saint Louis, MO) added to medium. Cells were stimulated with LPS for 16 hrs at 37°C in a 5% CO₂ incubator. As a control, an equal number of cells were incubated for the same period in medium alone.
Phenotypic analysis of surface markers on DCs – DCs derived from spleen and colic LNs of *H. hepaticus*-infected and non-infected mice, were analyzed for maturation-associated surface markers, before and after *in vitro* LPS stimulation, as described previously with minor modifications (8). Fc receptors were blocked with anti-mouse CD16/32 (eBioscience, San Diego, CA) antibody in FACS buffer. Cells were then stained with various combinations of directly-labeled antibodies: FITC-conjugated anti-CD40 (HM40-3), Phycoerythrin-Cy7 (PE-Cy7)-conjugated anti-CD90.1 (HIS51) and PerCP-Cy5.5-conjugated anti-CD11c (N418) from eBioscience, San Diego, CA; FITC-conjugated anti-B7.2 (CD86, GL1), PE-conjugated anti-I-A<b>^b^</b> (MHC II) (AF6-120.1), allophycocyanin (APC)-conjugated anti-B7.1 (CD80, 16-10A1), PE-CyTM7-conjugated anti-NK-1.1 (PK136) and PE-CyTM7-conjugated anti-CD19 (1D3) from BD Biosciences, San Jose, CA. Flow cytometry was performed on a FACS Canto (Becton Dickinson, San Diego, CA), and data were analyzed using BD FACS Diva Software (Becton Dickinson, San Diego, CA) or FlowJo software (TreeStar, Ashland, OR). DCs were identified as CD11c^+^, NK1.1<sub>low</sub>, CD19<sub>low</sub> and CD90.1<sub>low</sub> for analysis.

The CD11c^+^ DC population was recovered from spleen and colic LNs following collagenase treatment as described previously (12). CD11c^+^ DCs obtained by this method can be contaminated with natural killer cells, immature and mature B cells. These contaminating cells were removed from the CD11c^+^ population by negative selection of CD19^+^ mature B cells, CD90.1<sup>+</sup> immature B cells and NK1.1^+^ natural killer cells.

Intracellular staining for detection of cytokines produced by DCs – For detection of the proinflammatory cytokines produced by DCs after LPS stimulation, intracellular
staining was done as described above for IFN-γ. To prevent secretion of cytokines into
the culture supernatant, spleen and colic LN cells were treated with Golgi plug™ (final
concentration 1 µg/ml) for the last 8 hours of stimulation. DCs were identified as
CD11c⁺, NK1.1low, CD19low and CD90.1low using PerCP-Cy5.5- conjugated anti-CD11c,
PE-Cy™7-conjugated anti-NK-1.1, PE-Cy™7-conjugated anti-CD19 and PE-Cy™7-
conjugated anti-CD90 antibodies. For detection of proinflammatory cytokines, PE-
conjugated anti-IL-12/IL-23p40 (C17.8) (eBioscience, San Diego, CA) and APC-
conjugated anti-TNF-α (MP6-XT22) (BD Biosciences, San Jose, CA) antibodies were
used. Flow cytometry was performed on a FACS Canto and data were analyzed using BD
FACS Diva Software or FlowJo software.

**Statistical Analysis** – Statistical significance was determined with the tw- tail, unequal
variance t-test. Comparisons between groups were performed and p values < 0.05 were
considered to be significant.
Results

**Immune responses against intranasal HSV-1 infection** – We compared the HSV-1-specific antibody and T cell-mediated immune responses between *H. hepaticus*-infected and non-infected mice 1 week after intranasal HSV-1 infection. To determine the magnitude of HSV-1-specific antibody immune responses, we tested sera from mice using the plaque reduction assay. All sera from mice infected with HSV-1 contained anti-HSV-1 antibodies as indicated by a statistically significant decrease in the number of viral plaques when compared to sera from HSV-1-naive control mice (*p* < 0.05) (Figure 1). However, there was no difference between the numbers of plaques obtained with sera from *H. hepaticus*-infected and non-infected mice (Figure 1) indicating that *H. hepaticus* infection did not alter the magnitude of the antibody response to HSV-1.

To evaluate cell-mediated immune responses to HSV-1 infection, we determined the number of HSV-1-specific CD8$^+$ T cells in the draining LNs of mice. These cells were detected and quantified based on their ability to bind the HSV-1-specific peptide, gB$_{498-505}$ in the tetramer assay. In superficial cervical LNs, we found no difference in the percentage or absolute numbers of gB$_{498-505}$ peptide-specific CD8$^+$ T cells between *H. hepaticus*-infected and non-infected mice (Figure 2). In tracheobronchial LN, there was no difference in the percentage of gB$_{498-505}$ peptide-specific CD8$^+$ T cells between the two groups of mice (Figure 2A). However, we noticed a trend towards a decrease (*p* =
0.11) in the absolute number of gB_{498-505} peptide-specific CD8^{+} T cells in tracheobronchial LNs of *H. hepaticus*-infected mice (Figure 2B).

To compare the function of HSV-1-specific T cell-mediated immune responses between *H. hepaticus*-infected and non-infected mice, we quantified IFN-γ-producing HSV-1-specific CD8^{+} T cells in the draining LNs. As was expected, the percentage as well as absolute numbers of IFN-γ-producing CD8^{+} T cells in the superficial cervical and tracheobronchial LNs were higher (*p < 0.05*) when cells were stimulated with gB_{498-505} peptide as compared to OVA_{257-264} peptide, indicating that this was a HSV-1-specific immune response (Figure 3). In superficial cervical LNs, there was no difference in the percentage or absolute number of IFN-γ-producing gB_{498-505} peptide-specific CD8^{+} T cells between the two groups (Figure 3). In tracheobronchial LNs, there was no difference in the percentage of IFN-γ-producing gB_{498-505} peptide-specific CD8^{+} T cells in either group of mice (Figure 3A); however we noticed a decreasing (*p = 0.13*) trend in the absolute number of these cells in *H. hepaticus*-infected mice (Figure 3B).

To further evaluate the functional activity of HSV-1-specific T cells we used a CD107a degranulation assay. CD107a is present on the inner membrane of cytotoxic granules of T cells. In the process of degranulation in response to exposure to specific antigen, the membrane of the granule and the cell membrane fuse and CD107a is exposed on the surface of T cells. Thus, surface expression of CD107a can be used as a marker of antigen-specific cytotoxic T cells. As was expected, both the percentage and absolute numbers of CD107a^{+}/CD8^{+} T cells in the draining LNs was higher (*p < 0.05*) when cells were stimulated with gB_{498-505} peptide as compared to OVA_{257-264} peptide, indicating
that this response was a HSV-1-specific immune response (Figure 4). In the superficial cervical LNs, there was a trend toward a higher \((p = 0.07)\) percentage of \(gB_{498-505}\)-specific CD107a\(^+/\)CD8\(^+\) T cells in \(H. hepaticus\)-infected mice as compared to non-infected mice (Figure 4A). However, there was no difference in the absolute numbers of these cells in the superficial cervical LNs of either group of mice (Figure 4B). In tracheobronchial LNs, there was no difference in the percentage of \(gB_{498-505}\)-specific CD107a\(^+/\)CD8\(^+\) T cells between \(H. hepaticus\)-infected and non-infected mice (Figure 4A). However, we noticed a trend toward a decrease \((p = 0.07)\) in the absolute number of these cells in \(H. hepaticus\)-infected mice (Figure 4B).

Based on the assays that we used, we found that \(H. hepaticus\) infection had no statistically significant effect on the HSV-1-specific antibody and T cell-mediated immune responses in the superficial cervical and tracheobronchial LNs of HSV-1-infected mice. However, in tracheobronchial LNs, a decreasing trend \((0.07 \leq p \leq 0.13)\) in HSV-1-specific T cell-mediated immune responses was noticed in \(H. hepaticus\)-infected mice. In superficial cervical LNs, an increasing trend \((p = 0.07)\) in HSV-1-specific cytotoxic T cells was noticed in \(H. hepaticus\)-infected mice.
Figure 1 – Comparison of HSV-1-specific antibody responses between *H. hepaticus*-infected and non-infected mice. Virus neutralizing antibodies were quantified using the plaque reduction assay. Mean (± SE) PFUs of HSV-1 after neutralization of virus with 2-fold serial dilutions of sera obtained from HSV-1-infected mice. Sera from HSV-1-naive mice were used as control. n = 10 mice per group.
**Figure 2** – Quantification of HSV-1-specific CD8$^+$ T cells in superficial cervical and tracheobronchial LNs of *H. hepaticus*-infected and non-infected mice one week after intranasal HSV-1 infection. (A) Percentage of gB$_{498-505}$-specific CD8$^+$ T cells expressed in terms of total population of CD8$^+$ T cells and (B) absolute numbers of gB$_{498-505}$-specific CD8$^+$ T cells. Mean (± SE) are normalized to the percentage or absolute number of gB$_{498-505}$-specific CD8$^+$ T cells in LNs from non-infected mice within each experiment (n = 5 mice per group). Results were combined from 2 separate experiments. *p* value is from the paired t-test.
Figure 3 – Quantification of IFN-γ-producing, HSV-1-specific CD8+ T cells in the superficial cervical and tracheobronchial LNs of *H. hepaticus*-infected and non-infected mice after *in vitro* stimulation with OVA257-264 or gB498-505 peptide. (A) Percentage of IFN-γ-producing CD8+ T cells expressed in terms of total population of CD8+ T cells and (B) absolute number of IFN-γ-producing CD8+ T cells. Mean (± SE) are normalized to percentage or absolute number of IFN-γ-producing CD8+ T cells after gB498-505 peptide stimulation of lymphoid cells from non-infected mice within each experiment (n = 5 mice per group). Results were combined from 2 separate experiments. *p* value is from the paired t-test.
**Figure 4** – Quantification of HSV-1-specific CD107⁺/CD8⁺ cytotoxic T cells in the cervical and tracheobronchial LNs of *H. hepaticus*-infected and non-infected mice after *in vitro* stimulation with OVA_{257-264} or gB_{498-505} peptide. (A) Percentage of CD107⁺/CD8⁺ T cells expressed in terms of total population of CD8⁺ T cells and (B) absolute number of CD107⁺/CD8⁺ T cells. Mean (± SE) are normalized to the percentage or absolute number of CD107⁺/CD8⁺ T cells after gB_{498-505} peptide stimulation of lymphoid cells from non-infected mice within each experiment (n = 5 mice per group). Results were combined from 2 separate experiments. *p* values are from the paired t-test.
**Characteristics of DCs from colic LNs** – We measured the expression of maturation-associated surface markers CD40, CD80, CD86, and MHC II on the DCs derived from colic LNs of *H. hepaticus*-infected and non-infected mice by flow cytometry. Surface expression of CD40, CD86 and MHC II was significantly lower on DCs derived from *H. hepaticus*-infected mice as compared to DCs from non-infected mice (*p* < 0.05) (Figure 5A). Representative histograms showing the surface staining profile of CD11c^+^ DC population are presented in Figure 5B. Since the expression of these markers increase with maturation, this data suggest that *H. hepaticus* infection inhibits maturation of DCs in the colic LNs of mice. There was no difference in surface expression of CD80 between the two groups of mice.

We also measured the expression of maturation-associated surface markers on the DCs after *in vitro* LPS stimulation to induce their maturation. After LPS stimulation, DCs from both *H. hepaticus*-infected and non-infected mice showed an increase (*p* < 0.05) in surface expression of CD40, CD80 and CD86 in comparison to stimulation with medium alone (Figure 6). However, no difference was found between *H. hepaticus*-infected and non-infected mice (Figure 6). There was no increase in surface expression of MHC II in either group of mice when cells were stimulated with LPS (Figure 6). These data overall suggest that *H. hepaticus* has an inhibitory effect on the expression of CD40, CD86 and MHC II by DCs of the colic LN *in vivo* but that the effect is not permanent and is overcome when the DCs are exposed to proinflammatory stimuli *in vitro*.

We compared the expression of proinflammatory cytokines IL-12p40 and TNF-α by the DCs derived from the colic LNs of *H. hepaticus*-infected and non-infected mice
after *in vitro* stimulation with medium or LPS. There was no difference in the fluorescence intensity of IL-12p40 and TNF-α-positive DCs between the two groups of mice (data not shown). As expected, the percentage of IL-12p40- and TNF-α-producing DCs was higher after LPS stimulation compared to medium stimulation (Figure 7A). Interestingly, we found a lower percentage of IL-12p40 and TNF-α-producing DCs in the colic LNs of *H. hepaticus*-infected mice than in non-infected mice after medium and LPS stimulation (*p* < 0.05) (Figure 7A). Representative dot plots depicting the IL-12p40 and TNF-α-producing DCs are shown in Figure 7B. This is additional evidence that *H. hepaticus* inhibits maturation of DCs in the colic LNs. However, in the case of these proinflammatory cytokines, unlike the maturation-associated surface markers, the effect persists after the cells are collected from the mice and stimulated *in vitro*. 
Figure 5 – Expression of maturation-associated surface markers on DCs derived from the colic LNs. (A) Geometric mean fluorescence intensity (GMFI) (± SE) is normalized to the level of surface expression on DCs from non-infected mice (% baseline GMFI) within each experiment (n = 5-6 mice per group). Results were combined from 3 separate experiments. (B) Representative histograms depicting the staining profile for surface markers on DCs of *H. hepaticus-*infected (bold line) and non-infected (shaded area) mice. Dotted line represents unstained control. Asterisks indicate a significant difference ($p < 0.05$) between the indicated groups.
Figure 6 – Expression of maturation-associated surface markers by DCs derived from colic LNs of *H. hepaticus*-infected and non-infected mice after *in vitro* medium or LPS stimulation. GMFI (± SE) is normalized to the level of surface expression on DCs from non-infected mice after medium stimulation (% baseline GMFI) within each experiment (n = 5-6 mice per group). Results were combined from 3 separate experiments. Asterisks indicate a significant difference (*p* < 0.05) between the indicated groups.
**Figure 7** – Intracellular proinflammatory cytokine expression by DCs in the colic LNs of *H. hepaticus*-infected and non-infected mice after *in vitro* medium or LPS stimulation.

(A) Mean (± SE) percentage of cytokine-producing DCs is normalized to the percentage of cytokine-producing cells from non-infected mice after medium stimulation within each experiment (n = 5-6 mice per group). Results were combined from 3 separate experiments. (B) Representative dot plots depicting the intracellular staining profile for IL-12p40 and TNF-α in CD11c⁺ DCs population. Asterisks indicate a significant difference (p < 0.05) between the indicated groups.
Characteristics of the DCs from the spleen – When we measured the expression of maturation-associated surface markers on DCs derived from the spleen, in contrast to colic LNs, we found no difference in levels of CD40, CD80, CD86 and MHC II between *H. hepaticus*-infected and non-infected mice (Figure 8). *In vitro* LPS stimulation led to the expected increase (*p* < 0.05) in surface expression of CD40, CD80 and CD86 on DCs from both group of mice, however, increased expression was not seen for MHC II (Figure 9A). Surface expression of CD40, CD80, CD86 and MHC II reached higher (*p* < 0.05) levels on splenic DCs from *H. hepaticus*-infected mice than non-infected mice after LPS stimulation (Figure 9A). Expression of CD86 was higher in *H. hepaticus*-infected mice after medium stimulation as well (*p* < 0.05) (Figure 9A). Representative histograms of expression of surface markers on DCs are shown in Figure 9B.

As expected, LPS stimulation led to an increase (*p* < 0.05) in the percentage of TNF-α-producing DCs in the spleens of both groups of mice (Figure 10). However, there was no difference in either the percentage of TNF-α-producing DCs or the fluorescence intensity of those cells in *H. hepaticus*-infected or non-infected mice (Figure 10).
**Figure 8** – Expression of maturation-associated surface markers on DCs derived from the spleens of *H. hepaticus*-infected and non-infected mice as measured by flow cytometry. GMFI (± SE) is normalized to the level of surface expression on DCs from spleens of non-infected mice (% baseline GMFI) within each experiment (n = 5-6 mice per group). Results were combined from 3 separate experiments.
Figure 9A – Expression of maturation-associated surface markers on DCs derived from the spleens of *H. hepaticus*-infected and non-infected mice after *in vitro* stimulation with medium or LPS. GMFI (± SE) is normalized to the level of surface expression on DCs from non-infected mice after medium stimulation (% baseline GMFI) within each experiment (n = 5-6 mice per group). Results were combined from 3 separate experiments. Asterisks indicate a significant difference (p < 0.05) between the indicated groups.
Figure 9B – Expression of maturation-associated surface markers on DCs derived from the spleens after *in vitro* LPS stimulation. Representative histograms show DCs from *H. hepaticus*-infected (bold line) and non-infected mice (shaded area) after LPS stimulation. Dotted line represents unstained controls.
Figure 10 – Intracellular proinflammatory cytokine expression by DCs derived from the spleens of *H. hepaticus*-infected and non-infected mice after *in vitro* stimulation with medium or LPS. Mean (± SE) of percentage of TNF-α-producing DCs is normalized to the percentage of TNF-α-producing cells from non-infected mice after medium stimulation within each experiment (n = 5-6 mice per group). Results were combined from 3 separate experiments. Asterisks indicate a significant difference (p < 0.05) between the indicated groups.
Discussion

Although it has been 15 years since the first reports of *H. hepaticus* were published, infections with *H. hepaticus* or related enterohepatic *Helicobacter* species remain common in mouse colonies at research facilities. Commercial breeders produce *Helicobacter*-free animals, but for a variety of reasons – expense being paramount – non-commercial facilities often still have many strains of *Helicobacter*-infected mice. The primary method of monitoring for *Helicobacter*, PCR of feces, is more costly than serological monitoring used for the common viral infections. Elimination of *Helicobacter* by rederivation, even by a non-surgical method such as neonatal transfer, is time consuming and expensive.

We initiated this study to determine whether and how *H. hepaticus* infection could influence immunological studies at our institution. We employed sensitive flow cytometry-based assays to measure specific markers and functional characteristics of cells of the immune system. We chose a mouse strain commonly used in immunological research, the C57BL/6 strain, to increase the general applicability of our results. The C57BL/6 mouse strain is considered relatively resistant to development of lesions when infected with *H. hepaticus* (31).
*H. hepaticus* infection did not have a significant effect on the antibody or T cell-mediated immune responses against HSV-1 infection in mice (Figure 1, 2, 3 and 4). After intranasal inoculation, the virus replicates in the nasal mucosa. It then travels via peripheral nerves to the brain and spinal cord. C57BL/6 mice are resistant to HSV-1-induced lesions and the infection is cleared in 7 days by virus-specific, cell-mediated immunity (2). The advantage of using the HSV-1 model is that it is a very well characterized model of T cell-mediated immune responses. However, if a viral infection model that infected the gut or liver had been used, for example parvovirus or mouse hepatitis virus, it is possible we would have seen a difference in specific antibody and T cell-mediated immune responses between *H. hepaticus*-infected and non-infected mice. In fact, co-infection of mouse hepatitis virus and *H. hepaticus* in mice has been shown to increase the severity of pathology associated with mouse hepatitis virus (7).

We find it interesting that, while not reaching the level of statistical significance, there was a decreasing trend in HSV-1-specific T cell-mediated immune responses in the tracheobronchial LNs of the *H. hepaticus*-infected mice. There were lower absolute numbers of HSV-1-specific CD8\(^+\) T cells, IFN-\(\gamma\)-producing CD8\(^+\) T cells and CD107\(^{\text{+}}\)/CD8\(^{\text{+}}\) cytotoxic T cells as compared to non-infected mice (Figure 2B, 3B and 4B). The \(p\) value came closest to statistical significance for the decrease in absolute numbers of HSV-1-specific CD107\(^{\text{+}}\)/CD8\(^{\text{+}}\) cytotoxic T cells, for which the \(p\) value was 0.07 (Figure 4B). If a larger number of mice had been used per group, the differences would possibly have reached statistical significance. This decreasing trend was not seen for the percentage of HSV-1-specific CD8\(^{\text{+}}\) T cells relative to the total number of CD8\(^{\text{+}}\) T cells counted. This suggests that there may have been a decrease in the overall number of
CD8+ T cells in the tracheobronchial LNs of *H. hepaticus*-infected mice. Rashidi et al. demonstrated that portal, celiac and mediastinal LNs receive lymphatic drainage from the liver of the mice (24). The mediastinal LNs shown in that work correspond to the cranial mediastinal LNs using the nomenclature of Van de Broeck et al. (30). Having found decreased maturation-associated surface markers on DCs obtained from LNs that drain the colon of *H. hepaticus*-infected mice, we consider the possibility that the cells we collected from tracheobronchial LNs could have been affected by some component of *H. hepaticus* in the liver, either by unintentional collection of some cranial mediastinal LNs, which are anatomically very close, or if tracheobronchial LNs receive some degree of lymphatic drainage from the liver.

In superficial cervical LNs, an increasing trend (*p* = 0.07) was observed in the percentage of HSV-1-specific CD107a+/CD8+ cytotoxic T cells in *H. hepaticus*-infected mice (Figure 4A). Since this finding involved the analysis of a single parameter, we do not find this as interesting as the trend in the tracheobronchial LNs. The superficial cervical LNs do not receive lymphatic drainage from tissues colonized by *H. hepaticus*.

We found that DCs derived from the colic LNs of *H. hepaticus*-infected mice had lower expression of maturation-associated surface markers CD40, CD86 and MHC II as compared to DCs derived from the colic LNs of non-infected mice (Figure 5). After *in vitro* medium or LPS stimulation, the percentages of IL-12p40 and TNF-α-producing DCs was lower in colic LNs of *H. hepaticus*-infected mice than non-infected mice (Figure 7). These findings indicate that *H. hepaticus* can inhibit functional responses of the immune system and is supported by an *in vitro* study by Sterzenbach et al. (26). They demonstrated that treatment of mouse intestinal crypt epithelial cells with *H. hepaticus*
bacterium, lysate and soluble component LPS reduced secretion of the pro-inflammatory chemokine, macrophage inflammatory protein-2. Furthermore, *H. hepaticus* lysate and soluble component LPS antagonized the stimulatory effect of *E. coli* LPS and flagellin on the immune responses of mouse intestinal crypt epithelial cells. The effects we observed on DCs may be important factors that allow *H. hepaticus* to evade the host immune response and persistently colonize the mouse gut. In contrast to our findings, Mcbee et al. reported an up-regulation of co-stimulatory molecules on DCs from mesenteric LNs of *H. hepaticus*-infected C57BL/6 mice (19). Unfortunately, we cannot compare their finding with ours because their study was published in abstract form only.

It is possible that the inhibitory cytokine IL-10 played a role in inhibition of DCs from colic LNs of *H. hepaticus*-infected mice. IL-10 has been shown to inhibit surface expression of CD80 and CD86 on murine bone marrow-derived DCs (17) and MHC II trafficking to the plasma membrane of antigen-presenting cells (15). Therefore, lower expression of CD86 and MHC II on the DCs derived from the colic LNs of *H. hepaticus*-infected mice (Figure 5) could have resulted from higher IL-10 production. We were not able to detect a difference in CD80 expression on DCs from the colic LNs, however, because surface expression of CD80 was below the detection limit by flow cytometry.

A greater level of IL-10 production could also account for the lower percentage of IL-12p40-producing DCs in the colic LNs of *H. hepaticus*-infected mice (Figure 7). IL-10 is known to inhibit expression of IL-12p40 gene transcription (34). The kinetics of IL-10 expression in the colic LNs of *H. hepaticus*-infected mice are not known but it has been shown that in the cecal mucosa, *H. hepaticus*-infected C57BL/6 mice have higher levels of IL-10 mRNA for up to 14 days post infection than do uninfected mice (21). IL-
10 has been shown to be important in maintaining low levels of IL-12p40 in the colon of 
*H. hepaticus*-infected C57BL/6 mice and in preventing the development of colitis (16). 
*H. hepaticus*-infected C57BL/6 mice deficient in IL-10 had higher expression of IL-
12p40 and developed colitis. Intestinal inflammation subsided when the latter mice were 
treated with anti-IL-12p40, demonstrating the importance of IL-12p40 to induction and 
maintenance of colitis. The inhibitory effect of IL-10 on IL-12p40 was mediated by 
p50/p105 subunit of NF-κβ (29).

Unlike DCs derived from the colic LNs, there was no decrease in the expression 
of maturation-associated surface markers on DCs derived from the spleen of *H. 
hepaticus*-infected mice (Figure 8). Furthermore, after *in vitro* LPS stimulation, DCs 
from the spleen of *H. hepaticus*-infected mice had higher levels of CD40, CD80, CD86 
and MHC II than did non-infected mice (Figure 9). One possible reason for the different 
responses of DCs from the two sources may be related to the different routes by which 
antigens reach the tissues. Antigens reach the spleen only through the blood stream (23) 
whereas the colic LNs receive lymphatic drainage directly from the large intestine, the 
major site of *H. hepaticus* colonization. Since it has been shown that the related human 
pathogen *H. pylori* translocates from the stomach to the draining gastric LNs (13), 
perhaps *H. hepaticus* can travel to the colic LNs to exert an inhibitory effect on DCs.

Another possible reason for the different responses is that the populations of DCs 
themselves differ between the LNs and spleen (33). In the spleen, 100% of the DCs are 
blood-derived or resident and are mostly immature with low levels of expression of MHC II. Whereas, in the LNs approximately 50% of the DCs are blood derived and rest of the 
DCs are migratory DCs. Migratory DCs travel from the peripheral tissues to the draining
LN;S; their maturation is triggered by encounter of pathogen products at the periphery. While the presence of a different DC population in itself would not explain higher expression of maturation-associated markers on the splenic-derived DCs from *H. hepaticus*-infected mice (Figure 9), it is possible that *H. hepaticus* induces some proinflammatory mediator that travels via blood from the site of infection to the spleen. Such a process could account for the differing effects of *H. hepaticus* infection we observed on DCs at the local level of the draining LN and the systemic level of the spleen. This possibility will require further investigation. It would be interesting to determine if a stimulatory effect can be observed on DCs from LNs that receive no lymphatic drainage from tissues colonized by *H. hepaticus*, such as superficial cervical or popliteal LNs.

On DCs from both the colic LNs and spleen, we observed a very high level of expression of MHC II with medium stimulation. We did not see up-regulation of MHC II after *in vitro* LPS stimulation (Figure 6 and 9A). If the MHC II expression level did increase after LPS stimulation, we were unable to detect it because expression levels were already near the upper limit of detection by flow cytometry.

In conclusion, we have shown that *H. hepaticus* infection of mice influence parameters of the immune system as measured by widely used assays. Future studies will be needed to determine the mechanisms by which this occurs. It is important to utilize *H. hepaticus*-free mice in order to prevent confounding variables from interfering with immunological research.
References


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APPENDIX

Review of literature

**Introduction** – *Helicobacter hepaticus* was described for the first time in 1994, as the cause of chronic active hepatitis, which was associated with high incidence of hepatocellular neoplasms in the A/JCr mice of long term toxicology study. *H. hepaticus* persistently colonized the hepatic bile canaliculi of mice, causing chronic active hepatitis and which was associated with a high incidence of hepatocellular neoplasms in infected A/JCr mice (51). *H. hepaticus* also colonized the cecal and colonic mucosa of SCID/NCr and A/JCr mice (14).

*H. hepaticus* is a Gram-negative, curved to spiral-shaped bacterium with bipolar-sheathed flagella. The bacterium grows at 37°C under microaerobic and anaerobic conditions, rapidly hydrolyzes urea, is catalase and oxidase positive, reduces nitrate to nitrite, and is resistant to cephalothin and metronidazole (14).

Although rodents from commercial breeders produce *H. hepaticus*-free animals, there are still many mouse colonies at public and private research institutions that carry *H. hepaticus*. A recent survey found *H. hepaticus*-infected mice in 59% of commercial and academic institutions tested in Canada, Europe, Asia, Australia, and the United States (45).
Pathology – *H. hepaticus* persistently colonized the hepatic bile canaliculi, cecal and colonic mucosa of mice (14, 51). In infected A/JCr mice, *H. hepaticus* was consistently isolated from the lower bowel but only sporadically from the liver. In liver, *H. hepaticus* was detected only in bile canaliculi. *H. hepaticus* infected mice, particularly males, developed chronic hepatitis; oval cell, Kupffer cell, and Ito cell hyperplasia; hepatocytomegaly; and bile duct proliferation. The inflammatory and necrotizing lesions were progressive and involved the hepatic parenchyma, portal triads, and intralobular venules. Hepatic adenomas developed, but only in male A/J mice. Infected mice developed sustained anti-*H. hepaticus* serum IgG antibody responses and elevated alanine aminotransferase levels (15).

*H. hepaticus* infection in SCID/NCr mice was characterized by hepatitis, proliferative typhlitis, colitis and these lesions were progressive with age. Liver lesions in SCID/NCr mice consisted of Kupffer, Ito, and oval cell hyperplasia along with multifocal to coalescing coagulative hepatocyte necrosis with minimal to mild accumulations of monocytic cells and neutrophils. Proliferative typhlitis in *H. hepaticus*-infected SCID/NCr mice was characterized by moderate to marked mucosal epithelial cell hyperplasia with mild monocytic and neutrophilic infiltration. *H. hepaticus*-infected SCID/NCr mice developed minimal to mild colitis with mucosal epithelial cell hyperplasia of the colon, which was most marked in older mice (28). Higher number of Warthin-Starry-positive *H. hepaticus* were observed in the liver parenchyma of SCID/NCr mice as compared to A/JCr mice (15, 28). *H. hepaticus* infected SCID/NCr mice did not develop serum IgG response against *H. hepaticus* as compared to A/JCr mice (28).
Impact on Research – In 1998, results from 12 national toxicology programs, 2-year carcinogenesis studies were confounded by *H. hepaticus* infection of the B6C3F1 mouse colonies. Many of the male B6C3F1 mice from 9 of these studies had *H. hepaticus*-associated hepatitis. The incidence of hepatocellular and hemangiosarcoma in male B6C3F1 mice was higher in affected studies as compared from unaffected studies. The incidence of hepatocellular neoplasms was higher in the males exhibiting *H. hepaticus*-associated hepatitis (24).

Colonization dynamics of altered Schaedler flora (ASF) was influenced by *H. hepaticus* infection in the intestines of immunocompetent Swiss-Webster mice (18). *H. hepaticus* infection led to increase in aerotolerant *Lactobacillus* species strain, ASF360 in the jejunum and ilea and decrease in *Clostridium* species strain, ASF502 in the cecum. The normal flora in the cecal mucosa of a mice changed significantly following *H. hepaticus* infection (26). Two days after *H. hepaticus* infection, *H. hepaticus* comprised a minor component of the mucosa-associated microbiota, but within 14 days, *H. hepaticus* became the dominant member of the microbial community.

*H. hepaticus* infection led to an increase in incidence of gall stone formation in immunocompetent C57BL/6 mice on a lithogenic diet (33). The incidence of cholesterol gall stone in *H. hepaticus*-infected mice was 40% higher than in non-infected mice. In another study, *H. hepaticus*-infected, immunocompetent, BALB/cJ mice displayed a statistically significant increase in cholesterol gallstone prevalence as compared to non-infected mice (81% vs. 39%) (34).
H. hepaticus infection can influence in vitro studies using cell lines. H. hepaticus infection of HEp-2 cell line influenced the expression of the 17 proteins by these cell lines (38). These 17 proteins participated in several biological functions including amino acid metabolism, cell growth and proliferation, stress response, protein translation and modification. H. hepaticus infection led to the enlargement, distension and elongation of HEp-2 cells. This shows that the presence of H. hepaticus has broad effects on the physiology of HEp-2 cells. Non-frozen tumor cell lines transmitted H. hepaticus to SCID mice, but the cryopreserved tumor cell lines did not (23). This finding suggests that H. hepaticus has the ability to spread via biomaterials and that freeze-thawing is able to reduce the numbers of organisms to levels insufficient to infect mice.

H. hepaticus infection of mice can alter the pathogenesis of other pathogenic organisms. Infection of gamma interferon-deficient mice with mouse hepatitis virus (MHV) induces a multisystemic infection, with multifocal hepatic necrosis, acute necrotizing and inflammatory lesions of the gastrointestinal tract, and acute peritonitis and pleuritis with adhesions on the serosal surfaces of the viscera. Although co-infection of gamma interferon-deficient mice with H. hepaticus and MHV induced lesions similar to those associated with MHV alone, the pathogenesis of the MHV infection was modified (8). H. hepaticus reduced the severity of MHV-induced lesions during the acute phase of infection, and exacerbated hepatitis and meningitis at the later time point.

Clinical Signs – Adult immunocompetent mice infected with H. hepaticus were usually asymptomatic. H. hepaticus infection in immune dysregulated mice caused inflammatory bowel disease leading to diarrhea and/or rectal prolapsed (50).
**Genetics** – Susceptibility to *H. hepaticus* infection depends on the genetic makeup of the mice. Mouse strains like A/JCr, BALB/CAnNCr, SJL/NCr, B6C3F1, SCID/NCr, and C3H/HeNCr were susceptible to *H. hepaticus* infection (14, 51). Among these strains of mice, immunocompetent A/JCr mouse strain and an immuno-deficient SCID/NCr strain were the most hepatitis-prone strains. C3H/HeNCr, SJL/NCr and BALB/cAnCr were susceptible to *H. hepaticus* induced hepatitis and hepatic tumors (49, 51). In B6C3F1 mice, *H. hepaticus* infection was associated with a higher incidence of hepatocellular carcinoma and hemangiosarcoma (24). On the other hand, C57BL/6NCr strain was very resistant to *H. hepaticus* induced hepatitis (51). Nonetheless, *H. hepaticus* bacterial load was more in the cecum of hepatitis resistant C57BL/6NCr mice as compared to hepatitis prone A/JCr mice (54). *H. hepaticus*-induced hepatitis has dominant inheritance. *H. hepaticus* infected F1 hybrid mice derived from susceptible, A/JCr and resistant, C57BL/6 matings (AB6F1 and B6AF1 mice) as well as parental strains developed significant hepatitis (17).

**Age** – In the immunocompetent susceptible mouse strain, A/JCr, *H. hepaticus* infection led to chronic hepatitis and hepatocellular carcinoma (15). The lesions became more severe with age in A/JCr mice and hepatocellular carcinoma was most prevalent 18 months after infection (15). The lesions associated with *H. hepaticus*-infection became more severe with age in various strains of mice (15, 16, 28).

**Zoonotic importance** – Human patients with chronic liver diseases had increased antibody levels to *H. hepaticus* as compared with the population and blood donors
without liver disease, indicating a possible role of *H. hepaticus* in the natural course of chronic liver diseases in humans (48).

**Immune responses** – *H. hepaticus* infection in A/JCr mice resulted in chronic active hepatitis characterized by perivascular, periportal, and parenchymal infiltrates of mononuclear and polymorphonuclear cells. A/JCr mice infected with *H. hepaticus* developed secretory IgA and systemic IgG2a antibody by 4 weeks post infection, which persisted through 12 months post infection. Splenocytes from infected mice proliferated and produced more IFN-γ than IL-4 or IL-5 when cultured with *H. hepaticus* outer membrane proteins. The predominantly IgG2a antibody response in the serum and the *in vitro* production of IFN-γ in excess of IL-4 or IL-5 were consistent with a Th1 type of immune response (55).

A Th1 type of mucosal immune response was produced by *H. hepaticus* infection in the cecal mucosa of A/JCr mice (37). There was up-regulation of Th1 type of immune related genes, including interferon-inducible protein 10, monokine induced by gamma interferon and macrophage-induced protein 1 alpha in the cecal mucosa of *H. hepaticus*-infected A/JCr mice as compared to non-infected mice. The expression of these proinflammatory genes in A/JCr mice was biphasic that corresponded to acute and chronic phase of disease (typhlitis) (36). The chronic mucosal inflammation in the cecum of *H. hepaticus*-infected mice was mediated by Th1 type of immune response as there was up-regulation of IL-12/23p40 and IFN-γ. Furthermore, treatment with anti-IL-12/23p40 and anti-IFN-γ antibodies resolved the inflammation in the cecum of *H. hepaticus*-infected A/JCr mice. Higher expression levels of Th1 type of cytokines, IFN-γ
and IL-12p40 in the cecal mucosa persisted until 3 month after *H. hepaticus* infection in AJ/Cr mice. However, C57BL/6 mice, which are resistant to *H. hepaticus* induced lesions, had higher expression of IFN-γ only, which waned at 1 month post-infection (36).

Sub-clinical *H. hepaticus* infection in mice had immuno-modulatory effects (35). *H. hepaticus* infection in C57/BL6 mice induced a Th1 type of immune response against the immuno-tolerant soluble ovalbumin, when administered orally. Also, *H. hepaticus* infection caused up-regulation of the co-stimulatory molecules on the dendritic cells isolated from the draining mediastinal lymph nodes.

Intact *H. hepaticus* bacteria activated innate immunity via Toll-like receptor 2 but not Toll-like receptor 4 (32). Macrophages from both wild-type and Toll-like receptor 4-deficient mice produced a robust cytokine secretion response (IL-6 and MCP-1) when stimulated with intact *H. hepaticus*. In contrast, macrophages from Toll-like receptor 2 -deficient mice were profoundly unresponsive to intact *H. hepaticus* stimulation, failing to secrete cytokines even at high (100:1) bacterium-to-macrophage ratios. This study suggests that Toll-like receptor 2 may be the dominant innate immune receptor for recognition of *H. hepaticus*.

*H. hepaticus* infection had an inhibitory effect on the innate immune responses of mouse intestinal epithelial cells (44). It was demonstrated that the innate immune responses of intestinal epithelial cells to lipopolysaccharide (LPS) via Toll-like receptor 4 and to flagellin-mediated activation via Toll-like receptor 5 were reduced by *H. hepaticus* infection through soluble bacterial factors. In particular, *H. hepaticus* lysate and the
soluble component LPS antagonized Toll-like receptor 4-and Toll-like receptor 5-mediated immune responses of intestinal epithelial cells.

**Model for Inflammatory bowel disease** – *H. hepaticus* infection in immuno-deficient mice induced inflammatory bowel disease that was manifested clinically as rectal prolapsed (50). Immuno-deficient mice developed various degrees of chronic proliferative typhlitis, colitis, and proctitis, usually without concomitant hepatitis. Immuno-deficient, IL-10 knockout mice infected with *H. hepaticus* developed severe colitis, which was associated with Th1 type of immune response (27). Il-12p40 played a crucial role in development of colitis in this model, as treatment with anti-IL-12p40 antibody led to marked reduction in *H. hepaticus*-induced colitis (27). Treatment with IL-10 prevented the development of colitis in recombinant activating gene-2 knockout (Rag-2\(^{+/-}\)) mice which was mediated by reduced expression of IL-12p40, TNF-α and IP-10 mRNA in the colon (46). The inhibitory effect of IL-10 on IL-12p40 was mediated by p50/p105 subunit of NF-κβ. SCID mice developed inflammatory bowel disease with adoptive transfer of CD4\(^{+}\) T cells expressing high levels of CD45RB (CD45RB(high) CD4\(^{+}\) T cells) (6). These mice did not develop inflammatory bowel disease in germfree conditions. The combination of *H. hepaticus* infection and CD45RB(high) CD4\(^{+}\) T-cell reconstitution resulted in severe disease expression similar to that observed in human inflammatory bowel disease.
**Male vs. Female** – Male A/JCr mice infected with *H. hepaticus* developed more severe lesions as compared to female A/JCr mice (15). At one month post infection with *H. hepaticus*, there was no significant difference in cecal lesion (typhlitis) between the female and male A/JCr mice. At 3 months after *H. hepaticus* infection, cecal lesion (typhlitis) were significantly more severe in female as compared to male A/JCr mice (29).

Colonization levels of *H. hepaticus* in the cecum and colon of male outbred Swiss Webster mice were approximately 1,000-fold higher than in female outbred Swiss Webster mice at 16 weeks post infection (19). Higher incidence of hepatocarcinogenesis in male mice with chronic hepatitis was maternally imprinted and androgen-independent (21). There was no significant difference in the hepatitis and hepatocarcinogenesis in four groups (surgical castration, chemical castration, castration followed by dihydrotestosterone supplementation, or sexually intact controls) of male A/JCr mice.

Differential susceptibility of male and female mice to *H. hepaticus* induced lesions was explained by difference in cytokine expression between male and female mice (29). At 1 month post *H. hepaticus* infection, female A/JCr mice expressed higher levels of IL-10 and macrophage inflammatory protein-1alpha (MIP-1α) in the cecal tissue as compared to infected male mice. At 3 months post infection, infected female A/JCr mice expressed significantly more IFN-γ, TNF-α, IL-10, MIP-1α, interferon-inducible protein of 10 kDa, and monokine induced by gamma interferon mRNA in the cecal tissue as compared to infected male mice.
**Pathogenic proteins of *H. hepaticus*** – *H. hepaticus* produces a cytolethal distending toxin (CDT) expressed by CdtA, CdtB, and CdtC genes. Inactivation of the CdtB gene abolished the ability of *H. hepaticus* to colonize jejunum, ileum, cecum, and colon of outbred Swiss Webster female mice at both 8 and 16 weeks post infection, whereas CdtB deficient *H. hepaticus* colonized jejunum, ileum, cecum, and colon of males at 8 weeks post infection and then was eliminated by 16 weeks post infection (19). Wild-type *H. hepaticus* was detected in the corresponding intestinal regions of both male and female mice at 8 and 16 weeks post infection. Even in IL-10 deficient mice CDT deficient *H. hepaticus* was no longer detectable at 4 month after infection (39). Whereas, wild type *H. hepaticus* persisted for 8 months in IL-10 deficient mice. IL-10 deficient mice infected with wild-type *H. hepaticus* exhibited severe typhlocolitis at 8 months after infection, while IL-10 deficient mice infected with the CDT-deficient mutant developed minimal cecal inflammation at 8 months after infection. It has been shown that CDT has immunomodulatory properties (21). A/JCr mice infected with wild type *H. hepaticus* had activation of the inflammatory NF-κB pathway and up-regulation of proinflammatory cytokines but not in the CDT-deficient mutant-infected mice.

A 70 kb pathogenomic island named HHG11, with low G + C content was found for *H. hepaticus* (5). Male A/JCr mice infected with HhG and HhNET developed less-severe hepatitis than infected with Hh3B1. *H. hepaticus*: type strain Hh3B1, contained the complete pathogenomic island, and strains HhNET and HhG, lacked all or large parts of HHG11, respectively.

Urease has been described as a pathogenomic and immuno-modulatory protein of *H. hepaticus* (20). In A/JCr mice, urease deficient *H. hepaticus* had reduced colonization
in liver as compared to wild type *H. hepaticus*. Urease-deficient *H. hepaticus* induced less severe hepatitis and lower hepatic mRNA levels of proinflammatory cytokines, IFN-γ and TNF-α as compared to the wild type *H. hepaticus*. Furthermore, urease-deficient *H. hepaticus* induced significantly lower total IgG, Th1-associated IgG2a and Th2-associated IgG1 immune responses as compared to the wild type *H. hepaticus*.

**Control of *H. hepaticus* infection in mouse colonies** – Caesarian delivery of pups or embryo transfer has been used to eliminate *H. hepaticus* infection from mouse colonies. These methods are costly and complex, and require special expertise, making them difficult to carry out on a large scale. Cross-fostering is a viable, economical method for rederivation of mouse colonies contaminated with pathogens such as *H. hepaticus*(52). Neonatal mice from *H. hepaticus*-infected dams were immersed in a dilute iodine solution and transferred to disease-free foster mothers within 48 h of birth (52). At 4 to 6 weeks after neonatal transfer, 95% of foster mothers (which served as sentinels for the transferred pups) tested free of *H. hepaticus*. In another study, pups fostered by this method remained free of *H. hepaticus* up to 89 days of age (47). Neonatal transfer of newborn mice onto *H. hepaticus*-free foster dams must be done within 24 hours of birth to remain free of *H. hepaticus* (43). The removal of the male prior to parturition extended the fostering period from 24 hours to 4 days to yield *H. hepaticus*-free mice (9).

Clearing of the mice colonies from *H. hepaticus* infection by treatment is possible but is very time consuming. An eight-week-long treatment of mice with medicated feed containing four antibiotic compounds (amoxicillin, clarithromycin, metronidazole, omeprazole) was effective in clearing *H. hepaticus* (25). In another study, amoxicillin or
tetracycline-based triple therapy (amoxicillin or tetracycline in combination with metronidazole and bismuth) given by oral gavage, 3 times daily for 2 weeks eradicated *H. hepaticus* in 8 to 10-week old A/JCr mice (12). Amoxicillin-based triple therapy administered in the diet or by oral gavage was effective in eradicating *H. hepaticus*. However, this amoxicillin-based triple therapy administered in the water was not effective in eradicating the *H. hepaticus* from the mouse colonies. Although, amoxicillin is commonly included in treatment regimen for *H. hepaticus* infection in mice, it is not effective against *H. hepaticus*. Recently, hefA, a *H. hepaticus* gene associated with amoxicillin resistance has been identified (2).

Horizontal transmission of *H. hepaticus* infection between the mice housed in close proximity can be prevented in a very economical way by the use of good husbandry practices (53). Use of microisolator caging, forceps to transfer mice, and adherence to the practice of changing only one cage at a time in a sequence that considers potential differences in the *H. hepaticus* infection status of different groups of mice prevented horizontal transmission of *H. hepaticus*. Individually ventilated and static microisolator cages were better than open top cages in controlling the spread of *H. hepaticus* infection (3). This control depends on the use of good husbandry practices. Using individually ventilated cage system, mice were maintained *H. hepaticus* free up to 12 months with *H. hepaticus*-infected mice in the adjacent cage.

Soiled bedding transfer from the colony cage to sentinel mice cage was an effective means to detect *H. hepaticus* infection (30). *H. hepaticus* infection in the sentinel mice was detected by fecal PCR at 2-4 weeks after the exposure. On the other
hand, *H. hepaticus* specific IgG antibodies were detected by ELISA at 4-6 weeks after the exposure of sentinel mice.

**Diagnostic tests** – Culture and PCR detection of *H. hepaticus* in the liver of mice was relatively more sensitive than detection by immunofluorescence-labelling or silver-staining of *H. hepaticus* in liver sections (13). *H. hepaticus* detection by fecal culture followed by filtering of fecal material through 0.45-micron-pore-size filter gave reproducible results but required extended periods of time of about 1 to 3 weeks to obtain a definitive answer (41). In 1995 Shames et al. developed a novel rapid, fecal PCR technique for *H. hepaticus* detection. In this technique, bacteria were isolated from fecal material in the presence of polyvinylpyropyrolidone and lysed by treatment with Chelex 100. The PCR was performed with Tth polymerase supplemented with a polymerase enhancer. Primers used in this assay were based on *H. hepaticus* specific conserved sequence region of the 16S r DNA. This PCR assay using Tth polymerase had a 100-fold increase in sensitivity over Taq polymerase.

A *Helicobacter* genus-specific PCR assay was developed by Riley et al. in 1996 (40). This PCR assay was based on 16S r RNA sequence and detected as little as 5 pg of *H. hepaticus, H. bilis, or H. muridarum* DNA in the cecal contents. The assay amplified the expected 374-base pair product from all three rodent *Helicobacter* species. However, this PCR based assay was not able to differentiate between *H. hepaticus, H. bilis, or H. muridarum*. So an extra step was included to differentiate between these species. *MboI, MaeI, and HhaI* restriction enzyme analyses of the amplified PCR product was able to differentiate among the murine *Helicobacter* species, *H. hepaticus, H. bilis, or H.*
muridarum. In 1997, Beckwith et al. demonstrated that PCR assay based on 16S r RNA can also detect Helicobacter in the feces of infected mice (1). The lower limit of detection of H. hepaticus, in the presence of fecal DNA was 100 fg. This fecal PCR assay was highly specific for Helicobacter species.

In 1999 Livingston et al. developed an ELISA to detect H. hepaticus (31). ELISA was based on the use of 44 kilo Dalton recombinant outer membrane protein of H. hepaticus as an antigen. ELISA was able to differentiate H. hepaticus from other Helicobacter species like H. muridarum and H. bilis. ELISA had similar specificity but lower sensitivity to another ELISA that used a detergent extract of H. hepaticus as the antigen. Both ELISAs performed with a high specificity (98%); however, the detergent extract-based ELISA performed with a higher sensitivity (89%) than the recombinant protein-based ELISA (sensitivity, 66%).

Restriction fragment-length polymorphism (RFLP) analysis followed by Helicobacter species specific PCR amplification of a 1,200-base pair DNA fragment coding for 16S rRNA was used to distinguish 11 enterohepatic Helicobacter species (42). RFLP assay involved restriction digestion by four restriction endonucleases: Alu I, Hinf I, Hha I, and Dde I.

H. hepaticus forms a spreading film on selective agar and is not amenable to routine quantitative counts of organisms in tissues using a colony forming unit method. In 2001 Ge et al. developed a fluorogenic PCR-based assay to quantitatively detect H. hepaticus in mouse ceca and feces (22). This assay used a pair of primers and a probe generated from the H. hepaticus cdtB gene (encoding subunit B of the H. hepaticus cytolethal distending toxin). The sensitivity for detection of H. hepaticus chromosomal
DNA prepared from pure culture was 20 fg, which was equivalent to approximately 14 copies of the *H. hepaticus* genome. *H. hepaticus* present in feces and cecal samples from *H. hepaticus*-infected mice was readily quantified. The selected PCR primers and probe did not generate fluorescent signals from eight other *Helicobacter* species (*H. canis, H. cineadi, H. felis, H. mustelae, H. nemestrinae, H. pullorum, H. pylori, and H. rodentium*).

In 2002, another fluorogenic nuclease PCR assays that specifically detect *H. hepaticus* was developed to eliminate post-PCR processing, enhanced specificity, and provided quantitative data on starting template concentration. The primers and probes used in this assay were based on the urease gene of the *H. hepaticus* (10). This fluorogenic PCR assay detected a minimum of 10 copies of target template, had a comparable or greater sensitivity when compared directly with corollary gel detection PCR assays, and detected only targeted species when numerous *Helicobacter species* and other enteric bacteria were analyzed.

In 2004, a duplex PCR assay was developed by Bourgade et al. for detection of false-negative results during routine *Helicobacter* species feces analysis (4). This assay used *Lactobacillus* group-specific primers in addition to *Helicobacter* genus specific primers. *Lactobacillus* species forms part of the normal intestinal flora of laboratory rodents. This assay was able to eliminate false negatives due to PCR inhibitors present in the feces.

In 2005, fluorescence in situ hybridization (FISH) probes specifically for *H. hepaticus* were developed by Chan et al. (7). This probe was based on 16S-RNA gene alignments. Using FISH probe, it was found that *H. hepaticus* was consistently located in the mid upper region of the crypts in cecum in a IL-10-knockout mice.
In 2005, a multiplex PCR was developed by Feng et al. which accurately and simultaneously detected five species of mouse helicobacters, \textit{H. bilis}, \textit{H. hepaticus}, \textit{H. muridarum}, \textit{H. rodentium}, and \textit{H. typhlonius} (11). This assay had high specificity and senstitivity. This assay detected as little as 0.5 ng of DNA in a single five-plex PCR in the feces. The primer pairs used in this assay were based on \textit{H. bilis} p17 protein, \textit{H. hepaticus} p25 protein, and \textit{H. muridarum} p30 protein, in combination with 16S rRNA sequences that were specific for \textit{H. rodentium} and \textit{H. typhlonius}. 
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