Immunomodulation by subclinical persistent infection with *Helicobacter hepaticus*

By

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ABSTRACT

Recognition of polymicrobial infections is becoming important for understanding differential host responses to environmental exposures, vaccines, as well as therapeutics. *Citrobacter rodentium* is a well-characterized model of infectious colitis with particular usefulness for modeling human diarrheal disease or inflammatory bowel disease. Infection with *Helicobacter hepaticus* is subclinical and persistent in C57BL/6 mice, but causes disease in susceptible strains and immunodeficient mice. To test the hypothesis that subclinical persistent infection modulates the host response to diarrheal disease a polymicrobial mouse model utilizing *H. hepaticus* and *C. rodentium* was developed and characterized.

Concurrent infection has been shown to modulate disease outcome through several mechanisms including: cross-reactivity between viral antigens; shifting T cell response from T_h1 to T_h2 by helminth infection; and induction of regulatory T cells that suppress host response. In this new model of polymicrobial infection, a new paradigm in which persistent infection prolonged the course of acute colitis associated with a deviation from T_h1-biased disease to T_h17 was observed. In addition, $Foxp3^+$ naturally-occurring regulatory T cells (nT_{reg}) were markedly increased during active colitis. The accumulation of nT_{reg} was sustained when mice were persistently infected with *H. hepaticus*, indicating on-going active colitis. Although persistent infection was able to modulate host response, protective immunity to a subsequent *C. rodentium* infection was not compromised. Persistent infection also modulated host response to soluble antigen by preventing induction of oral tolerance to single bolus, but not to continuous, high-dose antigen feeding.

Using *H. hepaticus* infection of C57BL/6 mice, models to investigate the immunomodulatory potential of persistent infection on immunogenic responses of protective immunity to enteric infection, host response to polymicrobial enteric infection, as well as tolerogenic responses to soluble antigen were developed. These models establish baselines for further investigation into the influences of persistent infection on host immune responses.

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Table of Contents

Chapter 1 ..... **8**

Introduction **8**

Chapter 2 ..... **14**

***Helicobacter hepaticus* causes persistent subclinical infection in C57BL/6 mice** **14**

Introduction 15

Results 15

Discussion 20

Figures 22

Chapter 3 ..... **30**

***Helicobacter hepaticus* reveals multiple mechanisms of IL-10-independent oral tolerance in C57BL/6 mice** **30**

Introduction 31

Results 33

Discussion 40

Tables and Figures 43

Chapter 4 ..... **49**

Persistent *Helicobacter hepaticus* infection does not prevent development of protective immunity to *Citrobacter rodentium* **49**

Introduction 50

Results 53

Discussion 61

Tables and Figures 64

Chapter 5 ..... **73**

Persistent subclinical *Helicobacter hepaticus* infection modulates host response to *Citrobacter rodentium* causing prolonged disease **73**

Introduction 74

Results 75

Discussion 84

Figures 88

Chapter 6 ..... **99**

Conclusions **99**

Chapter 7 ..... **104**

Materials and Methods **104**

References **112**

List of Figures and Tables

Figure 2-1. *H. hepaticus*-specific IgG1 and IgG2c antibodies are generated as a result of *H. hepaticus* infection.

Figure 2-2. C57BL/6 mice infected with *H. hepaticus* for 18 months do not develop colonic or cecal disease.

Figure 2-3. MLN cytokine expression in C57BL/6 mice did not change after 2 months post-infection with *H. hepaticus.*

Figure 2-4. Colonic cytokine mRNA expression exhibit tremendous biological variation in *H. hepaticus* infected and uninoculated mice, as such no differences were found.

Figure 2-5. Cecal cytokine expression in *H. hepaticus* infected C57BL/6 mice was comparable to uninoculated counterparts, with a modest increase in IL-17 expression at 2 months post-infection.

Figure 2-6. No change in cecal cytokine expression was observed in *H. hepaticus* infected C57BL/6 mice between 2 MPI and 18 MPI.

Figure 2-7. Expression of cecal cytokines was consistently elevated in A/J mice over C57BL/6 mice in both uninoculated and *H. hepaticus* infected.

Figure 2-8. At 18MPI *H. hepaticus*-specific IgG1 and IgG2a/c titers are comparable between males and females of both A/J and C57BL/6 mice, but IgG2c titers in C57BL/6 mice are lower than IgG2a titers in A/J mice.

Table 3-1. Cecal and colonic lesions only developed in a few *H. hepaticus* infected IL-10-deficient mice.

Figure 3-1. A single high dose of ovalbumin reduced IFN-γ production upon re-stimulation of PLN from *Helicobacter*-free mice, but not *H. hepaticus* infected C57BL/6 mice.

Figure 3-2. Both single and continuous high-dose feeding of ovalbumin reduce OVA-specific IgG2c serum titers after systemic ovalbumin immunization, but persistent H. hepaticus infection prevents the titer reduction from single high-dose feeding.

Figure 3-3. Neither *H. hepaticus* infection nor feeding of a single 25mg dose of OVA changed the number of $Foxp3^+$ cells in cecum or colon of C57BL/6 mice, but IL-10-deficient mice infected with *H. hepaticus* had an elevated number of $Foxp3^+$ cells in the cecum and colon.

Figure 3-4. In IL-10-deficient mice, continuous high-dose feeding of ovalbumin reduce OVA-specific IgG2c serum titers after systemic ovalbumin immunization, which is unaffected by persistent *H. hepaticus* infection.

Figure 3-5. Continuous feeding of a high dose of ovalbumin reduced IFN-γ production upon re-stimulation of PLN from *Helicobacter*-free and *H. hepaticus* infected C57BL/6 mice.

Table 4-1. Re-challenge strain of *C. rodentium* did not cause cecal or colonic disease and was unaffected by *H. hepaticus* infection.

Figure 4-1. Immunization with *C. rodentium* sonicate generates *C. rodentium*-specific circulating antibodies.

Figure 4-2. Immunization did not consistently prevent or reduce bacterial burden or weight loss during *C. rodentium* challenge.

Figure 4-3. Reduced colonic disease in mice with decreased bacterial burden.

Figure 4-4. *C. rodentium* infection provides protection from re-infection and associated weight loss.
Figure 4-5. Prior *C. rodentium* infection prevents colonic disease development.
Figure 4-6. Persistent *H. hepaticus* infection does not affect protection from decreased bacterial burdens in convalescent mice.
Figure 4-7. No increases in cecal or colonic $Foxp3^+$ cell numbers were observed with *C. rodentium* re-challenge despite persistent *H. hepaticus* infection.
Figure 4-8. Colonic mRNA cytokine expression was not altered by either *H. hepaticus* or *C. rodentium* infection.

Figure 5-1. Persistent *H. hepaticus* infection impairs recovery of weight loss without changing kinetics of *C. rodentium* bacterial shedding.
Figure 5-2. Pathogen-specific immunoglobulin titers are not affected by persistent *H. hepaticus* infection.
Figure 5-3. *C. rodentium* infection causes marked colon disease that is prolonged by persistent *H. hepaticus* infection.
Figure 5-4. Persistent *H. hepaticus* infection maintains elevated numbers of Foxp3 expressing cells in colonic tissue as a result of *C. rodentium* infection.
Figure 5-5. Persistent *H. hepaticus* infection suppresses the T_h1 pro-inflammatory cytokine profile induced after *C. rodentium* infection resulting in higher IL-17 (T_h17) expression at 4 WPI.
Figure 5-6. Changes in colonic mRNA expression levels of other colitis-associated factors were either not affected by *C. rodentium* infection or concurrent persistent *H. hepaticus* infection.
Figure 5-7. Colonic disease severity is positively correlated with IFN-γ expression, not IL-17 expression.
Figure 5-8. Early during *C. rodentium* infection cecal disease was apparent, yet when the cecal apex was not included in evaluation persistent *H. hepaticus* infection prevented disease.
Figure 5-9. $Foxp3^+$ cell numbers in the cecum did not change with *H. hepaticus*, *C. rodentium*, or concurrent infection.
Figure 5-10. *C. rodentium* infection caused modest changes of cytokine expression in the MLN and the cecum with slight suppression of pro-inflammatory cytokines seen with persistent *H. hepaticus* infection.
Figure 5-11. Cecal disease severity at 1 WPI is positively correlated with proinflammatory cytokine expression, and negatively correlated with anti-inflammatory cytokine expression.

Chapter 1

Introduction

Helicobacter hepaticus is a microaerophilic gram-negative spiral organism that specifically infects mice (26, 85). First isolated from livers of SCID/NCr mice with active, chronic hepatitis, and mucosa of the cecum and colon in SCID/NCr as well as A/JCr mice (26), *H. hepaticus* primarily resides in the cecal mucosa (15, 31). Although infection of most strains of immunocompetent mice show limited signs of clinical disease, infection of susceptible mouse strains causes inflammation of the large intestines and liver leading to hepatocellular carcinoma (4, 27, 37, 68, 85, 86, 88). In contrast, immunodeficient mice, particularly those lacking adaptive immune functions, develop an IBD-like disease (13, 17, 19, 39, 40, 43, 46, 78, 84). Due to the limited disease development in immunocompetent mice, little is known about their host response to *H. hepaticus* infection, and most of the published data relate to their use as control mice for knockout studies.

Although *H. hepaticus* preferentially infects the cecal mucosa of mice and becomes a dominant member of the microflora by 2 weeks post-inoculation (WPI) (38), infections in immunocompetent hosts suggest both sex and strain differences with regards to both colonization and host response. Analysis of cecal bacterial burden using quantitative PCR revealed higher bacterial loads in male compared to female outbred Swiss Webster mice (31) and C57BL/6 mice at 3 months post-inoculation (MPI) (15). However, in another study of C57BL/6 mice taken to 6 MPI there was not a gender difference in cecal bacterial load (86). This same study compared so-called hepatitis-resistant C57BL/6 and hepatitis-susceptible A/JCr mice finding higher cecal

bacterial levels in the C57BL/6 mice (86). Cecum has the highest *H. hepaticus* burden, however by qPCR *H. hepaticus* can be found in colon tissue of immunocompetent mice (31), but is rarely quantifiable in livers of C57BL/6 mice (31). Although hepatitis-prone male A/JCr mice often have elevated quantifiable liver burdens, and therefore, have been shown to have higher bacterial numbers in their livers than female A/JCr mice (68).Liver and large intestinal histological changes associated with *H. hepaticus* infection have mostly been reported in immunodeficient mice and A/JCr hepatitis-susceptible mice. Liver lesions have been reported as early as 3MPI in both male and female A/JCr mice (47). Similarly, no gender difference in hepatitis was found in A/JCr mice after 6 months of infection (86). In contrast, liver involvement was not found in C57BL/6 mice at 6 MPI (86). Cecal lesions in C57BL/6 and A/JCr mice have been evaluated as early as 1 MPI, at which time neither C57BL/6 nor A/JCr mice had cecal disease (47). Appearance of cecal lesions was reported at 3 MPI in both male and female A/JCr mice (47), however another study found no typhlitis in either C57BL/6 or A/JCr mice at 6 MPI (86). By 12 MPI chronic typhlitis develops in A/JCr mice, but studies of other aged immunocompetent mice are lacking, particularly C57BL/6 mice (88). As controls for immune knockout mice, immunocompetent 129Sv/Ev and FVB mice were evaluated at 8 and 10 MPI, respectively, at which times no cecal or colonic lesions were observed (18, 52).

Both innate and adaptive immune cells have been implicated in *H. hepaticus* disease development. An early study found large intestinal lesions that were attributable to natural infection with *H. hepaticus* in SCID and Nude mice of different strain backgrounds, which are deficient in functional T and B cells (84). Further studies have gone on to characterize the requirements for disease development in RAG1- or RAG2-deficient mice that also lack

functional T and B cells in addition to the contribution of different T cell subsets to pathogenesis (8, 18, 19, 39-41, 53, 54).

H. hepaticus-induced disease in RAG-deficient mice varies in time-to-development depending on the strain background with 129Sv/Ev RAG-deficient mice developing severe typhlocolitis by 2 MPI, whereas few lesions were observed in C57BL/10 RAG-deficient mice (18, 39, 54). This innate, T cell-independent intestinal disease involved increased numbers of lamina propria leukocytes (LPL) with enrichment for neutrophils rather than monoyctes or NK cells (54). Typhlocolitis in *H. hepaticus* infected 129Sv/Ev RAG-deficient mice was associated with increased expression of cytokines IL-12/23p40, IL-23p19, IL-6, TNF-α, IP-10, IFN-γ, MCP-1, IL-1β, KC, IL-17 (18, 36). Treatment of *H. hepaticus* infected mice with anti-IL-12/23p40 or TNF-α reduced disease pathology (54). Previously thought to be only a subunit of IL-12, IL-12/23p40 subunit, the recent recognition that is shared with IL-23 lead to finding increased IL-23 production with *H. hepaticus*-induced innate disease (36). When *H. hepaticus* infected 129Sv/Ev RAG-deficient mice were treated with anti-IL-23p19 antibody, more dramatic disease reduction was achieved than with anti-IL-12/23p40 (36). Attenuated disease due to anti-IL-23p19 treatment was accompanied by reduced colon cytokine expression, indicating IL-23 triggers a cascade of proinflammatory cytokines leading to development of innate disease (36). Comparable reduction of *H. hepaticus* induced innate disease observed with anti-IL-23 treatment is also found after addition of regulatory T cells to RAG-deficient infected mice (36, 40, 41, 53, 54).

Before Foxp3 was a well-established marker for naturally-occurring regulatory T cells (nT_{reg}), $CD4^+$ cells expressing low levels of CD45RB along with CD25 ($CD4^+CD45RBlowCD25^+$) were used as a regulatory T cell-containing subset of cells. Transfer

of these cells into 129Sv/EvRAG-deficient mice prior to *H. hepaticus* infection, ablated innate intestinal disease development (18, 54). The influx of neutrophils in the lamina propria observed with *H. hepaticus* infection was prevented with transfer of $CD4^+CD45RBlowCD25^+$ regulatory T cells, indicating that neutrophils are major contributors to cytokine production and disease pathology. When 129Sv/EvRAG-deficient $CD4^+CD45RBlowCD25^+$ transfer recipients infected with *H. hepaticus* were treated with either anti-TGF-β or anti-IL-10R, the ability of the transferred regulatory T cells to attenuate inflammation was prevented (54).

The important role of regulatory T cell populations in *H. hepaticus* induced disease was further accentuated by their prevention of T cell-dependent disease. When either effector T cells ($CD4^+CD45RBhigh$) were transferred into 129Sv/EvRAG-deficient mice or $CD4^+IL\text{-}10^{-/-}$ cells were transferred into C57BL/10 RAG-deficient mice, *H. hepaticus* infection caused typhlocolitis. Co-transfer of $CD4^+CD45RBlowCD25^+$ cells inhibited *H. hepaticus*-induced disease development in both models (41, 54). Similar to T cell-independent disease administration of anti-IL-10R antibodies prevented regulatory T cell suppression of T cell dependent disease (41). The important role of IL-10 in *H. hepaticus* infection is highlighted by the development of intestinal lesions from infection of either wild type C57BL/6 mice receiving anti-IL-10R antibody treatment or C57BL/6 IL-10-deficient or C57BL/10 IL-10-deficient mice (8, 39-43, 64).

The IBD-like disease that develops in IL-10-deficient mice on either C57BL/6 or C57BL/10 strain background occurs between 1 and 2 MPI. Cecal lesions are characterized by epithelial hyperplasia, goblet cell loss, and moderate to severe inflammation with lymphocyte, neutrophil and macrophage infiltration of the lamina propria. Mild to moderate inflammation of the colon and rectum also occur, typically more severe in the proximal colon (8, 43).

Upregulation of MHC-II along with an influx of CD3+ cells in the colon is observed with immunohistochemical staining (8, 42). Increased intestinal cytokine production or mRNA expression of IL-17, IFN-γ, TNF-α, IL-12/23p40, and iNOS have been found, compared to uninfected IL-10-deficient control mice (42, 64). Intervention by cytokine depletion (anti-IL-12/23p40, anti-TNF-α, anti-IFN-γ or double anti-TNF-α+anti-IFN-γ antibody treatment) only showed efficacy for anti-IL-12/23p40 (42, 43). *H. hepaticus* infected IL-10-deficient mice treated with anti-IL-12/23p40 at 1 MPI for one month showed reduced cecal inflammation consisting of fewer inflammatory infiltrates in the lamina propria and less epithelial hyperplasia compared with control antibody treated mice (42, 43). mRNA transcripts of IFN-γ, TNF-α, and iNOS were decreased in anti-IL-12/23p40 treated mice, but not anti-TNF-α, anti-IFN-γ or anti-TNF-α + anti-IFN-γ treated mice (42). Unlike IL-10/IFN-γ double knockout mice that were still susceptible to *H. hepaticus* intestinal disease, IL-10/IL-12/23p40 double knockout mice were comparable to uninfected IL-12/23p40 and wild type C57BL/10 controls, indicating IL-12/23p40 plays a critical role in disease development (42).

Recently, the role of IL-10 signaling during *H. hepaticus* infection using anti-IL-10R antibody treatment was used to dissect the roles of IL-12 versus IL-23 in *H. hepaticus* disease development. Wild type C57BL/6 mice given anti-IL-10R treatment develop comparable disease to IL-10-deficient mice that can be blocked by co-administration with anti-IL-12p40 (40). Neither IL-12/23p40-deficient nor IL-12p35-deficient mice develop disease when infected with *H. hepaticus*, however when treated with anti-IL-10R antibody, IL-12p35 mice develop cecal and colonic disease comparable to wild type treated mice; indicating IL-12 is not required for *H. hepaticus* disease development. IFN-γ has a minor role in disease development, as IFN-γ-

deficient mice only develop comparable typhlitis, not colitis when treated with anti-IL-10R antibody (40).

Despite robust immune activation demonstrated by immunopathology and tissue cytokines in immunodeficient mice, very little evidence of immune activation in immunocompetent mice, particularly disease-resistant C57BL/6 mice, exists. Although the immune system appears to be ignorant of *H. hepaticus* in wild type C57BL/6 mice, recognition does occur, as serum *H. hepaticus*-specific immunoglobulin titers and fecal sIgA are detectable (43, 87). Serum titers in C57BL/6 mice are even comparable to C57BL/6 IL-10-deficient (43). Yet, there is little evidence for immune activation at the site of infection. Compared to IL-10-deficient mice, MHC-II expression in the cecum and colon of wild type C57BL/6 mice was minimal regardless of infection (8). Of many cytokines evaluated, only IL-17A (IL-17) had increased expression in *H. hepaticus* infected C57BL/6 mice compared with uninfected mice (40).

H. hepaticus is well adapted to persist in its natural host. Many knockout lines of mice demonstrate the need for adaptive immunity in controlling mucosal inflammation and resulting damage. Basis for these studies is the hypothesis that such adaptive immune responses in subclinical persistent infection can influence the immune responses, either tolerogenic or immunogenic, to oral tolerance to soluble antigen, protective immunity from immunization or prior infection, and concurrent acute enteric infection.

Chapter 2

Helicobacter hepaticus causes persistent subclinical infection in C57BL/6 mice

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Introduction

Similar to the human pathogen *Helicobacter pylori*, *H. hepaticus* is highly prevalent in its natural host: mice. Although eliminated from most commercial vendors, *H. hepaticus* is enzootic in many academic research facilities. Additionally, *H. hepaticus* infection, like *H. pylori* in humans, is life-long and subclinical. For *H. hepaticus,* the exception is susceptible immunocompetent strains (A/JCr) in which it causes typhlocolitis, hepatitis, and liver cancer (24, 28, 37, 69, 85, 86). Both of these Helicobacter infections have been suggested to act through similar mechanisms to remain subclinical, which is via careful regulation of host-microbe interactions via regulatory T cells (16, 48, 65). Further evidence of this mechanism is that in several strains of knockout mice, particularly those lacking adaptive immune functions, an inflammatory bowel disease-like (IBD-like) condition develops (8, 9, 36, 39, 40, 42, 43, 46, 84). If IL-10 competent T cells are transferred into infected mice of an IBD-prone strain, the proinflammatory events initiated by the *H. hepaticus* infection are contained, whereas cells deficient in IL-10 have no effect on *H. hepaticus*-induced disease (18, 19, 41, 53). Since *H. hepaticus* infection is most commonly studied in immune-deficient strains of mice, *H. hepaticus* infected C57BL/6 mice were followed over time to determine whether disease progressed due to alteration in immune regulation. Therefore, whether C57BL/6 mice have subclinical *H. hepaticus* infection both histologically and immunologically after 2 or 18 months of infection was determined.

Results

C57BL/6 mice infected with *H. hepaticus* show no weight change after 3 months of infection

Infection of male and female immunocompetent C57BL/6 mice with *H. hepaticus* for 2 to 3 months has not been previously characterized. Studies of shorter duration (1 month) (40) and longer duration (6 months) (86) using *H. hepaticus* inoculated C57BL/6 mice as controls have been reported. Between 2 and 3 months post-inoculation (MPI) *H. hepaticus* had no effect on weight, as weight gain in infected mice was not significantly different from uninoculated controls (Chapter 5, Figure 1 A). Establishment of infection in these mice was confirmed with all-*Helicobacter* PCR on feces (data not shown). To verify that the immune system did in fact respond to infection with *H. hepaticus*, antibody titers were measured.

H. hepaticus-infected mice make an _H. hepaticus_-specific serum IgG1 and IgG2c response that is sustained for at least 11 weeks post-inoculation

Mice infected with *H. hepaticus* mount an adaptive immune response to *H. hepaticus* antigens, including serum IgG, T_h1, and T_h17 cell populations (40, 43, 87). To confirm that the adaptive response to *H. hepaticus* was present and comparable throughout the duration of infection, the serum IgG1 and IgG2c response against *H. hepaticus* was monitored at 3, 8, and 11 weeks post-inoculation (WPI) as well as 18 months post-inoculation (MPI) in wild type C57BL/6 and 8 WPI in C57BL/6 IL-10-deficient mice. *H. hepaticus*-specific IgG1 and IgG2c titers were detectable by 3 WPI (data not shown). Titers increased between 3 and 8 WPI, but had stabilized by 8WPI, as titers for both IgG1 and IgG2c were comparable between 8 and 11 WPI (Figure 2-1). IgG2c titers specific to *H. hepaticus* were comparable between IL-10-deficient and wild type mice at 8 WPI, however IL-10-deficient mice had lower *H. hepaticus*-specific IgG1 titers than wild type mice, $P < 0.05$ (Figure 2-1). These titers were not normalized to total isotype in the serum, and if normalized, may not be significantly different. At 18 MPI *H.*

hepaticus-specific titers remain detectable (Figure 2-8) and normalized titers were not different from 2 to 3 MPI (Chapter 5). Additionally, *H. hepaticus*-specific sIgA titers of between 0.1 and 1% of total sIgA in cecal contents were measured after 8 weeks of infection (Chapter 5).

Infection with *H. hepaticus* does not cause cecal or colonic lesions in C57BL/6 mice

As expected in wild type C57BL/6 mice, infection with *H. hepaticus* for up to 11 weeks did result in cecal or colonic lesions (Chapter 3 and Chapter 5). The median colonic disease index from mice infected with only *H. hepaticus* for 11 weeks was 0.25 with a range of [0.00-1.00] compared with 0.0 (0.0-0.0) in uninoculated mice, $P > 0.05$ (from Chapter 5 experiment). In these same mice, cecal disease was also not significantly different (1.0 [0.5-2.0]) compared with 1.75 [0.50-2.00] in uninoculated mice, $P > 0.05$. After 18 months of infection C57BL/6 mice did not exhibit appreciable colonic or cecal lesions compared with age-matched uninoculated mice, $P > 0.05$ (Figure 2-2). In contrast, marked cecal disease was present in A/J mice at 18 MPI, $P > 0.001$ (Figure 2-2).

***H. hepaticus* infection increases IL-17 expression in the cecum, but other cytokines were unchanged in cecum, colon or mesenteric lymph node**

After 2 to 3 months of infection with *H. hepaticus*, cytokine mRNA expression in the mesenteric lymph node (MLN) was not different from uninoculated mice (Figure 2-3). Similarly, in distal colon tissue neither proinflammatory nor anti-inflammatory cytokine message levels were distinguishable from uninoculated control mice despite the colon being a tissue infected with *H. hepaticus*, $P > 0.05$ (Figure 2-4). The lack of change in cytokine mRNA expression in either MLN or colon tissue substantiate the subclinical nature of *H. hepaticus*

infection. Since the cecum is thought to be the primary niche for *H. hepaticus*, cecal cytokine expression was also evaluated. At 2 to 3 MPI, cecal expression of IL-6, TNF-α, IL-1β, MCP-1, IFN-γ, IL-17, IL-12/23p40, IL-10, TGF-β, and Foxp3 were compared to uninoculated mice. The only cytokine with differential expression in the cecum was IL-17, which was ~10-fold higher than uninoculated age-matched mice, $P < 0.05$ (Figure 2-5). As no lesions were apparent, despite elevated IL-17 expression, the presence of $Foxp3^+$ regulatory T cells in the local tissue was also assessed.

H. hepaticus infection does not increase local tissue $Foxp3^+$ cells

Several studies have implicated the involvement of multiple, and not mutually exclusive regulatory T cell populations in *H. hepaticus* infection (14, 41, 53). However staining of cecum and colon for the transcription factor Foxp3, which is commonly used as a marker for naturally-occurring regulatory T cells, revealed no significant increase in $Foxp3^+$ cells in either cecum distal colon after infection with *H. hepaticus* for 2 to 3 months (Chapter 5, Figure 5-4 and 5-9). This does not rule out changes in the populations of other regulatory T cells for which there are no exclusive markers, particularly IL-10 expressing T_R1 cells, thought to play a role in containing clinical *H. hepaticus* disease (41).

Cecal cytokine expression does not change after 18 months of *H. hepaticus* infection in C57BL/6 mice

Cecal cytokine expression after infection with *H. hepaticus* for 18 months was measured to verify whether the tissue responses to infection remained unaltered. Although IL-17 had increased expression after 2 to 3 months, it was not evaluated at 18 MPI. Cytokine measured

include innate cytokines (IL-6 and TNF-α) known to be important in *H. hepaticus* infection of immunocompromised mice, anti-inflammatory cytokines (IL-10 and TGF-β) and Foxp3. At 18 MPI, innate cytokines were unchanged compared to age-matched uninoculated mice, 3- to 4-month old uninoculated mice, and 3- to 4-month old mice infected with *H. hepaticus* for 2 months, $P > 0.05$ (Figure 2-6). Similarly, the anti-inflammatory cytokines and Foxp3 expression were also unaltered, $P > 0.05$ (Figure 2-6). Since C57BL/6 mice have been shown have lower susceptibility to *H. hepaticus*-induced disease compared with A/JCr mice, cytokine expression was measured in cecums of age-matched A/J mice with and without *H. hepaticus*.

A/J mice have higher basal expression of cytokines at old-age than C57BL/6 mice, which decrease with *H. hepaticus* infection

Comparison of cecal cytokines and Foxp3 from age-matched C57BL/6 and A/J mice not inoculated with *H. hepaticus* showed strain-specific differences in which aged A/J mice had higher basal expression of IL-6, TNF-α, TGF-β, IL-10, and Foxp3 (Figure 2-7). However, *H. hepaticus* infected (18 MPI) A/J mice consistently had lower expression of all 5 transcripts than uninoculated A/J mice, $P < 0.01$ (Figure 2-7). When mice infected with *H. hepaticus* for 18 months from each strain were compared, A/J mice had increased levels of TNF-α, $P < 0.05$ (Figure 2-7). Whether strain differences in host response to *H. hepaticus* were also detectable in adaptive immunity, serum antibody titers were measured.

A/J and C57BL/6 mice show strain differences in *H. hepaticus*-specific IgG2a/c titers

To determine whether a change in the adaptive immune response to *H. hepaticus* is partially responsible for differences in strain susceptibility to *H. hepaticus* induced disease late

during infection (18 MPI), serum immunoglobulin titers were measured. There was no difference in IgG1 titers specific to *H. hepaticus* between A/J and C57BL/6 mice 18 months after infection (Figure 2-8). A/J and C57BL/6 mice express different T_h1- dependent isotypes of IgG (IgG2a and IgG2c, respectively), therefore their respective titers were measured and when compared, *H. hepaticus*-specific titers in A/J mice (normalized to total IgG2a) was over and order-of-magnitude higher than *H. hepaticus*-specific IgG2c in C57BL/6 mice, $P < 0.001$ (Figure 2-8). Since *H. hepaticus* liver disease in A/J can be sex-specific, antibody titers were separated into male and female mice (47, 86). No difference was observed between male and female mice in either C57BL/6 or A/J mice.

Discussion

Many aspects of host response to infectious agents were evaluated in C57BL/6 mice at early and late time points post-inoculation with *H. hepaticus*. Together, these studies confirm that *H. hepaticus* infection in C57BL/6 mice is completely subclinical. C57BL/6 mice make a specific immune response to *H. hepaticus,* as both IgG1 and IgG2c titers were detectable as early as 3 WPI through 18 MPI. However, not only was there an absence of intestinal lesions with scores not significantly different from uninoculated *Helicobacter*-free control mice, but cytokine expression remained unchanged out to 18 MPI, indicating the host and pathogen have adapted to prevent host damage while maintaining a niche for *H. hepaticus* to survive.

An initial study comparing aged disease-susceptible A/J and disease-resistant C57BL/6 mice indicate strain differences with regard to immune response at the tissue level exist. Lower expression levels of cytokines in *H. hepaticus* infected versus uninoculated A/J mice may indicate an adaptive host response to attempt to limit immune responses without respect to

function. Additionally, other proinflammatory cytokines, such as IFN-γ, not measured in this study, could be preferentially increased. Although this study did not give conclusive results, they are suggestive that further studies into strain differences in immune responses to *H. hepaticus* would be worthwhile. Overall, these studies show that infection of C57BL/6 mice with *H. hepaticus* is subclincal and remains subclinical throughout the life of the animal.

Figures

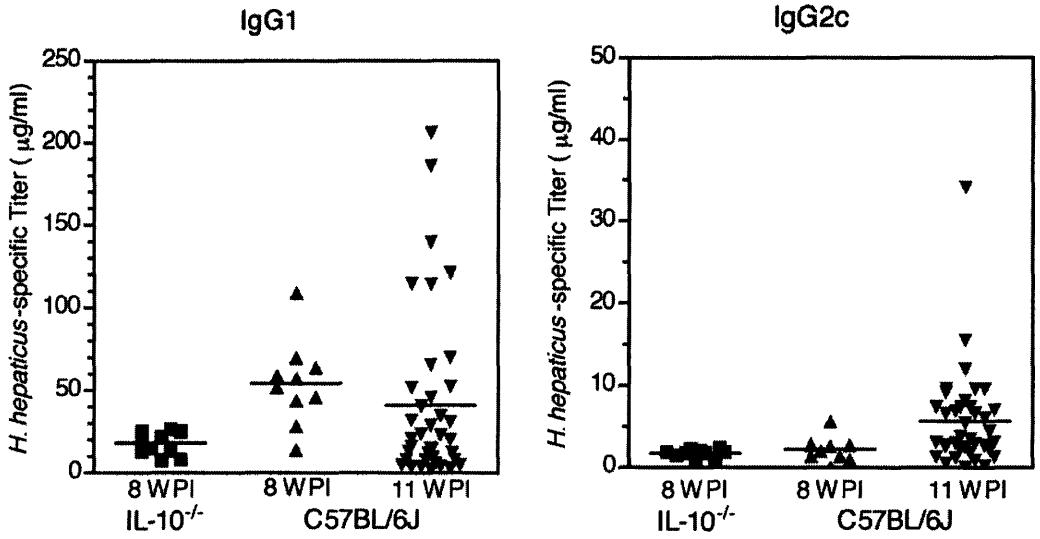

Figure 2-1. *H. hepaticus*-specific IgG1 and IgG2c antibodies are generated as a result of *H. hepaticus* infection. C57BL/6 mice persistently infected with *H. hepaticus* generated both IgG1 and IgG2c anti-*H. hepaticus* titers that were equivalent between 8 WPI and 11WPI. C57BL/6 IL-$10^{-/-}$ mice had lower IgG1 titers (not normalized) than C57BL/6 mice at 8 WPI, but *H. hepaticus*-specific IgG2c titers were comparable. One-way ANOVA with Bonferroni post-test; * $P < 0.05$.

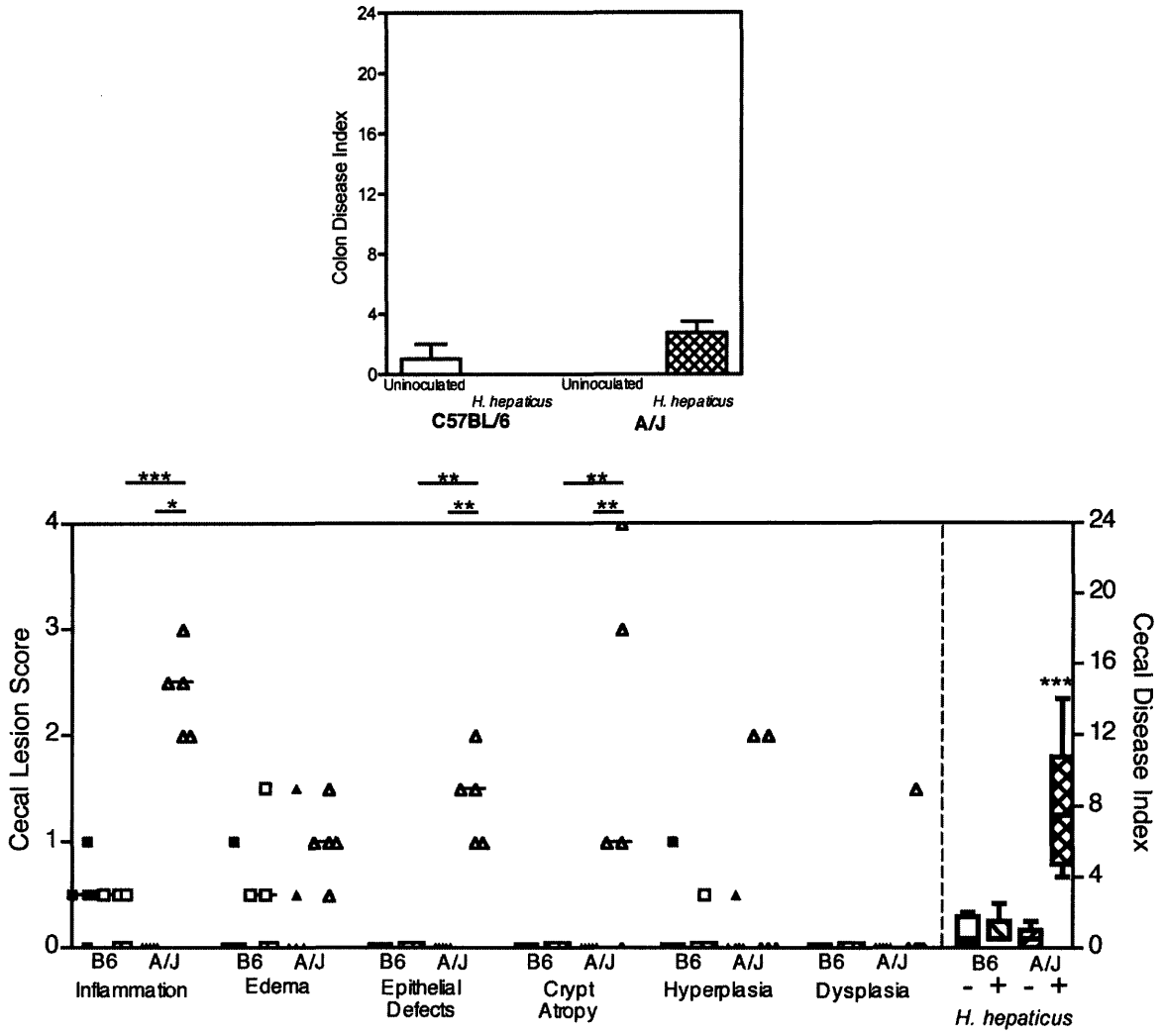

Figure 2-2. C57BL/6 mice infected with *H. hepaticus* for 18 months do not develop colonic or cecal disease. Colonic disease was not present in C57BL/6 mice infected with *H. hepaticus* for 18 months. Substantial colonic disease was also not present in *H. hepaticus* infected A/J mice after 18 months of infection. A few C57BL/6 mice had minimal cecal lesions, however they were comparable to uninoculated age-matched C57BL/6 mice, $P > 0.05$. In contrast A/J mice infected with *H. hepaticus* for 18 months had mild to moderate cecal lesions with overall markedly worse cecal disease, $P < 0.001$. Kruskal-Wallis with Dunn's post-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

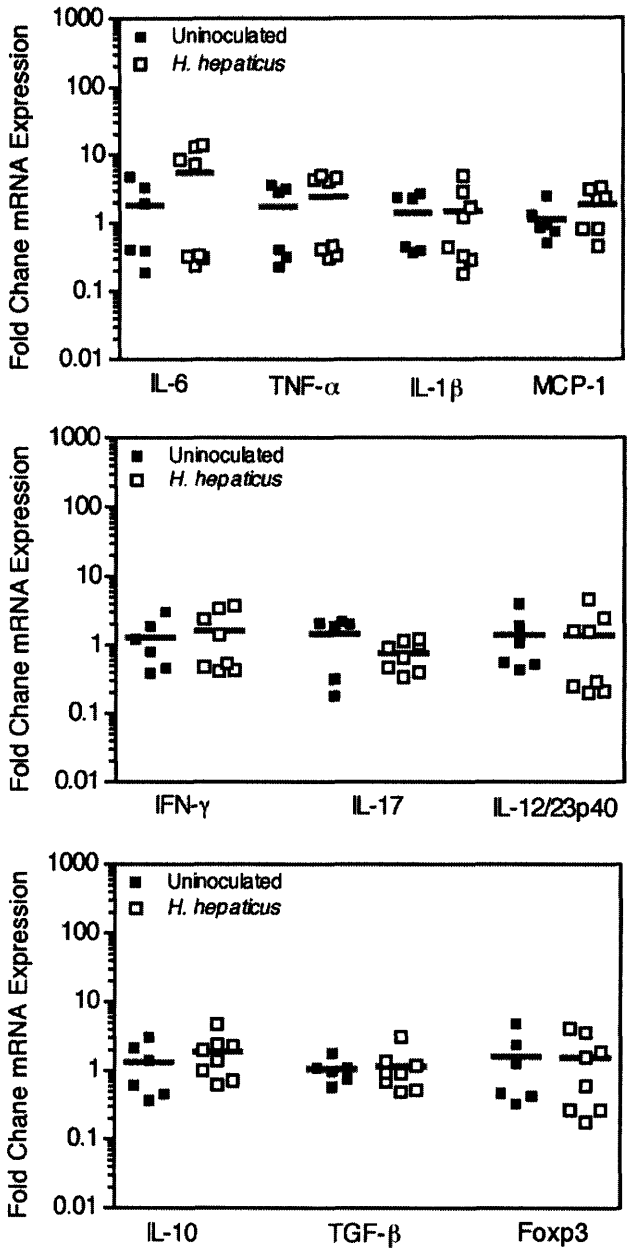

Figure 2-3. MLN cytokine expression in C57BL/6 mice did not change after 2 months post-infection with *H. hepaticus*. Innate, pro-inflammatory, and anti-inflammatory cytokine expression in the MLN of *H. hepaticus* infected C57BL/6 mice was indistinguishable from age-matched uninoculated mice. Unpaired, two-tailed t test with Welch's correction when necessary; $P < 0.05$ was considered significant.

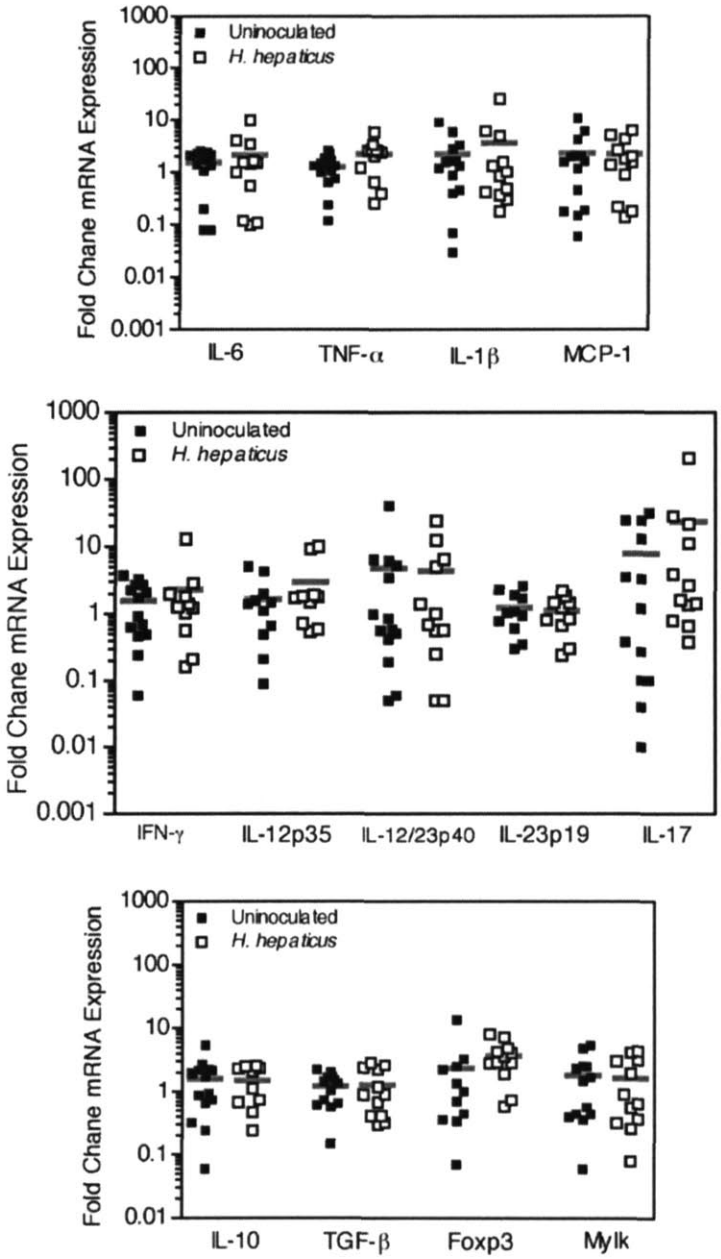

Figure 2-4. Colonic cytokine mRNA expression exhibit tremendous biological variation in *H. hepaticus* infected and uninoculated mice, as such no differences were found. Young (3 to 4 months old) *Helicobacter*-free mice had over two orders of magnitude difference in colonic cytokine expression levels. After 2-3 months of *H. hepaticus* infection, no changes in colonic cytokines were found. Unpaired, two-tailed t test with Welch's correction when necessary; $P < 0.05$ was considered significant.

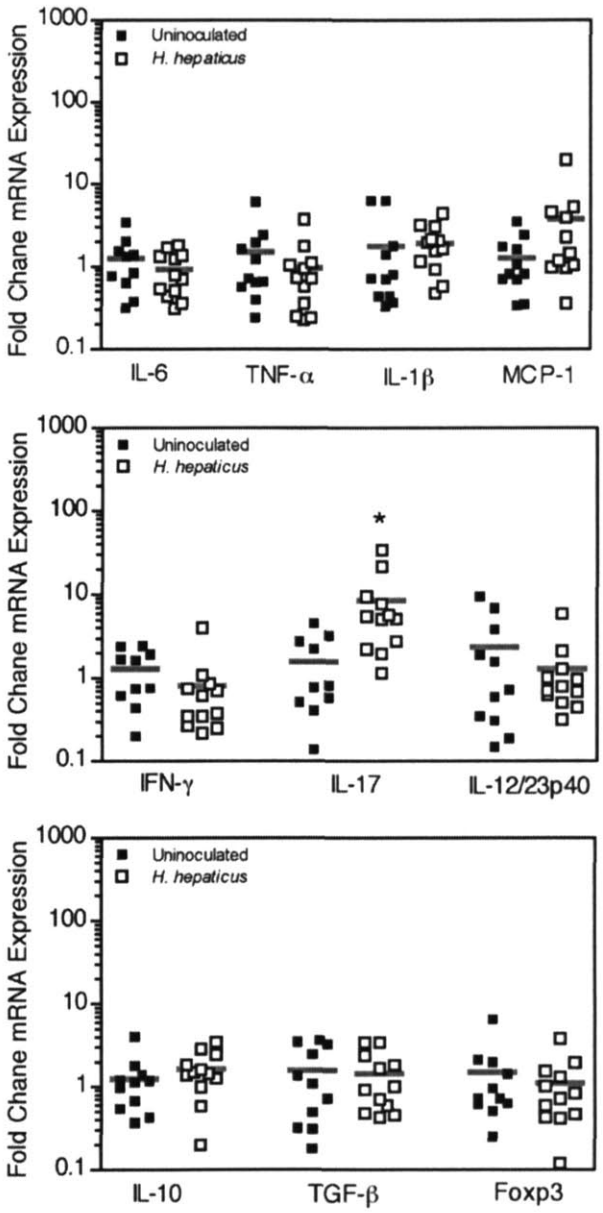

Figure 2-5. Cecal cytokine expression in *H. hepaticus* infected C57BL/6 mice was comparable to uninoculated counterparts, with a modest increase in IL-17 expression at 2 months post-infection. Most pro- and anti-inflammatory cytokine expression levels measured were similar, regardless of *H. hepaticus* infection. IL-17 was the only cytokine with elevated expression in *H. hepaticus* mice. Unpaired, two-tailed t test with Welch's correction; $*P < 0.05$.

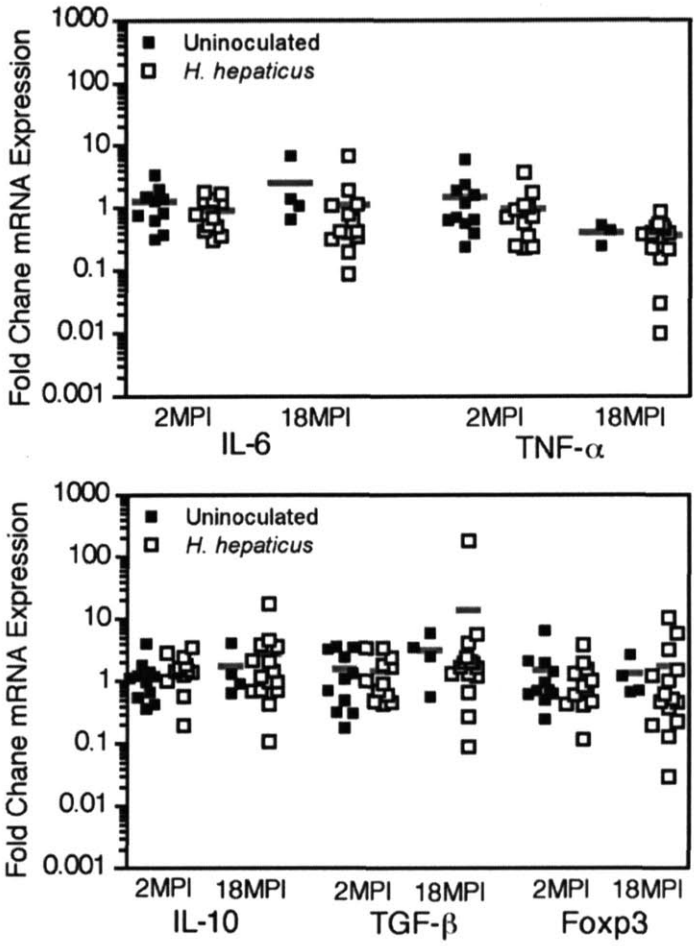

Figure 2-6. No change in cecal cytokine expression was observed in *H. hepaticus* infected C57BL/6 mice between 2 MPI and 18 MPI. Cytokines produced by the innate immune system (IL-6 and TNF-α) were not different between cecums of uninoculated and *H. hepaticus* infected mice at 18 MPI. Cecal expression of regulatory T cell-associated genes (IL-10, TGF-β, and Foxp3) was comparable between uninoculated and *H. hepaticus* infected mice at 18 MPI. No temporal change in cecal expression levels of IL-6, TNF-α, IL-10, TGF-β, or Foxp3 was observed between 2 MPI and 18 MPI. One-way ANOVA with Bonferroni post-test; $P < 0.05$ was considered significant.

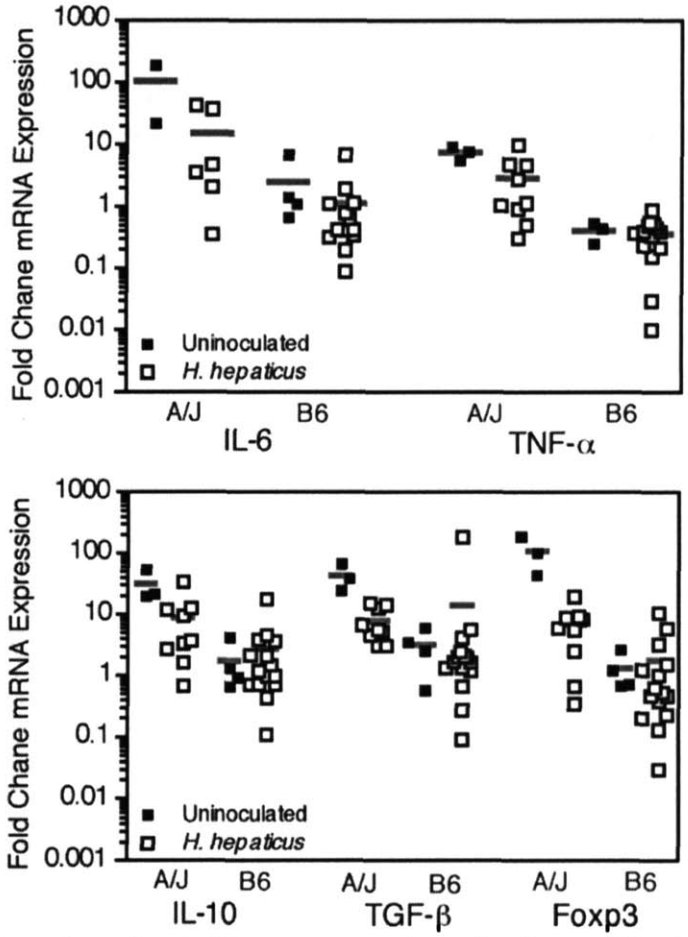

Figure 2-7. Expression of cecal cytokines was consistently elevated in A/J mice over C57BL/6 mice in both uninoculated and *H. hepaticus* infected. Basal cecal IL-6, TNF-a, IL-10, and Foxp3 expression was increased in uninoculated A/J over uninoculated C57BL/6 mice (IL-6 $P < 0.01$; TNF-a $P < 0.001$; IL-10 $P < 0.001$; Foxp3 $P < 0.001$). *H. hepaticus* infected C57BL/6 mice had no change in cecal cytokine expression after 18 months of infection. Compared with uninoculated A/J mice infected A/J mice had lower cecal expression of all cytokines measured ($P < 0.01$, except Foxp3 $P < 0.001$). Among infected mice of both strains, only cecal TNF-a expression was different with higher expression in A/J mice ($P < 0.05$). One-way ANOVA with Bonferroni post-test.

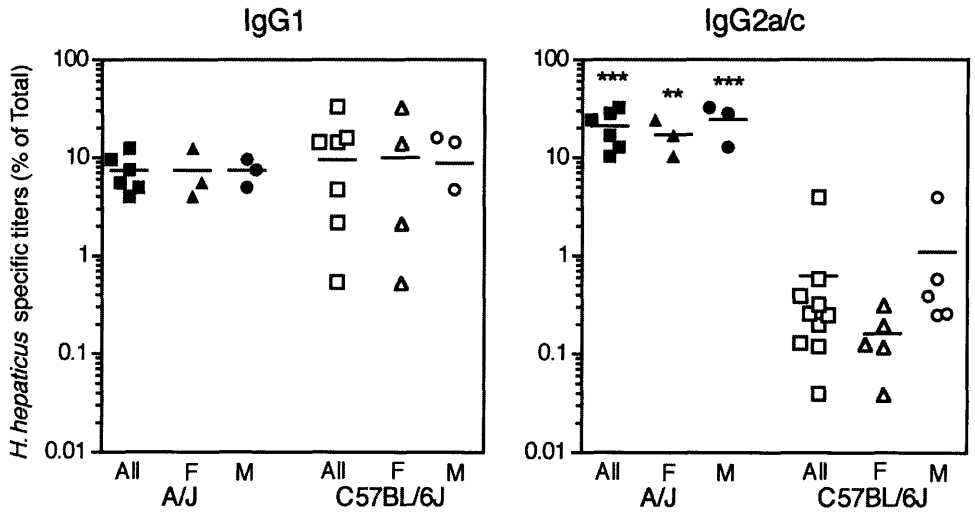

Figure 2-8. At 18 MPI *H. hepaticus*-specific IgG1 and IgG2a/c titers are comparable between males and females of both A/J and C57BL/6 mice, but IgG2c titers in C57BL/6 mice are lower than IgG2a titers in A/J mice. As a percentage of total IgG1 *H. hepaticus* specific titers were equivalent between males and females of both A/J and C57BL/6 strains of mice 18 MPI. Titers of the T_h1-dependent isotype IgG2a/c were strain dependent as A/J mice produce higher *H. hepaticus*-specific IgG2a than *H. hepaticus*-specific IgG2c in C57BL/6 mice. One-way ANOVA with Bonferroni post-test; ** $P < 0.01$, *** $P < 0.001$ (compared with corresponding group in C57BL/6 mice).

Chapter 3

Helicobacter hepaticus reveals multiple mechanisms of IL-10-independent oral tolerance in C57BL/6 mice

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Introduction

Antigen can engender tolerance or immunity, the outcome of which can be determined by the presence of an adjuvant. When presented to the immune system in the context of an adjuvant, antigen exposure results in antigen-specific immunity. Conversely, tolerance or non-responsiveness to the antigen develops when no adjuvant is present during antigen presentation. In addition, immunity depends on the route and site of exposure in terms of the type and magnitude of the resultant immune response. Unlike parenteral antigen immunization in the presence of an adjuvant, which typically elicits a strong immunogenic response, oral immunization of antigen may lead to different responses depending on the presence of adjuvant, including: local secretory IgA (sIgA) production in the mucosa, systemic priming with production of serum antibodies and cell-mediated immunity, or systemic or local non-responsiveness to the antigen upon subsequent encounter (21). Although immunity (humoral and cell-mediated responses) develops for most mucosal infections, a robust adaptive immune response against commensal microbiota and food antigen is not desirable. The normal gut is remarkably adept at limiting immune responses to such antigens. Therefore, development of mucosal non-responsiveness either via tolerance or ignorance to external non-self antigens, which gain access via a natural route (orally) under physiological conditions (non-inflammatory), is beneficial to the host. Consequently, specific suppression of cellular or humoral immune responses to an antigen previously delivered by the oral route is a natural immunologic event termed "oral tolerance" (21).

Current dogma suggests that the mechanism behind oral tolerance induction depends on the feeding dose and duration employed. At least two mechanisms exist for oral tolerance, which are not mutually exclusive. High-dose feeding protocols are thought to lead to clonal

deletion or anergy of antigen-specific $CD4^+$ T cells (12, 21, 29, 56, 76). While low-dose continuous feeding protocols have been shown to generate regulatory T cells that mediate suppression of antigen-specific $CD4^+$ T cells via cell-cell contact or secretion of suppressive cytokines TGF-β and IL-10 (20, 22, 59, 95). However, well-controlled comparisons of feeding protocols have not been published, and the same feeding protocol may, in fact, induce multiple mechanisms of oral tolerance (21, 56).

Oral delivery of the same antigen that induces oral tolerance, when co-delivered with an adjuvant leads to immunity. Hence, whether incidental enteric infection can act as an adjuvant producing immunity instead of tolerance was investigated. Previous studies by Nagler-Anderson et al. have demonstrated prevention of oral tolerance induction due to infection with the helminth *Heligsomoides polygyrus* (71). The proposed mechanism is via a T_h2-polarized adjuvant effect as a result of the T_h2-biased infection. This infection adjuvanticity was mediated through up-regulation of co-stimulatory molecules on local antigen presenting cells (APC) (72).

To determine whether an immunomodulatory effect of subclinical persistent infection with *H. hepaticus* alters systemic tolerogenic responses to orally delivered antigen, *H. hepaticus* infected C57BL/6 mice were fed a single high dose of ovalbumin (OVA) or PBS as a control, then subjected to peripheral immunization with OVA in Complete Freud's Adjuvant (CFA). Additionally, it was determined whether *H. hepaticus* infection would prevent oral tolerance induced via continuous feeding of high doses of antigen (1% OVA in drinking water). In *Helicobacter*-free C57BL/6 mice, both feeding doses resulted in tolerance induction, although continuous feeding was more efficient at reducing the peripheral recall response to OVA. Persistent *H. hepaticus* infection in PBS-fed mice resulted in a modest reduction of OVA-specific recall responses in both experiments. *H. hepaticus* infection affected the outcome of

single high-dose feeding by preventing tolerance induction, but not with continuous antigen feeding. Induction of oral tolerance in mice lacking IL-10 was evaluated since regulatory T cells have been implicated in oral tolerance induction specifically with continuous feeding regimens (20, 23, 59, 73). Oral tolerance via either a single or continuous feeding of a high dose of OVA in C57BL/6 mice is IL-10 independent.

Results

Modest oral tolerance induction in C57BL/6 mice when soluble antigen delivered via a single high dose

Oral tolerance via a single 25mg oral dose of OVA has been demonstrated in BALB/c mice (70-72). The feasibility of oral tolerance induction in C57BL/6 mice using the same method of administration was evaluated. One week after feeding, mice were immunized with OVA in CFA. Three weeks after immunization, cells from the draining popliteal and inguinal lymph nodes (PLN) were re-stimulated with OVA in vitro to measure the magnitude of response via production of cytokines. PLN of *Helicobacter*-free mice re-stimulated with OVA produced less IFN-γ when fed a single high-dose of OVA than when fed PBS, $P < 0.001$ (Figure 3-1 A). In contrast to IFN-γ, a T_h1 cytokine, IL-13, a T_h2 cytokine, was low and not different between feeding groups (Figure 3-1 B). In addition to cytokine secretion in a recall response, primary evidence of oral tolerance *in vivo* was evaluated by production of OVA-specific antibodies after immunization with OVA. In *Helicobacter*-free mice, OVA-specific IgG2c titers were reduced when mice had been fed a single high dose of OVA compared with PBS fed mice, $P < 0.001$ (Figure 3-2 A). No difference was observed in OVA-specific IgG1 titers, which were low compared with IgG2c (Figure 3-2 B). Immunization with antigen using CFA as an adjuvant is

known to produce a robust T_h1-biased immune response. Therefore, the order of magnitude greater OVA-specific T_h1-dependent cytokine production and serum IgG2c antibody titers were anticipated. In similar regard, the previous studies in BALB/c mice used incomplete Freud's adjuvant (IFA) to generate a T_h2-biased immune response (2). Immunization after feeding boosts the immune response allowing for easier detection of differences in OVA-specific titers. Use of C57BL/6 mice, thought to be T_h1-prone, to investigate the effects of *H. hepaticus* infection, also thought to be more T_h1-prone than T_h2, lead to use of CFA rather than IFA for an adjuvant. With this caveat, in C57BL/6 mice single high-dose oral administration of OVA partially, but not completely, prevents an immunogenic response to immunization with OVA in CFA.

Persistent *H. hepaticus* infection in C57BL/6 mice prevents induction of oral tolerance via a single high dose of soluble antigen

In comparison to *Helicobacter*-free PBS-fed mice, IFN-γ production was consistently lower in PLN cells from *H. hepaticus* infected PBS-fed mice (Figure 3-1 A). This is an interesting, but as yet unexplained phenomenon. Although, immune responses in OVA-fed mice were equivalent in *Helicobacter*-free and *H. hepaticus* infected mice, the effect of infection on immune response to OVA in CFA influenced the interpretation of the results. Therefore, in *Helicobacter*-free mice, OVA feeding suppressed subsequent immune responses to OVA. In contrast, with *H. hepaticus* infection immune responses to OVA were primed by OVA feeding as PLN IFN-γ production from *H. hepaticus* infected OVA-fed mice was higher than PBS fed counterparts, $P < 0.01$ (Figure 3-1 A). As shown in Figure 1 B, IL-13 production was not different between treatment groups. *In vivo* evaluation of oral tolerance by OVA-specific serum

IgG2c antibody titers demonstrated that tolerance was not induced in *H. hepaticus* infected OVA-fed mice, as titers in *H. hepaticus* infected mice fed OVA were comparable to those fed PBS, $P > 0.05$, (Figure 3-2 A). As seen in uninfected mice, serum IgG1 titers specific to OVA were not different between treatment groups (Figure 3-2 B).

Continuous delivery of high doses of antigen effectively induces oral tolerance

PLN cells from *Helicobacter*-free mice given OVA-free drinking water produced IFN-γ (4.32 +/- 0.14 ng/ml) upon re-stimulation *in vitro* (Figure 5 A). In contrast, continuous feeding of 1% OVA drinking water were tolerized, as PLN cells from *Helicobacter*-free mice did not produce IFN-γ (0.03 +/- 0.03 ng/ml) when stimulated *in vitro*, $P < 0.001$ (Figure 3-5 A). This tolerogenic effect of OVA feeding on T_h1 cytokine production did not production tolerance of the T_h2 cytokine IL-13 (Figure 3-5 B). *In vivo* measurement of oral tolerance through OVA-specific IgG2c titers was also suppressed in mice that received 1% OVA water compared with OVA-free drinking water, $P < 0.01$ (Figure 3-2 C). The T_h2-dependent IgG1 OVA-specific titers were not different between feeding groups (Figure 3-2 D). Since robust oral tolerance was induced in C57BL/6 mice by continuous feeding of 1% OVA water, the effect of *H. hepaticus* infection on induction of oral tolerance via this feeding regimen was assessed.

Persistent *H. hepaticus* infection does not prevent oral tolerance via continuous high dose OVA feeding

In contrast to results from single high dose feeding, *H. hepaticus* infected mice continuously fed a high dose of OVA did not produce IFN-γ in re-stimulated PLN cells, $P < 0.001$ compared to *H. hepaticus* infected PBS-fed mice (Figure 3-5 A). Similar to *Helicobacter*-

free mice, no difference in OVA-specific production of IL-13 was found (Figure 3-5 B). The OVA-specific serum IgG2c antibody response appeared tolerized in *H. hepaticus* infected OVA-fed mice, however it was only statistically significant when compared with *Helicobacter*-free PBS fed mice, $P < 0.01$, not with *H. hepaticus* infected PBS-fed mice, $P > 0.05$ (Figure 3-2 C). Analogous to *Helicobacter*-free mice, OVA-specific IgG1 titers were not tolerized in 1% OVA fed *H. hepaticus* infected mice (Figure 3-2 D). Interestingly, *H. hepaticus* infection decreased IFN-γ production in PLN of drinking water mice, similar to the finding in *H. hepaticus* infected PBS-fed controls in the single high dose tolerance studies, warranting further study into the effects of *H. hepaticus* on systemic responses to parenteral immunization (Figure 3-5 A). A lack of inflammation in the lower bowels of the mice in both feeding experiments was confirmed, as it could be a mechanism by which *H. hepaticus* prevented oral tolerance induction and instead primed the immune system for increased IFN-γ production. Additionally, numbers of naturally-occurring regulatory T cells, which express Foxp3, in the cecum and colon were assessed because studies have suggested they play a role in continuous feeding oral tolerance.

Neither active inflammation nor tissue Foxp3$^+$ T cells were associated with induction or prevention of oral tolerance via a single high-dose of OVA in wild type C57BL/6 mice

Histological evaluation of cecum and colon revealed tissue lesions, including inflammation, in *H. hepaticus* infected mice were not significantly different from *Helicobacter*-free mice (Table 3-1). Thus, *H. hepaticus* infection primed for a T_h1 response to a single high dose of orally administered OVA in the absence of a visible intestinal inflammatory response. Whether a single high dose of OVA increased numbers of naturally-occurring regulatory T cells (Foxp3$^+$ nT_{reg}) present in cecal or colon tissue was determined. Foxp3$^+$ cell numbers were not

increased in local tissue of OVA fed (11 $Foxp3^+$ cells per mm^2 cecum and 2 $Foxp3^+$ cells per mm^2 colon) compared with PBS fed *Helicobacter*-free mice (18 $Foxp3^+$ cells per mm^2 cecum and 2 $Foxp3^+$ cells per mm^2 colon, Figure 3-3 A). In PBS fed mice, *H. hepaticus* infection caused a decrease in the number of cecal, but not colonic $Foxp3^+$ cells per mm^2 tissue (6 and 3, respectively). Compared with *Helicobacter*-free OVA fed mice, *H. hepaticus* infected mice had a trend for increased cecal numbers of $Foxp3^+$ cells that was not significant, however no change in colonic $Foxp3^+$ cell numbers were seen in single dose OVA fed *H. hepaticus* infected mice (Figure 3-3 A). An increase in cecal $Foxp3^+$ cells was seen in *H. hepaticus* infected mice when they were fed OVA, $P < 0.05$. Changes in cecal $Foxp3^+$ cells numbers, although significantly different were not associated with either tolerance or infection. Since Foxp3 is not an exclusive marker for all regulatory T cell subsets, such as IL-10 secreting induced regulatory T cells (T_r1), the role of the suppressive cytokine IL-10 was evaluated.

Induction of oral tolerance via a single high dose of soluble antigen in IL-10-deficient mice suggests IL-10 independent mechanism of oral tolerance

To evaluate the role of IL-10 in oral tolerance induced via a single high dose of OVA, a pilot study was conducted in which C57BL/6 IL-10-deficient mice were fed either PBS or OVA. Results of this study are preliminary, as group size was small and study was not repeated due to limited availability of *Helicobacter*-free C57BL/6 IL-10-deficient mice. Most likely because of technical reasons, IFN-γ production was not detected in PLN cells from *Helicobacter*-free PBS fed mice. However, in this pilot study, *in vivo* tolerance was evaluated, along with the effect of *H. hepaticus* infection on oral tolerance induction. In *Helicobacter*-free mice, OVA feeding via a single high dose did not result in a decrease of OVA-specific IgG2c or IgG1 (Figure 3-4 A and

B). However, there was a trend for lower IgG2c titers in the *Helicobacter*-free OVA-fed mice compared with PBS-fed. Additionally, in contrast to wild type mice, IL-10-deficient mice infected with *H. hepaticus* showed a trend for oral tolerance after OVA feeding (Figure 3-4 A). Given the limited number of animals in this pilot study, the results suggest that a single high dose of OVA does result in tolerance in C57BL/6 IL-10 deficient mice. Additionally, *H. hepaticus* infection appeared to have no affect on tolerance induction.

Oral tolerance induced by continuous feeding of high doses of antigen does not depend on IL-10

Continuous feeding of 1% OVA has been shown to induce regulatory T cells, therefore induction of oral tolerance via this feeding protocol was attempted in C57BL/6 mice deficient in IL-10 (23, 59). IL-10-deficient mice are known to develop spontaneous colitis after 2 months of age on the C57BL/6 background, depending on microbiota status and facilities, therefore histological evaluation of cecum and colon was conducted. At the time of evaluation, 3 months of age, there were no consistent histological changes observed in either the cecum or colon of *Helicobacter*-free mice (Table 3-1). *In vitro* OVA re-stimulation assays had inconsistent results in repeated studies (data not shown) possibly due to high variation in T_h1 responses from the lack of IL-10 or technical issues. However, *in vitro* antibody data were consistent. OVA-specific IgG2c titers were tolerized in mice fed 1% OVA water compared with that received only drinking water, $P < 0.05$ (Figure 3-4 C). Similar to studies in wild type mice, OVA-specific IgG1 titers in mice continuously fed 1% OVA were not significant (Figure 3-4 D). Since 1% OVA in the drinking water induced oral tolerance in C57BL/6 IL-10 deficient mice, the ability of *H. hepaticus* infection to prevent induction was evaluated.

H. hepaticus infected IL-10 deficient mice are orally tolerized to OVA given continuously at a high dose

Infection of C57BL/6 IL-10 deficient mice with *H. hepaticus* has previously been shown to result in typhlocolitis beginning around 2 months post-inoculation (43, 64). To reduce the likelihood of typhlocolitis, all experiments in IL-10 deficient mice were shortened to only one month of persistent *H. hepaticus* infection at the time of OVA feeding, which resulted in limited mild to moderate cecal and colonic lesions in 2 of 9 mice (Table 3-1). Comparison of OVA-specific IgG2c titers revealed tolerance induction in *H. hepaticus* infected mice fed 1% OVA drinking water when compared with infected mice given OVA-free water (Figure 3-4 C) and no difference in OVA-specific IgG1 titers (Figure 3-4 D).

Foxp3$^+$ cell numbers in cecum or colon of IL-10-deficient mice were only affected by *H. hepaticus* infection, not OVA feeding protocol

Induction of oral tolerance in *Helicobacter*-free C57BL/6 IL-10-deficient mice via a single high dose of OVA was not mediated by an increase in numbers of naturally occurring regulatory T cells (Foxp3$^+$) in either the cecum or colon, $P > 0.05$ (Figure 3-3 B). Additionally, oral tolerance induction by continuous high-dose feeding in IL-10-deficient mice did not change the mucosal Foxp3$^+$ cell numbers (Figure 3-3 B). Several IL-10-deficient mice infected with *H. hepaticus* had elevated numbers of Foxp3$^+$ cells giving the infected groups increased numbers of Foxp3$^+$ cells in the cecum (Figure 3-3 B). However, only the single feeding groups were different from each other (5 Foxp3$^+$ cells per mm^2 cecum in *Helicobacter*-free and 22 Foxp3$^+$ cells per mm^2 cecum in *H. hepaticus* infected, $P < 0.05$). Similarly, colons of *H. hepaticus*

infected mice fed a single high dose had elevated Foxp3$^+$ cells compared with *Helicobacter*-free counterparts, $P < 0.05$. Although, when compared to *H. hepaticus*-infected PBS fed mice there was no significant differences in either cecum or colon.

Discussion

Oral tolerance is a means for the host to prevent undesirable immunogenic responses to antigens in frequent contact with mucosal immune compartments. Using oral tolerance as a tool to observe the effects of a persistent infection on the mucosal immune response has provided evidence for modulation of the natural tolerogenic response by persistent enteric infection. *H. hepaticus* was able to prevent induction of tolerogenic responses to a single high dose of OVA. Upon a single feeding of 25mg of OVA to *Helicobacter*-free mice, T_h1-dependent IFN-γ recall response to OVA *in vitro* was halved resulting in a modest tolerance induction. In contrast, this recall response in *H. hepaticus* infected mice was not tolerized, instead the infection primed for increased IFN-γ production. *In vivo* antibody titers confirmed the prevention of tolerance induction as *H. hepaticus* infected mice had comparable OVA-specific antibody titers regardless of OVA feeding. These results indicate that the presence of a persistent enteric infection such as *H. hepaticus* can potentiate the host's response of oral tolerance to an antigen when exposed once at a high dose.

Dose and duration of exposure to an antigen can affect the mechanism of oral tolerance induction, either anergy/clonal deletion or active suppression by one or multiple regulatory T cell subsets (12, 21, 22, 29, 95). Therefore, the ability of *H. hepaticus* to prevent oral tolerance was evaluated at a comparable high dose of OVA, but for a continuous 5-day duration. In contrast to a single exposure to soluble oral antigen, continuous feeding of the antigen caused more robust

tolerance demonstrated by a complete inhibition of IFN-γ production during *in vitro* recall response, as well as suppression of OVA-specific antibodies. Tolerance induced by this feeding protocol was not altered by *H. hepaticus* infection, indicating that continuous exposure to high doses of soluble antigen leads to tolerogenic responses in spite of a persistent enteric infection.

Given the fact that *H. hepaticus* infection revealed different mechanism for bolus and continuous feeding oral tolerance, the role of IL-10 in each was investigated. Single feeding was only attempted once, and although in the one experiment the trend that oral tolerance can be induced in IL-10-deficient mice did not reach significance. Tolerance to soluble OVA through continuous feeding was also intact in IL-10-deficient mice. Although in studies using a different model antigen and continuous low-dose feeding, similar results were found in IL-10-deficient mice (33). With tolerance inducible in IL-10 deficient mice, whether T_h1 responses to *H. hepaticus* infection affected tolerance be evaluated, particularly because IL-10 deficient mice develop disease when infected with *H. hepaticus*.

Mice deficient in IL-10 also lack active suppression of immune responses, particularly towards T_h1 cells, thereby any effect of this suppression during either tolerance induction or *H. hepaticus* infection are negated. Since the single dose experiment was not repeated, conclusive statements cannot be formed. However they suggest that IL-10 may play a role in *H. hepaticus* preventing oral tolerance via single exposure to a high dose of antigen because the *in vivo* antibody response has a trend towards tolerance in the *H. hepaticus* OVA-fed mice. In this model, IL-10 is likely involved in the host response to *H. hepaticus* instead of tolerance induction, as tolerance was intact.

Continuous exposure of soluble OVA at a high dose resulted in immunologic tolerance to OVA, even in IL-10-deficient mice infected with *H. hepaticus*. Although the duration of

infection in the experiment was shortened in order to decrease the likelihood of *H. hepaticus* induced disease in IL-10 deficient mice, in one of two experiments half of the infected mice had developed disease by 2 MPI. However, both studies showed comparable results even though some mice exhibited active intestinal inflammation. Active inflammation creates an environment in the mucosa that is amenable to immunogenic, not tolerogenic responses. Yet, in IL-10-deficient mice infected with *H. hepaticus* a robust tolerogenic response resulted from continuous antigen exposure. This finding is intriguing, but requires carefully controlled studies that also examine the local mucosal responses in more detail.

Taken together, these studies support the notion that duration of exposure to a soluble antigen does cause different immunologic responses that all lead to induction of the phenomenon of oral tolerance. Additionally, in certain instances, such as a single high level of exposure to an antigen, persistent enteric infection can interfere with the natural mucosal immune responses, thereby preventing tolerance induction. Further studies characterizing the mucosal responses to these different exposures, both antigen and infection, will aid in understanding the interplay between host and pathogen with regard to tolerogenic versus immunogenic responses.

Tables and Figures

Table 3-1. Cecal and colonic lesions only developed in a few *H. hepaticus* infected IL-10-deficient mice. *H. hepaticus* infection of C57BL/6 mice did not result in cecal or colonic lesions. No *Helicobacter*-free IL-10-deficient mice had cecal or colonic lesions at 3-4 months of age. Some IL-10-deficient mice infected with *H. hepaticus* for 2 months developed cecal and colonic lesions.

C57BL/6

	Inflammation	Edema	Epithelial Defects	Hyperplasia	Dysplasia
Uninfected/PBS: Cecum	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Colon	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Uninfected/25mg OVA: Cecum	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Colon	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Uninfected/1% OVA: Cecum	N/A	N/A	N/A	N/A	N/A
Colon					
H. hepaticus/PBS: Cecum	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Colon	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
H. hepaticus/25mg OVA: Cecum	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Colon	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
H. hepaticus/1% OVA: Cecum	N/A	N/A	N/A	N/A	N/A
Colon					

C57BL/6 IL-10$^{-/-}$

	Inflammation	Edema	Epithelial Defects	Hyperplasia	Dysplasia
Uninfected/PBS: Cecum	0 (0-0)	0 (0-0.5)	0 (0-0)	0 (0-0)	0 (0-0)
Colon	0 (0-0.5)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Uninfected/25mg OVA: Cecum	0 (0-0)	0 (0-0.5)	0 (0-0)	0 (0-0)	0 (0-0)
Colon	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Uninfected/1% OVA: Cecum	0 (0-0)	0 (0-0.5)	0 (0-0)	0 (0-0)	0 (0-0)
Colon	0 (0-1.0)	0 (0-0)	0 (0-0)	0 (0-1.0)	0 (0-0)
H. hepaticus/PBS: Cecum	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0.5)	0 (0-0)
Colon	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
H. hepaticus/25mg OVA: Cecum	0 (0-2.0)	0 (0-1.0)	0 (0-1.0)	0 (0-2.5)	0 (0-0.5)
Colon	0 (0-0)	0 (0-0)	0 (0-1.0)	0 (0-2.0)	0 (0-0)
H. hepaticus/1% OVA: Cecum	0 (0-3.0)	0 (0-1.0)	0 (0-1.0)	0 (0-2.0)	0 (0-0)
Colon	0 (0-2.5)	0 (0-1.0)	0 (0-2.0)	0 (0-2.0)	0 (0-0.5)

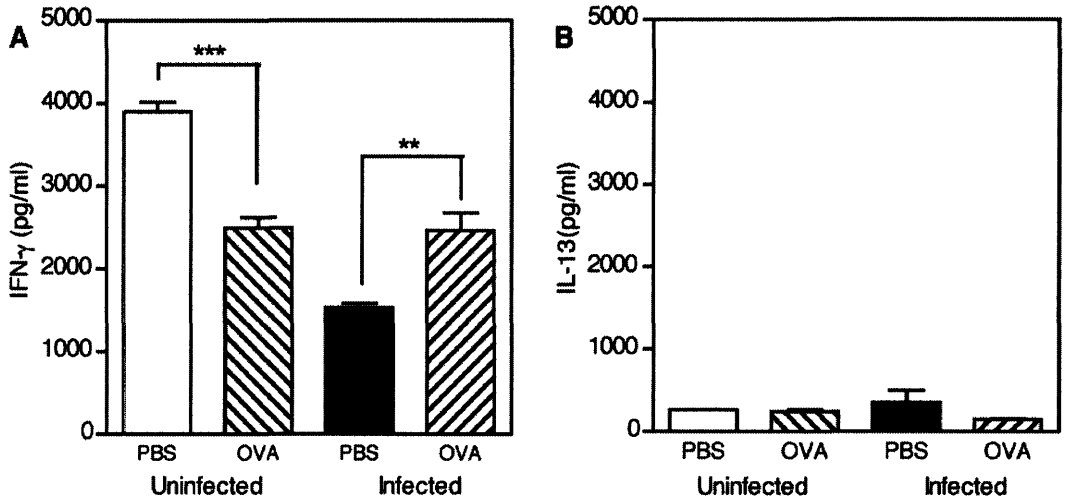

Figure 3-1. A single high dose of ovalbumin reduced IFN-γ production upon re-stimulation of PLN from *Helicobacter*-free mice, but not *H. hepaticus* infected C57BL/6 mice. Cells of the popliteal and inguinal lymph nodes (PLN) from *Helicobacter*-free mice fed PBS produced IFN-γ, but PLN from mice fed a single 25mg dose of OVA had decreased IFN-γ production (**A**). Infection with *H. hepaticus* reduced the IFN-γ production by PLN from mice fed PBS. Compared with *H. hepaticus* infected mice fed PBS, feeding of a single 25mg dose of OVA to *H. hepaticus* infected mice resulted in increased IFN-γ production. Regardless of *H. hepaticus* infection, a single 25mg dose of OVA did not affect IL-13 production by PLN (**B**). One-way ANOVA with Bonferroni post-test; ** $P < 0.01$, *** $P < 0.001$.

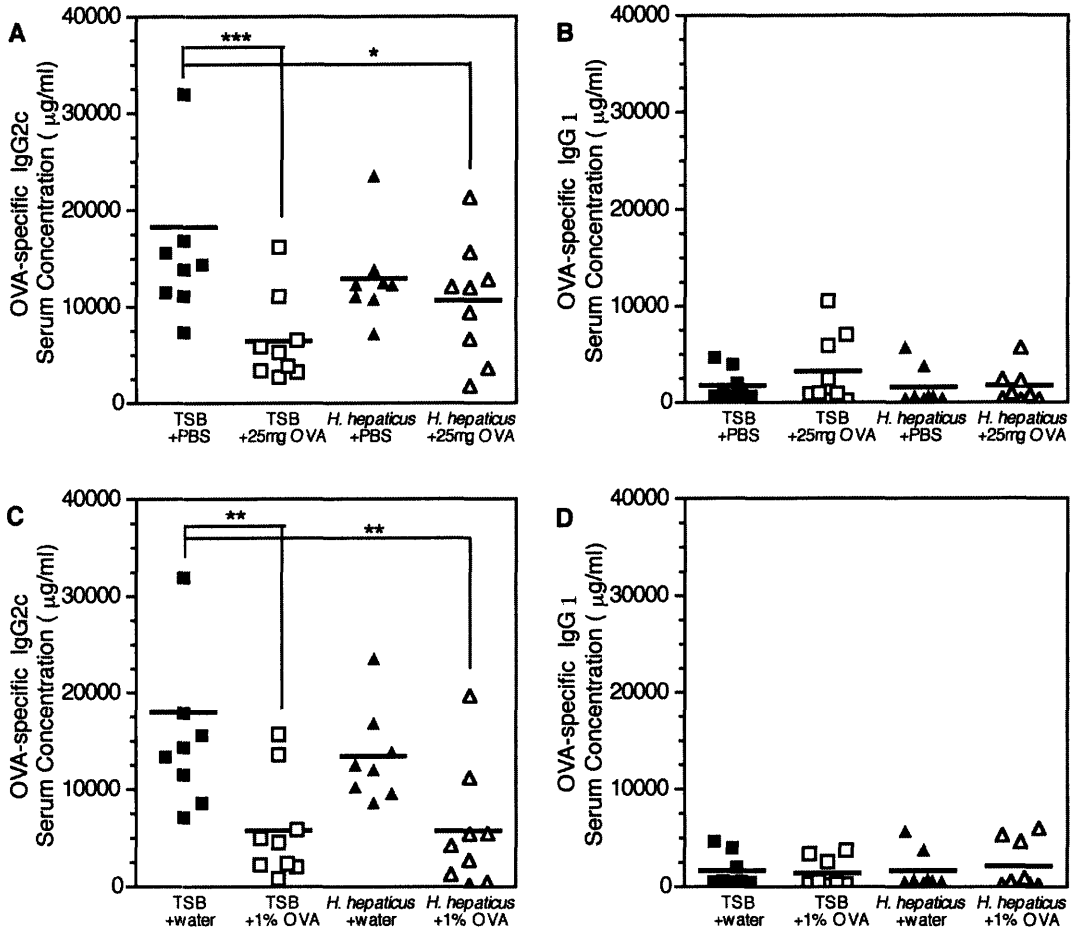

Figure 3-2. Both single and continuous high-dose feeding of ovalbumin reduce OVA-specific IgG2c serum titers after systemic ovalbumin immunization, but persistent H. hepaticus infection prevents the titer reduction from single high-dose feeding. Systemic OVA-specific IgG2c titers (A), but not IgG1 titers (B) were reduced in mice fed a single 25mg dose of OVA, however when mice were persistently infected with *H. hepaticus* feeding of a single 25mg dose of OVA did not result in decreased OVA-specific IgG2c titers. Continuous feeding of 1% OVA water suppressed OVA-specific IgG2c titers (C), but not IgG1 titers (D). *H. hepaticus* infection had no affect on OVA-specific IgG2c or IgG1 titers when mice were continuously fed 1% OVA. One-way ANOVA with Bonferroni post-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

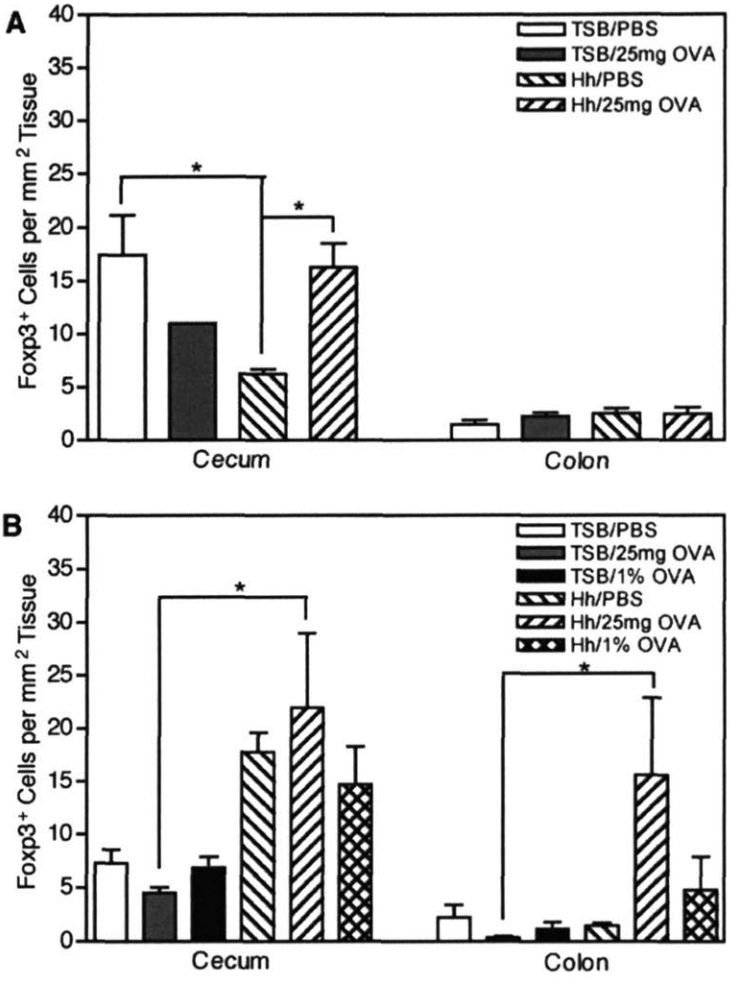

Figure 3-3. Neither *H. hepaticus* infection nor feeding of a single 25mg dose of OVA changed the number of Foxp3$^+$ cells in cecum or colon of C57BL/6 mice, but IL-10-deficient mice infected with *H. hepaticus* had an elevated number of Foxp3$^+$ cells in the cecum and colon. Numbers of Foxp3$^+$ cells in the cecum and colon of C57BL/6 mice remained stable regardless of OVA feeding or *H. hepaticus* infection in the colon, but not the cecum (**A**). A lack of IL-10 did not increase the number of Foxp3$^+$ cells in cecum or colon, regardless of feeding protocol (**B**). *H. hepaticus* infection in IL-10-deficient mice caused an increase in Foxp3$^+$ cells in the cecum and colon (**B**). One-way ANOVA with Bonferroni post-test; * $P < 0.05$.

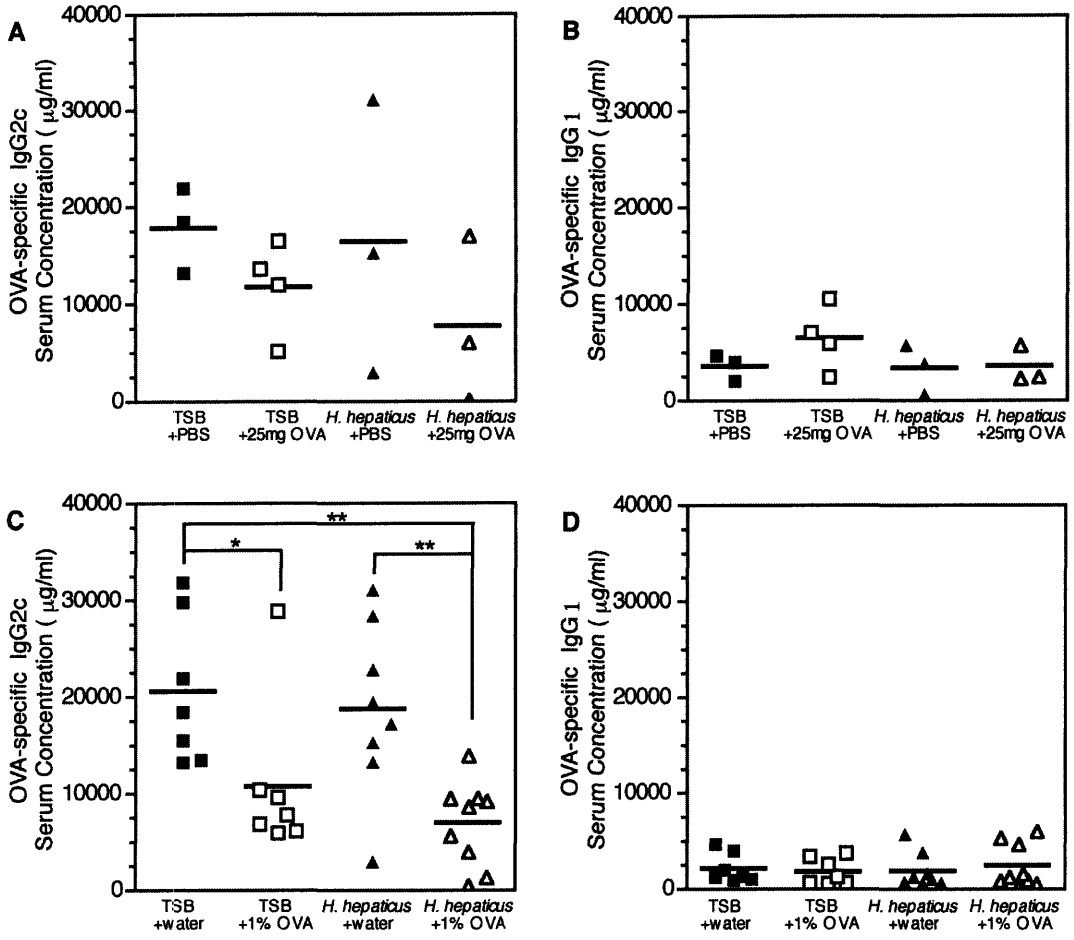

Figure 3-4. In IL-10-deficient mice, continuous high-dose feeding of ovalbumin reduce OVA-specific IgG2c serum titers after systemic ovalbumin immunization, which is unaffected by persistent *H. hepaticus* infection. With a small number of IL-10-deficient mice, feeding a single 25mg dose of OVA showed a trend for reduced systemic OVA-specific IgG2c titers (**A**), but not IgG1 titers (**B**). When IL-10-deficient mice were persistently infected with *H. hepaticus,* feeding of a single 25mg dose of OVA also had a trend for decreased OVA-specific IgG2c titers. Continuous feeding of 1% OVA water to IL-10-deficient mice suppressed OVA-specific IgG2c titers (**C**), but not IgG1 titers (**D**). Persistent *H. hepaticus* infection of IL-10-deficient mice continuously fed 1% OVA had no affect on OVA-specific IgG2c or IgG1 titers. One-way ANOVA with Bonferroni post-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

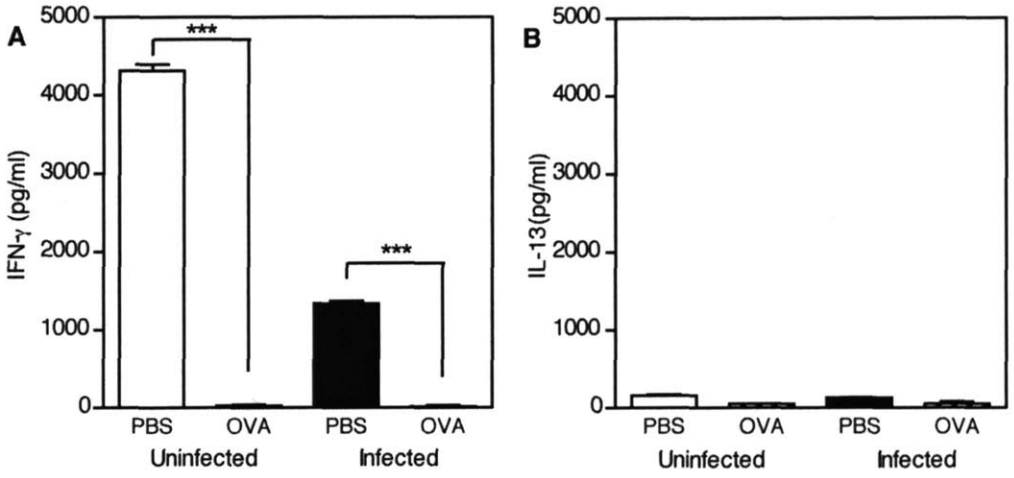

Figure 3-5. Continuous feeding of a high dose of ovalbumin reduced IFN-γ production upon re-stimulation of PLN from *Helicobacter*-free and *H. hepaticus* infected C57BL/6 mice. PLN from *Helicobacter*-free mice fed PBS produced IFN-γ, but PLN from mice fed 1% OVA water had decreased IFN-γ production (**A**). Infection with *H. hepaticus* reduced the IFN-γ production by PLN from mice fed PBS. Compared with *H. hepaticus* infected mice fed PBS, feeding of 1% OVA water to *H. hepaticus* infected mice also suppressed IFN-γ production. Regardless of *H. hepaticus* infection, feeding of 1% OVA water did not affect IL-13 production by PLN (**B**). One-way ANOVA with Bonferroni post-test; *** $P < 0.001$.

Chapter 4

Persistent *Helicobacter hepaticus* infection does not prevent development of protective immunity to *Citrobacter rodentium*

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Introduction

Children are at highest risk for acute diarrheal disease with an estimated 17% of mortality in children under 5 years of age (7). Vaccines against some of the most common causes of diarrhea have not been successfully developed, partly due to a lack of efficient induction of mucosal immune responses. In the mucosa, typically the primary site of infection and disease, an effective vaccine will induce secretion of high concentrations of pathogen-specific sIgA, IgM, and IgG, along with mucosal cell-mediated immunity (35, 61). Engendering strong protective humoral and cell-mediated responses at the desired mucosal site is not trivial. Induction of protective immune responses to enteric pathogens usually requires mucosal vaccination, as it is more efficient than parenteral immunization at inducing immune responses in the mucosa. Due to the difficulty in producing mucosal vaccines, only a few (six as of 2005) mucosal vaccines have been approved for use in the United States or elsewhere, including vaccines against enteric infections with poliovirus, rotavirus, *Salmonella typhi*, and *Vibrio cholerae* (35, 61).

One strategy to improve mucosal vaccine efficacy is through alternative formulation or administration route of antigens and adjuvants. Prime/boost strategies have been widely studied for both parenteral and oral delivery as a method to increase cell-mediated responses (44, 91). Parenteral priming with the antigen(s) of interest followed by boosting with the same antigen with a different adjuvant allows for presentation of the same antigen to the immune system in two different contexts, which can lead to synergistic effects on immunity (44, 91). Application of this immunization strategy to oral vaccination decreases the tendency for oral non-responsiveness through tolerance induction or ignorance, as well as enhancing the mucosal immune response. This prime/boost strategy with oral vaccination may offer a solution to

overcome the barrier of decreased immunogenicity observed with many oral vaccines in developing countries (45).

A possible, but as yet largely unstudied, reason for reduced oral vaccine efficacy is the effects of persistent bacterial infection on vaccine efficacy (45). Establishing an animal model in which both immunization efficacy to an enteric infection, along with the effects of a unrelated persistent infection could be examined will help address the issue of reduced vaccine efficacy. In such a model, biomarkers for efficacious vaccination during concurrent infection could be defined, as well as how concurrent mucosal infection influences immunogenic responses to unrelated pathogens. Compared with the number of pathogens to which humans are exposed, a single persistent infection is reductionist, yet, it would provide a more realistic scenario than current vaccine models potentially leading to improved oral vaccines for use in developing countries.

The use of animal models to study vaccines or interventions for diarrheagenic *E. coli* infections, a common enteric pathogen in developing countries, is difficult as there is a lack of an appropriate mouse model. Recently, *Citrobacter rodentium*, a murine pathogen related to enteropathogenic *E. coli* (EPEC), has proven useful for studying virulence and pathogenesis of acute diarrheal disease through *in vitro* and *in vivo* studies. *C. rodentium* clinical disease is similar, although not identical, to EPEC including loose stool progressing to diarrhea in susceptible strains of mice with associated weight loss and poor body condition (34, 49, 51, 60). Although C3H/HeJ and FVB strains of mice succumb to death from *C. rodentium* infection within 2 weeks, little is known about disease with respect to immunology in these strains, partly because of the high mortality (3, 82). Conversely, much more is known about the immunology of *C. rodentium* infection in C57BL/6 mice, in which *C. rodentium* causes self-limiting cecal and

colonic disease including hyperplasia, edema, destruction of the epithelial barrier, and inflammation. (34, 49, 51, 60) Without confounding factors, such as a compromised immune system, C57BL/6 mice recover from *C. rodentium* infection around three weeks after acquisition. (49, 60) Similar to EPEC infection in humans, mortality rates in C57BL/6 mice infected with *C. rodentium* are much greater early in life. Susceptibility to infection and disease during adulthood, makes *C. rodentium* a useful model for EPEC vaccination, since vaccine efficacy study are typically conducted in adult populations. To date, only two studies have evaluated protective immunity to *C. rodentium.* In one study using C3H/HeJ mice, three-dose intranasal or subcutaneous immunization with purified protein antigen (Int280α) with or without adjuvant followed by challenge with *C. rodentium* gave a 2-log protection from bacterial burden, at best, with a decrease in wet colon weight 13-16 days post-challenge (32). In the same study, protective immunity to *C. rodentium* was assessed with naïve and convalescent C3H/HeJ mice demonstrating that prior infection reduced bacterial loads and colon weight 11 days post-re-challenge. The second study found that prior infection with *C. rodentium* caused clearance of a subsequent *C. rodentium* within one week in both C57BL/6 pIgR-deficient mice (lack secretory IgA and IgM) and C57BL/6 mice (81). The field of EPEC vaccines would be significantly advanced by the development of an effective vaccination for *C. rodentium*, a mouse model for EPEC, particularly if produced in the presence of a persistent infection.

To determine whether a persistent infection, such as *H. hepaticus*, affects the efficacy of immunization against an acute pathogen, *C. rodentium*, an efficacious immunization method was needed. As *C. rodentium* is a mucosal enteric pathogen, antibodies that cross the epithelial barrier, such as sIgA and IgG, are key to the first line of protection. Although oral vaccines are preferred for ease of delivery, they typically have poor efficacy compared with their

subcutaneous or intramuscular counterparts. To verify whether parenteral or mucosal vaccination provides greater efficacy for *C. rodentium* immunization, two prime/boost vaccination strategies were tested: one utilizing parenteral and oral vaccination (subcutaneous [s.c.] prime followed by a three-dose oral boost) and one with only parenteral vaccination (s.c. prime followed by a single s.c. boost). Initially, these two immunization strategies were evaluated in *Helicobacter*-free mice to determine the effect on: 1) *C. rodentium*-specific antibodies; 2) disease development; 3) weight loss; and 4) bacterial burden. Neither immunization strategy provided enough protection to assess the effects of *H. hepaticus* infection, therefore prior *C. rodentium* infection was evaluated and used as the benchmark for protective immunity. Evaluation of the effects of a persistent infection on host response should include determination of whether the persistent infection prevents development of natural immunity (via recovery from prior infection). *H. hepaticus* infection did not affect the bacterial burden upon re-challenge with *C. rodentium.* Therefore, no other effects of persistent *H. hepaticus* infection observed, such as weight loss or colonic disease, since clinical disease associated with *C. rodentium* is correlated with bacterial burden.

Results

Immunization against *C. rodentium* produced *C. rodentium*-specific serum IgG1 and IgG2c

Generation of pathogen-specific immunoglobulins provides the host with an early, yet specific, line of defense against subsequent infection with the same pathogen. Therefore, for most pathogens an efficacious vaccine must cause the host to generate pathogen-specific immunoglobulins. *C. rodentium*-specific IgG1 and IgG2c in the serum accounted for 10% and 1%, respectively, of total immunoglobulin isotype when mice were immunized via subcutaneous

prime with alum as an adjuvant followed by three oral boosts with cholera toxin as an adjuvant (s.c. prime/oral boost) (Figure 4-1 A and B). Immunization of mice with a subcutaneous dose of *C. rodentium* homogenate in alum followed by a subcutaneous immunization with only homogenate (s.c. prime/s.c. boost) of *C. rodentium* homogenate resulted in comparable *C. rodentium*-specific titers (1% of total IgG2c and 10% of total IgG1) at 4 weeks post-primary immunization. Additionally, seroconversion between males and females within each group was similar. As expected for pre-*C. rodentium* challenge antibody titers, IgG1 titers were higher than IgG2c titers due to the T_h2-biased adjuvants (alum and cholera toxin) used for both subcutaneous and oral immunizations. Quantities of immunoglobulins that cross the mucosal barrier, as opposed to serum titers, are believed to be of importance for mucosal pathogens, such as *C. rodentium*, as initial defense occurs in the mucosa. Serum IgGs were measured as they are known to be required for eradication of *C. rodentium* (6, 50, 74, 81). The role of secretory IgA (sIgA), typically the most abundant immunoglobulin in the mucosa, is not clear, and could play a role as it was not measured. Since serum IgG1 and IgG2c were equivalent between vaccination groups, the efficacy of the immunizations was assessed via challenge with *C. rodentium.*

Subcutaneous prime/oral boost immunization reduced *C. rodentium* bacterial burden in 50% of mice

Upon challenge with *C. rodentium*, naïve mice had bacterial burdens of 10^8 CFU/g feces at 3 days post-inoculation (DPI) (Figure 4-2 A). By 6 DPI peak bacterial burdens of 10^9 CFU/g feces were reached, and this burden was maintained through 14 DPI, the end of the experiment. Mice that received the s.c. prime/s.c. boost immunization strategy were not protected from infection, as they had comparable bacterial loads to naïve mice (Figure 4-2 A). In the s.c.

prime/oral boost group the cage of male mice (M) had 10^7 CFU/g feces at 3 DPI, but shedding continued to increase reaching 10^9 CFU/g feces by 9 DPI. However, the female mice (F) given s.c. prime/oral boost immunization strategy were protected from sustained bacterial shedding with *C. rodentium* burdens below the detection threshold (10^3 CFU/g feces) by 9 DPI despite having 10^6 CFU/g feces at 3 DPI, $P < 0.001$ (Figure 4-2 A). Given the variable responses to the immunization strategies, weight was monitored for the duration of *C. rodentium* challenge.

Subcutaneous prime/oral boost immunization strategy reduced weight loss associated with *C. rodentium* clinical disease

Weight loss, used as a measure of clinical disease, in naïve mice challenged with *C. rodentium* occurred through the end of the study at 14 DPI. Between 3 DPI and 14 DPI naïve challenged mice lost 5% of their body weight compared with a 2% weight gain in mice unchallenged vehicle controls (received adjuvant with PBS only) as well as unchallenged s.c. prime/oral boost immunization controls, $P < 0.05$ (Figure 4-2 B). Subcutaneous prime/s.c. boost immunization did not prevent nor delay weight loss due to *C. rodentium* infection, as weight loss in these immunized challenged mice was comparable to naïve challenged mice (Figure 4-2 B). Both males and females that received the s.c prime/oral boost immunizations were not protected from weight loss attributable to *C. rodentium* infection; weight loss between 3 DPI and 14 DPI (2%) was intermediate for both males and females and was not statistically significant from naïve challenged, s.c. prime/s.c. boost challenged, immunized unchallenged or vehicle unchallenged mice (Figure 4-2 B). Histological evaluation of colon disease in challenged mice was completed to determine the extent of protection provided by each immunization strategy.

Early clearance of *C. rodentium* is associated with reduced colonic disease

At 14 DPI, challenge of naïve mice resulted in marked colon disease, represented by a colon disease index median of 7.75 (5.0-10.5) (Figure 4-3). Immunization via s.c. prime/s.c. boost did not protect mice from *C. rodentium* disease development (Figure 4-3). Colon disease (8.5 [6.5-10.0]) comparable to naïve challenged mice was also observed in the s.c. prime/oral boost M group. However, the s.c. prime/oral boost F group was protected from disease development (2.0 [0.0-3.5], $P < 0.05$) with only minimal lesions at 14 DPI, indicating immunization with *C. rodentium* homogenate has the potential to protect mice from subsequent infection.

The best vaccination efficacy was achieved with the subcutaneous prime/oral boost immunization strategy, which protected 50% of the mice from challenge with *C. rodentium.* In order to successfully evaluate the interaction of a persistent infection on vaccine efficacy, consistent protection in *Helicobacter*-free mice is desired. However, the development of natural protective immunity to *C. rodentium* with and without a persistent *H. hepaticus* infection will give a baseline for an ideal vaccine.

Prior *C. rodentium* infection prevents infection upon re-challenge

Naïve C57BL/6 mice challenged with *C. rodentium* had 10^7 CFU/g feces at 4 DPI that reached a maximum shedding burden of 1.5 x 10^8 CFU/g feces at 8 DPI (Figure 4-4 A). *C. rodentium* burden began to decline around 12 DPI as bacterial shedding had decreased to 10^8 CFU/g feces. Mice previously inoculated with *C. rodentium* six weeks earlier did not shed more than 10^3 CFU/g feces upon re-challenge, $P < 0.01$ (Figure 4-4 A). After peak burdens at 4 DPI, four orders of magnitude below naïve counterparts, the convalescent mice had no detectable

bacteria in their feces (<10^3 CFU/g feces in average) for the remainder of the study. To confirm this protection from sustained infection, weight of individual mice was monitored throughout the study.

Mice previously infected with *C. rodentium* have minimal weight loss that is rapidly re-gained

After initial weight loss between re-challenge and day 4 convalescent mice rapidly re-gained weight, weighing more than their body weight at the day of re-challenge until study end (Figure 4-4 B). Whereas, naïve mice lost comparable amounts of weight during the first four days of challenge followed by sustained weight loss through 11 DPI, $P < 0.01$ (Figure 4-4 B). To determine whether this protection from prolonged weight loss was a result of reduced colon disease severity, the histological lesions were evaluated.

Colon disease was less severe in convalescent mice

Substantial colon disease with a median disease index of 9.0 (8.0-10.5) was found at 11 DPI in naïve mice (Figure 4-5). In comparison, convalescent mice had minimal disease at 11 DPI with a disease index of 1.5 (0.0-4.0), $P < 0.001$ (Figure 4-5). None of the lesions evaluated (epithelial defects, inflammation, hyperplasia, edema, and dysplasia) was preferentially decreased nor constituted the sole component of the disease index of convalescent mice. Consistent generation of protective immunity to *C. rodentium* through prior infection provides a baseline from which to evaluate whether persistent *H. hepaticus* infection disrupts its development.

Using challenge of convalescent mice with *C. rodentium* as a model of protective immunity, mice infected persistently with *H. hepaticus* or *Helicobacter*-free mice were evaluated for development of protective immunity including bacterial burden, weight loss, and colon disease.

Persistent *H. hepaticus* infection does not prevent the development of protective immunity to *C. rodentium*

As primary infection controls for the second *C. rodentium* challenge, a group of mice were left uninoculated during the first *C. rodentium* inoculation, then challenged along with the convalescent mice. These *C. rodentium* naïve mice had bacterial burdens lower throughout the study than previous infections (peak burden of 10^8 CFU/g feces versus 10^9 CFU/g feces in other studies) indicating the strain of *C. rodentium* used for the second challenge was not comparable to other strains with regard to infectivity and resulting disease (Figure 4-6 A). Two strains were utilized to count only *C. rodentium* from the second infection. *Helicobacter*-free mice re-challenged with *C. rodentium* had more than two orders of magnitude lower bacterial shedding at 4 DPI followed by burdens below the threshold of detection (Figure 4-6 A). When mice had a persistent *H. hepaticus* infection and recovered from primary *C. rodentium* infection upon re-challenge they had comparable bacterial burdens (at the threshold of detection, 10^3 CFU/g feces) and clearance as *Helicobacter*-free mice (Figure 4-6 A). As confirmation of protective immunity, weight of individual mice was monitored.

Re-challenge strain of *C. rodentium* did not cause weight loss in naïve mice

The *C. rodentium* strain used for re-challenge in this study did not cause weight loss in mice naïve to *C. rodentium*, regardless of *Helicobacter* status (Figure 4-6 B), thereby making weight loss unusable as a readout. Throughout the 3 weeks of monitoring, weight of naïve mice challenged with *C. rodentium* was comparable to the primary challenge control mice, which had cleared their infection from 4 weeks earlier. Consistent with no weight loss in the naïve mice, convalescent mice re-challenged with *C. rodentium* did not have the initial weight loss observed in the pilot study. *H. hepaticus* persistently infected convalescent mice also had no weight loss during the 3 weeks post-re-challenge. The lack of weight loss does not preclude colonic disease development, therefore the pathogenicity of the re-challenge strain was evaluated by assessing cecal and colonic lesions.

No cecal or colonic lesions were present 3 weeks after re-challenge

In agreement with causing no weight loss, histological evaluation revealed no significant cecal or colonic lesions in naïve mice 3 weeks after challenge with *C. rodentium* (Table 4-1) even when persistently infected with *H. hepaticus*. Although the study was taken to 3 weeks post-inoculation (WPI), naïve mice inoculated with *C. rodentium* were expected to have histological evidence of infection, as another study has shown (see Chapter 5, Figure 5-3 and Figure 5-8). In fact, the cecum and colon appeared similar to the primary challenge control mice inoculated with only the first *C. rodentium* strain 7 weeks earlier (Table 4-1). Convalescent mice with or without a persistent *H. hepaticus* infection had no lesions from the re-challenge, as well. The lack of pathogenicity from the re-challenge strain made proper evaluation of whether *H. hepaticus* infection affects protection from *C. rodentium* disease development impossible. However, another marker for activation of the immune response at the site of infection is an

influx of Foxp3$^+$ T cells; therefore, numbers of Foxp3$^+$ cells were counted in cecal and colon tissue.

Foxp3$^+$ cell numbers were not different between infection groups in either cecum or colon

Numbers of Foxp3$^+$ cells in the cecum and colon of mice used as controls for the primary *C. rodentium* challenge were considered basal numbers, as 7 weeks had past since inoculation. Basal numbers of Foxp3$^+$ cells were 6 per mm^2 of cecum and 4 per mm^2 of colon (*Helicobacter*-free mice inoculated with *C. rodentium* 7 weeks earlier). Persistent *H. hepaticus* infection did not change these basal numbers (Figure 4-7 A and B). Based on another study (see Chapter 5, Figure 5-4 and Figure 5-9) numbers of Foxp3$^+$ cells were not expected to change in cecum 3 WPI, however an increase in the colon was expected during active disease 3 WPI with numbers close to 20-30 per mm^2 of colon. Consistent with histological evaluation, no increase in Foxp3$^+$ cell numbers was observed in cecum or colon of naïve mice at 3 WPI with the re-challenge strain, indicating little to no local activation of the immune system. Persistent *H. hepaticus* infection did not affect the Foxp3$^+$ cecal or colonic cell numbers at 3 weeks after *C. rodentium* challenge with the re-challenge strain (Figure 4-7 A and B). Similarly, cecal and colonic numbers of Foxp3$^+$ cells in convalescent mice with or without *H. hepaticus* were unchanged from basal numbers (Figure 4-7 A and B). As a final readout of local immune activation, colonic cytokine mRNA expression levels were measured.

No changes in colonic cytokine mRNA expression levels 3 weeks after inoculation with the re-challenge strain of *C. rodentium*

In another study, the colonic cytokine expression was evaluated weekly for four weeks after inoculation with *C. rodentium* and found significant changes in mRNA expression of both pro- and anti-inflammatory cytokines at 3 WPI. Therefore, as a highly sensitive measurement of immune activation in the colon after infection with *C. rodentium*, the colonic cytokine mRNA expression levels were measured in all infection groups. As shown in Figure 4-8, comparison of expression levels to *Helicobacter*-free mice used as controls for the primary *C. rodentium* challenge 7 weeks earlier revealed no changes in any of the cytokines, regardless of Helicobacter or Citrobacter status.

Discussion

Protection against infection by enteric pathogens can be induced by vaccination or by recovery from infection. In order to evaluate the effects of a persistent intestinal infection on the efficacy of a vaccine for an enteric diarrheal infection, an efficacious immunization method was needed. Initially, two strategies were evaluated in *Helicobacter*-free mice because no successful vaccination method has been reported to date. The subcutaneous prime/s.c. boost strategy to immunize against *C. rodentium* was not successful as evaluated by outcome of challenge with *C. rodentium*, despite serum immunoglobulins specific to *C. rodentium*. Subcutaneous prime/oral boost has potential to be an efficacious vaccine against *C. rodentium*, as 5 of 10 mice cleared the infection within a few days and had reduced disease. However this vaccination strategy needs to be re-evaluated with a follow-up study. The lack of protection observed in mice with serum IgGs after immunization is in contradiction to reports of protection provided by convalescent serum, specifically IgGs. One possibility is that mucosal immunoglobulins were not secreted into the mucosa in quantities large enough to provide protection. Alternatively, other IgG

isotypes not measured (IgG3 or IgG2b) may be of greater importance for protection. Previous reports of immunization strategies using mouse models of EPEC infection have not been successful at preventing infection or disease. However, anecdotal knowledge indicates mice previously infected with *C. rodentium* did not develop disease or symptoms of infection upon re-exposure, indicating mice do develop protective immunity to *C. rodentium.* Since immunization against *C. rodentium* could not be consistently achieved, and other studies have shown evidence of protective immunity to *C. rodentium*, prior infection with *C. rodentium* was evaluated as an alternative model for assessing the effects of *H. hepaticus* infection on immunity to an unrelated pathogen.

Prior infection with *C. rodentium* provided protective immunity to subsequent infections, which was virtually sterilizing, as only an initial loss of weight, corresponding with bacterial shedding, followed by minimal histological evidence of infection at two weeks after re-challenge was observed. These results confirm the anecdotal evidence and two studies stating convalescent mice were protected from high bacterial burdens (32, 81). Prior infection with a pathogen is a natural way to develop immunity to subsequent disease and typically provides better protection than immunization, although at the expense of disease-associated morbidity and mortality. Therefore, altering the development of natural immunity with another unrelated pathogen would be difficult, but could demonstrate the full extent of effects a concurrent infection might have on a host's immune system.

Issues with the pathogenicity of the re-challenge strain of *C. rodentium* made a thorough analysis of the effects of a persistent *H. hepaticus* infection on protective immunity to *C. rodentium* difficult to interpret. The re-challenge strain (DBS129) did not cause weight loss, cecal or colon disease, increased numbers of $Foxp3^+$ cells in colon tissue, or increased cytokine

expression. Despite a lack of pathogenicity, the study did determine that *H. hepaticus* infection did not affect protection from sustained bacterial burden. *C. rodentium*-induced disease and subsequent morbidity is closely linked with bacterial burden and duration of high burden. Therefore, in order for *H. hepaticus* to alter *C. rodentium*-induced disease, mice would need to lose protection from re-infection. Since comparable bacterial burdens were observed, the lack of effect on other disease parameters was uninformative. Persistent *H. hepaticus* infection did not affect the protective immunity to *C. rodentium* generated by a prior infection.

Notably, this is the first set of controlled studies to evaluate: 1) immunization strategies for *C. rodentium*, a mouse model of EPEC infection; 2) protective immunity to *C. rodentium* via prior infection; and 3) effects of a persistent intestinal infection on development of protective immunity. Together, these results provide a good baseline for future evaluation of vaccines for *C. rodentium* or EPEC infection. It is possible that *H. hepaticus* infection could influence a primary *C. rodentium* infection without altering protective immunity. Further study into the host response, with emphasis on the immune function, to *C. rodentium* would assist in the development of more efficient vaccines. Additionally, studying the modulation of host response to *C. rodentium*, a robust elicitor of mucosal immunity, with *H. hepaticus* infection will provide more information on cross-talk in mucosal immunity.

Tables and Figures

Table 4-1. Re-challenge strain of *C. rodentium* did not cause cecal or colonic disease and was unaffected by *H. hepaticus* infection. At 7 weeks post-*C. rodentium* infection mice had no cecal or colonic disease (TSB/Cr/LB) and mice also persistently infected with *H. hepaticus* no longer had cecal or colonic disease. Any cecal or colonic disease resulting from the re-challenge strain of *C. rodentium* had subsided by 3 WPI, which was unchanged by persistent *H. hepaticus* infection. Prior *C. rodentium* infection did not change the lack of cecal or colonic disease resulting from the re-challenge strain even with a persistent *H. hepaticus* infection.

	TSB/Cr/LB	TSB/LB/Cr	TSB/Cr/Cr	Hh/Cr/LB	Hh/LB/Cr	Hh/Cr/Cr
Cecum Disease Index	0.75 (0.5-1.0)	1.0 (0.0-1.5)	0.5 (0.0-1.0)	0.0 (0.0-1.0)	1.0 (0.5-1.0)	1.0 (0.0-1.0)
Colon Disease Index	0.25 (0.0-1.5)	0.5 (0.0-1.5)	0.0 (0.0-1.0)	0.0 (0.0-0.5)	0.0 (0.0-1.0)	0.0 (0.0-0.5)

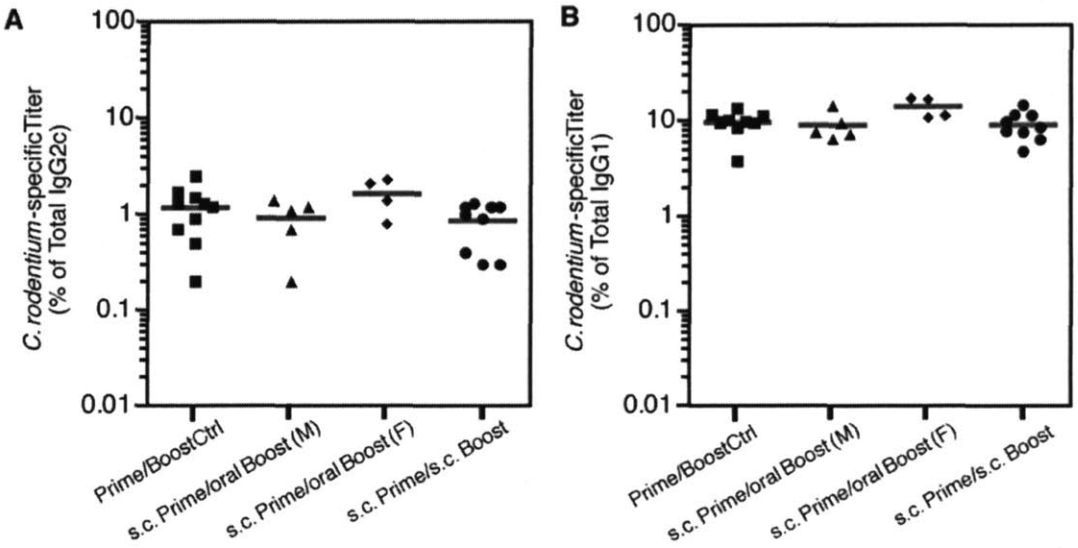

Figure 4-1. Immunization with *C. rodentium* sonicate generates *C. rodentium*-specific circulating antibodies. Pre-challenge *C. rodentium*-specific IgG2c **(A)** and IgG1 **(B)** serum titers were comparable between s.c. prime/s.c. boost and all s.c. prime/oral boost groups of mice. Gender did not affect pathogen-specific titer percentage of total isotype, shown for s.c. prime/oral boost challenge group. One-way ANOVA with Bonferroni post-test; $P < 0.05$ was considered significant.

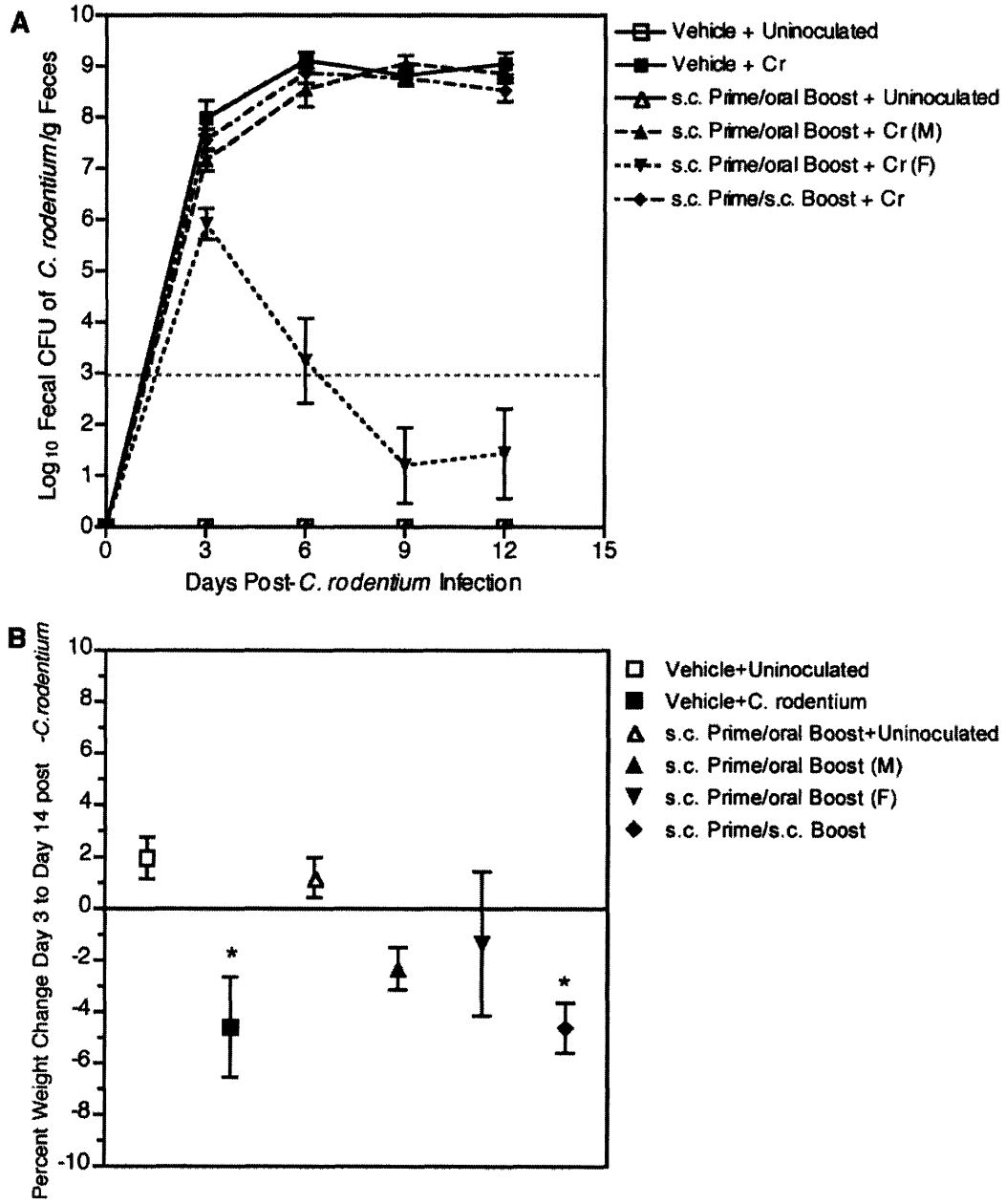

Figure 4-2. Immunization did not consistently prevent or reduce bacterial burden or weight loss during *C. rodentium* challenge. Fecal shedding of *C. rodentium* (**A**) in s.c. prime/s.c. boost and s.c. prime/oral boost male groups were equivalent to unimmunized mice. Subcutaneous prime/oral boost females cleared *C. rodentium* by 1 WPI. *C. rodentium* caused weight loss (**B**) between 3 DPI and 14 DPI in unimmunized and s.c. prime/s.c. boost challenged groups. Both males and females in the s.c. prime/oral boost group challenged with *C. rodentium* lost weight by 14 DPI, however not as much other challenged groups. One-way ANOVA with Bonferroni post-test, * $P < 0.05$.

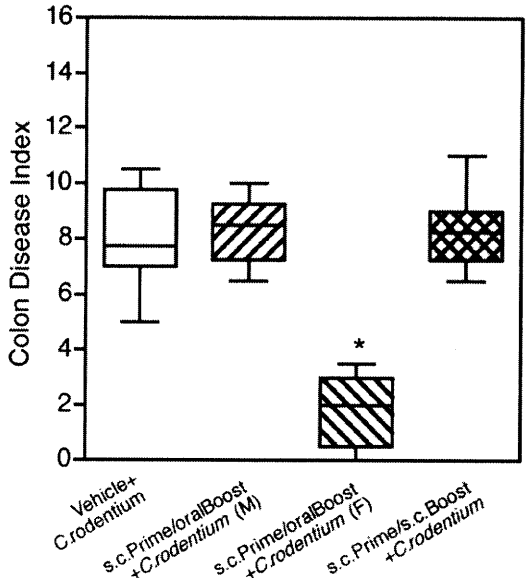

Figure 4-3. Reduced colonic disease in mice with decreased bacterial burden. *C. rodentium* challenge caused marked colon disease at 14 DPI in unimmunized mice. Only s.c. prime/oral boost females did not develop comparable colon disease. Kruskal-Wallis with Dunn's post-test; * $P < 0.05$.

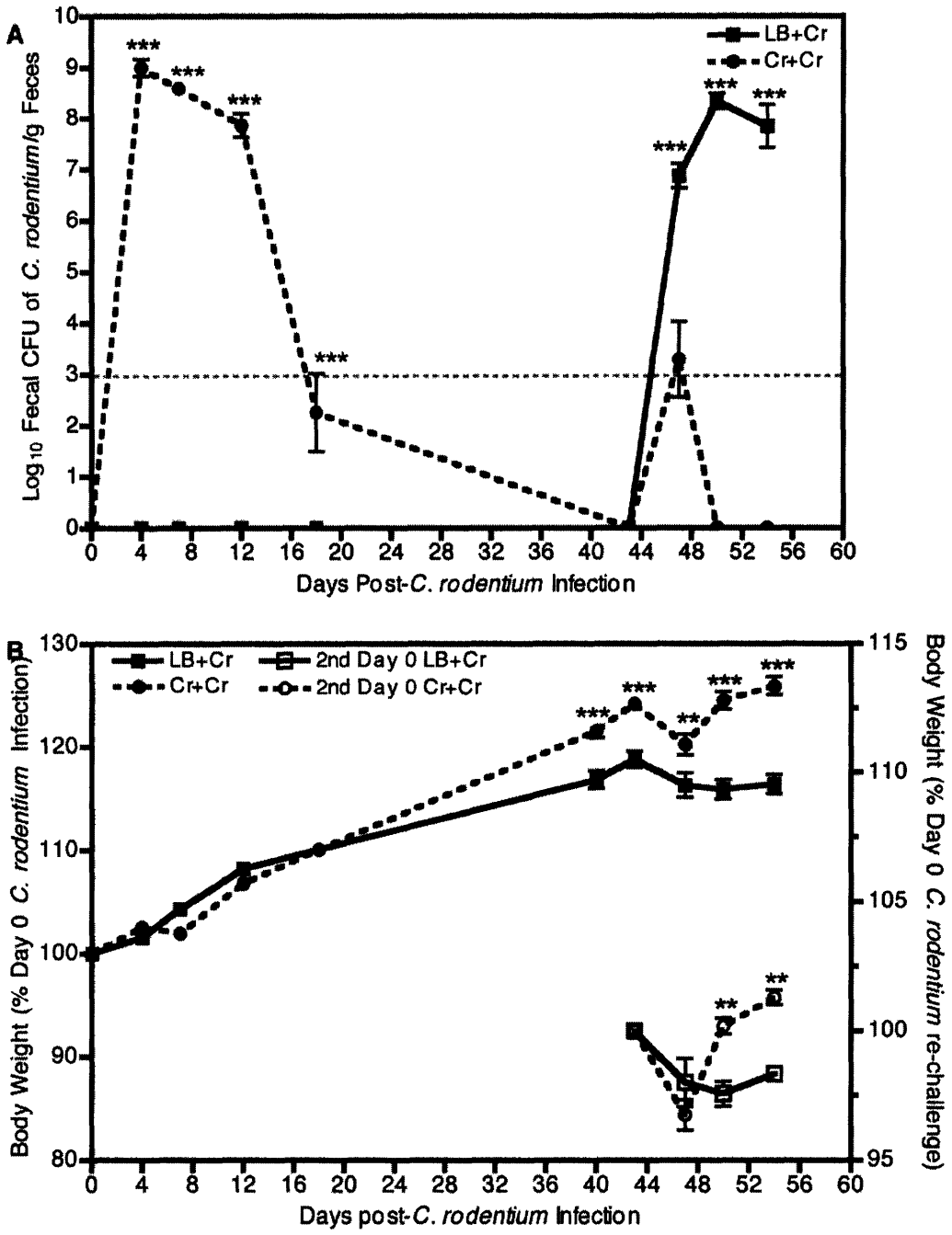

Figure 4-4. *C. rodentium* infection provides protection from re-infection and associated weight loss. Fecal shedding of *C. rodentium* **(A)** was near the threshold of detection (10^3 CFU/g feces) in mice previously infected with *C. rodentium* compared with greater than 6 logs higher burdens in naïve mice. Prior *C. rodentium* infection did not prevent an initial period of weight loss **(B)**, however convalescent mice gained weight afterwards compared with continued weight loss in naïve mice. Two-way ANOVA with Bonferroni post-tests; ** $P < 0.01$, *** $P < 0.001$.

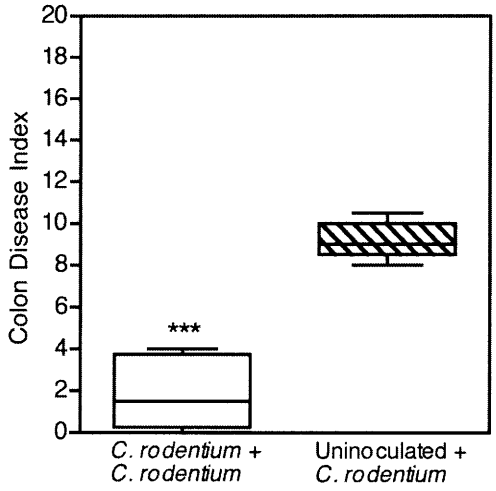

Figure 4-5. Prior *C. rodentium* infection prevents colonic disease development. Naïve mice challenged with *C. rodentium* develop intense colonic disease at 11 DPI. However, convalescent mice had only minor colonic disease. Mann-Whitney with two-tailed t test; *** $P < 0.001$.

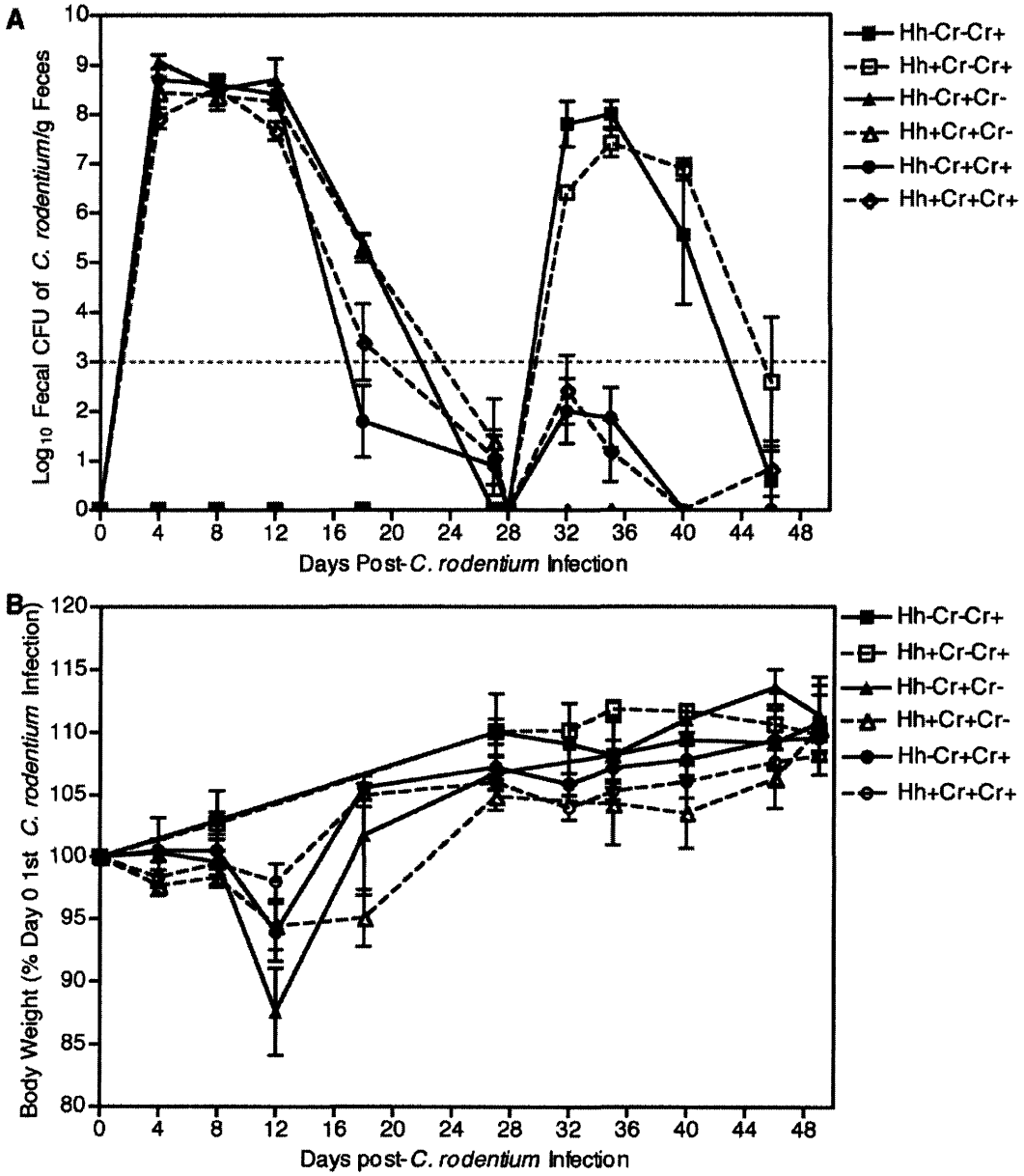

Figure 4-6. Persistent *H. hepaticus* infection does not affect protection from decreased bacterial burdens in convalescent mice. *C. rodentium* fecal shedding (**A**) was comparable in each group inoculated with *C. rodentium* regardless of *H. hepaticus* infection. *C. rodentium* infection associated weight loss (**B**) did not occur with the second challenge strain. Two-way ANOVA with Bonferroni post-tests; $P < 0.05$ was considered significant.

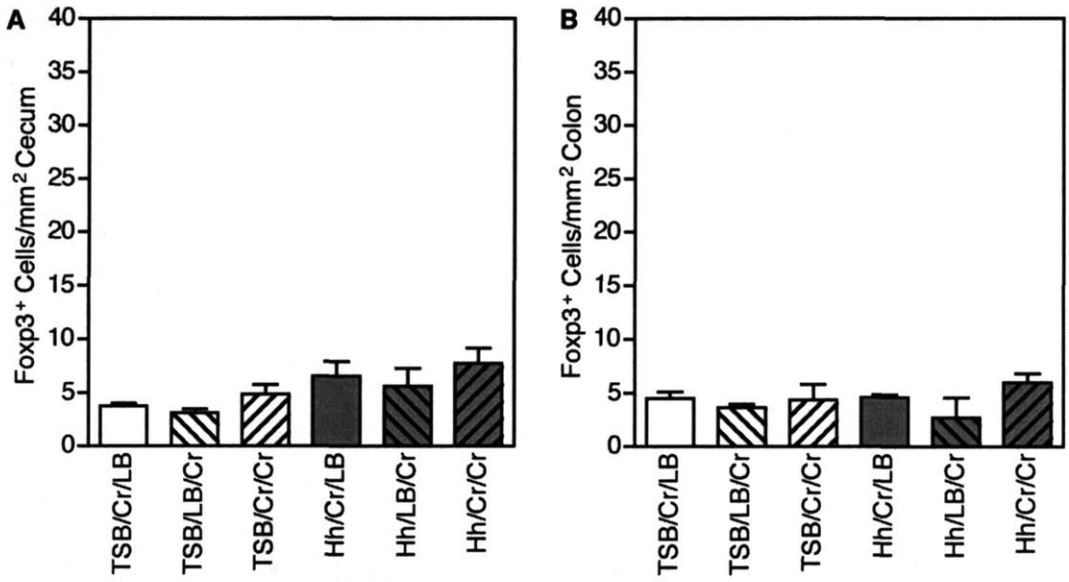

Figure 4-7. No increases in cecal or colonic Foxp3$^+$ cell numbers were observed with *C. rodentium* re-challenge despite persistent *H. hepaticus* infection. Mice persistently infected with *H. hepaticus* and inoculated with *C. rodentium* 7 weeks earlier had no increase in cecal (**A**) or colonic (**B**) Foxp3$^+$ cells. Regardless of *Helicobacter* status, the re-challenge strain of *C. rodentium* caused no increase in cecal or colonic Foxp3$^+$ cells 3 WPI. Convalescent challenged mice also had no change in cecal or colonic Foxp3$^+$ cells even with a persistent *H. hepaticus* infection.

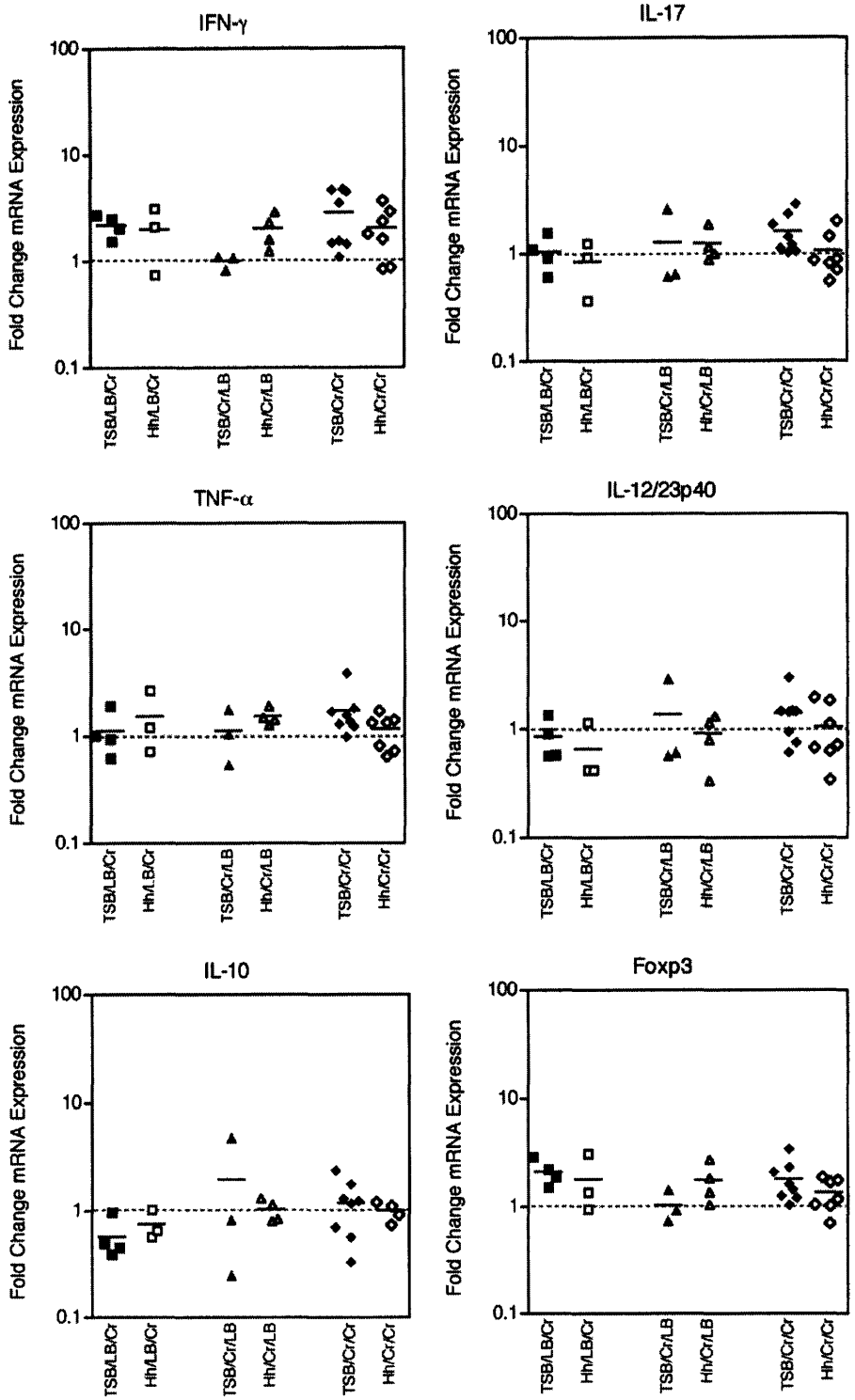

Figure 4-8. Colonic mRNA cytokine expression was not altered by either *H. hepaticus* or *C. rodentium* infection. Infection with *H. hepaticus* did not change pro-inflammatory or anti-inflammatory cytokine mRNA expression 7 WPI (TSB/Cr/LB versus Hh/Cr/LB). No increase in cytokine mRNA was observed upon challenge with the second strain of *C. rodentium* (TSB/LB/Cr), nor did *H. hepaticus* affect the mRNA levels. No mRNA cytokine response to the second challenge strain of *C. rodentium* was observed in convalescent mice regardless of *Helicobacter* status.

Chapter 5

Persistent subclinical *Helicobacter hepaticus* infection modulates host response to *Citrobacter rodentium* causing prolonged disease

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Introduction

Diarrheal disease caused by enteropathogenic *Escherichia coli* (EPEC) is a major health problem, particularly in developing countries where access to clean drinking water is limited. The frequent occurrence of EPEC infections in developing countries where a large fraction of the population also has a persistent infection, such as *Helicobacter pylori*, make a mouse model of polymicrobial enteric infection useful for understanding the effects of persistent infection on acute diarrheal disease. Recently, *Citrobacter rodentium*, a murine pathogen related to EPEC, has proven useful for studying virulence and pathogenesis of acute diarrheal disease through *in vitro* and *in vivo* studies. Similar to EPEC infection in humans, clinical disease in C57BL/6 mice is characterized by loose stool progressing to diarrhea in severe cases, weight loss that can lead to death, and poor overall body condition (34, 49, 51, 60). Histologically, *C. rodentium* infection causes self-limiting cecal and colonic disease, including hyperplasia, edema, destruction of the epithelial barrier, and inflammation, with variable degrees of severity depending on age at infection and concurrent infection status, such helminthes (34, 49, 51, 60). Without confounding factors, such as a compromised immune system, mice recover from the infection four to six weeks after inoculation (49, 60).

An estimated 50% of the world's population is infected with *Helicobacter pylori*, making it among the most common bacterial pathogens (63). Left untreated, infections with *H. pylori* are life-long and usually subclinical. In a small subset of people, complications develop, including peptic ulcers and neoplasia (less than 3%) (63). *Helicobacter hepaticus*, a related murine pathogen, serves as a good model of persistent enteric infection as it is also a life-long and subclinical infection (24, 28, 37, 69, 85, 86). Additionally, similar mechanisms have been suggested for both *Helicobacter* infections in that they remain subclinical through careful

regulation of host-microbe interactions via regulatory T cells (16, 48, 65). Studies in several strains of knockout mice, particularly those deficient in adaptive immune functions, indicate the host response to *H. hepaticus* includes regulation of the innate and adaptive immune responses because an inflammatory bowel disease-like (IBD-like) condition develops in these strains of mice when infected with *H. hepaticus* (8, 9, 36, 39, 40, 42, 43, 46, 84). When IL-10 competent T cells are transferred into infected mice, the proinflammatory events initiated by *H. hepaticus* infection are contained (18, 19, 41, 53).

A novel mouse model of polymicrobial infection was developed to characterize the immunomodulatory effects of persistent *H. hepaticus* infection on host response to the enteric diarrheal pathogen *C. rodentium* in C57BL/6 mice. Understanding alterations in host response to an acute infection attributable to concurrent persistent infection will be useful in developing a model for acute diarrheal disease in populations at risk for underlying infections. By characterizing clinical and immunological disease features throughout the course of the acute infection, important factors affected by persistent infection during the short-term as well as long-term can be determined.

Results

H. hepaticus prolongs clinical disease without affecting *C. rodentium* bacterial burden

In agreement with previous reports in C57BL/6 mice (50, 89, 90), *C. rodentium* bacterial burden, based on fecal shedding reached a maximum of 10^9 CFU/g feces between 1 and 2 weeks post-inoculation (WPI) (Figure 5-1 A). Clearance of *C. rodentium* occurred during the following week, as fecal shedding fell to undetectable levels ($<10^3$ CFU/g feces) by 3 WPI. Persistent infection with *H. hepaticus* had no significant effect on fecal shedding, $P > 0.05$ (Figure 5-1 A).

Recent studies (50, 89) have highlighted the cecum as a niche for *C. rodentium* early during infection. Bacterial burden in the cecal contents of mice at 1 WPI was not different between mice singly infected with *C. rodentium* ($3 \pm 5x10^7$ CFU/g cecal content) and mice concurrently infected with *H. hepaticus* ($4 \pm 2x10^7$ CFU/g cecal contents). In addition to confirming infection through bacterial burden, clinical disease was assessed by monitoring weight, since *C. rodentium* causes fluid imbalance and weight loss (3).

In contrast to infection with only *H. hepaticus*, in which weight was comparable to uninoculated mice, clinical weight loss due to *C. rodentium* was apparent within 1 WPI (Figure 5-1 B). *C. rodentium*-infected mice lost an average of 4% of their initial body weight by 12 days post-inoculation (DPI), whereas uninoculated mice had no change in body weight, $P < 0.01$. *Helicobacter*-free mice regained lost weight and were comparable to uninoculated mice by 21 DPI, which is consistent with previous reports in C57BL/6 mice (83). Weight loss in mice infected with *H. hepaticus* and inoculated with *C. rodentium* was comparable to *Helicobacter*-free mice with *C. rodentium* through 18 DPI. However, concurrent *H. hepaticus* infection limited weight gain, which became apparent at 3 WPI ($P < 0.01$ compared to *C. rodentium* singly-infected mice). Chronic impairment of weight gain was seen, as at 4 WPI the persistently infected mice had lower body weight than uninoculated mice, $P < 0.001$ (Figure 5-1 B). Given the effects on clinical disease, both immune response directed against *C. rodentium* and tissue lesions were evaluated.

C. rodentium-specific serum immunoglobulins are indistinguishable between _H. hepaticus_ infected and _Helicobacter_-free mice

Development of *C. rodentium*-specific immunity occurs during the first two weeks after inoculation with specific immunoglobulins detectable after two weeks (5). *C. rodentium*-specific immunoglobulins, particularly IgG isotypes are important for resolution of infection and prevention of prolonged bacterial burden-associated disease, as failure to develop systemic *C. rodentium*-specific IgGs leads to sustained infection, lesions, and increased mortality (5, 6, 50, 74). Three weeks post-*C. rodentium* inoculation, singly-infected mice had an average serum *C. rodentium*-specific IgM titer of 80 μg/ml or 5.2% of total serum IgM (Figure 5-2 C). The later-developing *C. rodentium*-specific IgGs had lower serum titers of 6 μg/ml or 0.7% of total IgG2c and 6μg/ml or 0.7% of total IgG1 (Figure 5-2 B). Secretion of *C. rodentium*-specific sIgA into the cecal lumen had not occurred by 1 WPI, as no samples had detectable sIgA in *C. rodentium*-specific ELISAs. Total sIgA in cecal contents of uninfected and *C. rodentium*-infected mice were equivalent (347 μg/g cecal content and 383 μg/g cecal content, respectively). At 3 WPI, subclinical infection with *H. hepaticus* had no significant effect on the serum titers of *C. rodentium*-specific IgM, IgG1, or IgG2c as shown in Figure 5-2 B and C. Cecal levels of total sIgA in mice concurrently infected with *H. hepaticus* and *C. rodentium* were elevated in comparison with all cohorts at 1 WPI, however the percentage of *H. hepaticus*-specific sIgA did not differ significantly (Figure 5-2 C).

Persistent *H. hepaticus* infection prolongs *C. rodentium*-induced colonic disease

To determine whether persistent *H. hepaticus* infection caused alterations in colon disease, the severity of lesions was assessed. Although *H. hepaticus* infection is used as a model of IBD in immune knockout mice (8, 9, 43), the subclinical nature of *H. hepaticus* infection in C57BL/6 mice was confirmed by the lack of colonic lesions in age-matched infected mice

(Figure 5-3 A and B). In contrast, colonic disease was apparent in *C. rodentium*-inoculated mice by 1 WPI consisting of mild inflammation, edema, epithelial destruction, and hyperplasia with a disease index of 4.0 (0.0-6.0) (Figure 5-3 A, $P < 0.001$ compared with uninoculated mice). Over the following week, appreciable colonic disease developed reaching a maximal severity of 9.0 (7.0-12.0) (Figure 5-3 A and C). Disease resolved in *Helicobacter*-free mice during weeks 3 and 4 post-inoculation reaching 2.0 (0.5-4.5) at 4 WPI (Figure 5-3 A and E). Concurrent *H. hepaticus* infection had no significant effect on maximal colonic disease development (Figure 5-3 A and D) with the highest disease index, also occurring at 2 WPI, of 8.5 (5.5-10.0). However, persistent *H. hepaticus* infection did prolong colon disease, as the disease index at 4 WPI was 4.5 (2.5-7.5) compared with 2.0 (0.5-4.0) in *C. rodentium* singly infected mice, $P < 0.001$ (Figure 5-3 A, E, and F). Since Foxp3$^+$ regulatory T cells (nT_{reg}) have been associated with active colitis (79), and *C. rodentium*-inoculated mice persistently infected with *H. hepaticus* had delayed weight gain and prolonged colitis, the colon was examined for nT_{reg} cells.

Foxp3$^+$ nT_{reg} accumulate in colon of *C. rodentium*-infected mice during active disease

Growing evidence of increased numbers of nT_{reg} cells at the sites of active mucosal disease (11, 65-67, 79), suggests the role of nT_{reg} cells at these sites is to contain the immune response. Therefore, to determine whether the delayed disease resolution was associated with an increased regulatory immune response, Foxp3$^+$ cells in the colon were counted. Single infection with *H. hepaticus* caused no increase in numbers of colonic Foxp3$^+$ cells (Figure 5-4 A). Similarly, early after *C. rodentium* inoculation (1 WPI) no significant increase in the number of colonic Foxp3$^+$ cells was observed. However, once marked colonic disease developed, *C. rodentium*-inoculated mice had an average of 10 and 21 Foxp3$^+$ cells/mm^2 of colon at 2 and 3

WPI, respectively, compared with 1 Foxp3$^+$ cells/mm^2 of colon in uninoculated mice (Figure 5-4 A and B, $P < 0.001$). By 4 WPI, colonic numbers of Foxp3$^+$ cells in *Helicobacter*-free mice were not significantly different from basal levels (Figure 5-4 A and D). Although accumulation of nT_{reg} cells occurred regardless of *Helicobacter* status at 2 and 3 WPI, increased numbers were observed earlier (1 WPI) in mice concurrently infected with *H. hepaticus* (2 Foxp3$^+$ cells per mm^2 in *C. rodentium* compared with 9 Foxp3$^+$ cells per mm^2 in *H. hepaticus* with *C. rodentium*, Figure 5-4 A). In striking contrast to a return to basal numbers in *Helicobacter*-free mice, at 4 WPI Foxp3$^+$ cells remained elevated in mice concurrently infected with *H. hepaticus*, $P < 0.001$ (Figure 5-4 A and E). Increases in nT_{reg} cell numbers during *C. rodentium* infection corresponded to active disease, similar to the findings in humans (79), indicating the increased presence of nT_{reg} cells could be a marker for active disease and could play a role in containing tissue damage. The colonic tissue cytokine expression profile was measured in order to determine whether a different cytokine expression pattern was associated with the prolonged colitis observed in mice with a persistent *H. hepaticus* infection.

C. rodentium induces colonic expression of both proinflammatory and anti-inflammatory cytokines mirroring colonic disease severity

C. rodentium has been reported to be a T_h1-inducing disease (6, 34, 75), however a recent study gives strong evidence for a major role of IL-17, produced by T_h17 cells, in disease development (55). Therefore, expression levels of cytokines and other factors thought to be involved in mucosal inflammation or colon disease were measured to characterize the basis of differences in clinical and pathological disease outcome. Two-way ANOVA revealed colonic transcripts for TNF-α, IFN-γ, IL-12p35, and IL-12/IL-23p40 (Figure 5-5), along with IL-6,

MCP-1, IL-1β, Foxp3, and IL-10 (Figure 5-6) had maximal expression in colons of mice infected with *C. rodentium* 2 WPI ($P < 0.001$, except IL-6 $P < 0.05$ compared with uninoculated mice). T_h2-associated cytokines IL-13 and IL-4 were not detected. With a few exceptions colonic transcripts of all genes measured followed a similar expression pattern: increased expression as disease became more severe, maximal expression at 2 WPI paralleling disease severity, decreased expression during disease resolution, and a return to near basal expression by 4 WPI. In contrast to this general pattern, expression of MLCK and IL-23p19 did not change over the course of infection, and IL-17 expression increased reaching a plateau at 3 WPI (Figure 5-5). In the study looking at IL-17 in *C. rodentium* infection (55), assessment was made at 8 DPI, and these results are in agreement as the current study does show higher colonic expression of IL-17 than IFN-γ at 1 WPI. Interestingly, when disease is evaluated for a longer duration, the pronounced flux in T_h1-associated cytokines confirms the dominant T_h1 nature of *C. rodentium*-induced colonic disease.

C. rodentium-induced disease correlates with T_h1 proinflammatory cytokines

To determine whether type 1 cytokines were associated with acute disease pathogenesis, correlation analysis of mRNA expression in individual mice and their disease index was performed with all animals from all times post-inoculation, regardless of infection status. Acute *C. rodentium*-induced disease correlated with colonic gene expression of T_h1 proinflammatory cytokines, but not with IL-17 (Figure 5-7). The analysis revealed a strong positive correlation between maximal disease severity and T_h1 cytokines TNF-α, IL-12p35, IL-12/23p40, and in particular IFN-γ (Spearman $r = 0.763$, $P < 0.0032$). In contrast, neither IL-23p19 nor IL-17 were significantly correlated with acute disease severity (IL-17: Spearman $r = 0.097$, $P > 0.05$). Since

cytokine expression was strongly correlated with disease, differences in expression during persistent *H. hepaticus* infection were evaluated.

H. hepaticus infection modulates T_h1-T_h17 cytokine expression in colon of *C. rodentium* infected mice

Comparison of *C. rodentium*-induced mRNA transcripts with or without concurrent *H. hepaticus* infection revealed that *H. hepaticus* infection did not change the temporal colonic cytokine expression pattern, except IL-17, of which the expression level continued to increase from 3 to 4 WPI. This profile is in contrast to equivalent expression between 3 and 4 WPI in *C. rodentium* singly-infected mice (Figure 5-5). Two-way ANOVA revealed no significant differences in *C. rodentium*-induced transcript levels at 1 WPI when mice were concurrently infected with *H. hepaticus* (Figure 5-5). However, maximal expression levels of IFN-γ and IL-12p35, observed at 2 WPI, were lower ($P < 0.05$) in mice with a persistent *H. hepaticus* infection indicating *H. hepaticus* infection suppresses the T_h1 response to *C. rodentium.* By 4 WPI, when T_h1 cytokines were approaching basal expression (Figure 5-5), IL-17 transcript levels continued to escalate in mice with a persistent *H. hepaticus* infection, reaching a 202-fold increase over uninoculated mice. Compared with *C. rodentium* singly infected mice, persistent *H. hepaticus* infection resulted in a modest increase in colonic IL-17 expression at 4 WPI (2-fold, $P < 0.05$). Overall, persistent infection with *H. hepaticus* modulates *C. rodentium*-induced cytokine expression by both slightly suppressing levels of T_h1 cytokines while allowing for a modest enhancement IL-17 response late. Increased IL-17 expression during prolonged disease in persistently infected mice, along with its association with chronic inflammation (93) and IBD (30, 62), indicate chronic disease may be mediated via IL-17.

Modest cecal disease appears early during *C. rodentium* infection, but is prevented by persistent *H. hepaticus* infection

Infection of the cecum by *C. rodentium* has been suggested as the initial site for activation of host response and disease development (89). As the primary location for *H. hepaticus*-induced cecal lesions, the ileocecolic junction evaluated for lesions. Additionally, the cecal apices were often removed for mRNA expression and could not be assessed in those studies. Compared to uninoculated mice (0.75 [0.0-1.5]), mice infected with *C. rodentium* developed evident cecal disease by 1 WPI with a disease index of 4.25 (1.5-9.0), $P < 0.001$ (Figure 5-8 A). During the second week after inoculation, disease was resolving (3.0 [0.5-5.5], yet still worse than uninoculated mice, $P < 0.001$. By 3 WPI, disease had resolved as there was no significant difference in overall cecal disease severity between uninoculated mice (1.5 [0.5-2.0]) and *C. rodentium* singly infected mice (2.0 [1.5-2.5]). Imaging studies have shown an initial accumulation of *C. rodentium* in the cecum near the cecal apex,(89) however, in an experiment where the apex was not removed, comparable disease developed at both the apex and ileocecolic junction at 1 WPI (Figure 5-8 B). In contrast to single infection with *C. rodentium*, persistent *H. hepaticus* infection during *C. rodentium* infection prevented cecal disease; at all time points, cecal disease was comparable to uninoculated mice and attenuated compared with *Helicobacter*-free *C. rodentium* inoculated mice, $P < 0.001$ (Figure 5-8 A). However, in the cecal apex the cecal disease seen in *Helicobacter*-free *C. rodentium* inoculated mice was similarly observed in *H. hepaticus*-infected mice 1 week after *C. rodentium* inoculation, $P > 0.05$ (Figure 5-8 B). Since regulatory T cells could be responsible for differences in disease severity, the numbers of $Foxp3^+$ nT_{reg} were counted in cecal tissue.

No changes in numbers of Foxp3$^+$ cells occurs in the cecum when mice are infected with *H. hepaticus* or *C. rodentium*

Cecal tissue was stained for Foxp3 expression to determine whether differences in *C. rodentium*-induced cecal disease when mice were persistently infected with *H. hepaticus* was associated with changes in tissue Foxp3$^+$ nT_{reg}. Generally, basal levels of Foxp3 expressing cells in the cecum were ~5-fold greater than basal colonic numbers. Compared with uninoculated mice, which had between 5 and 10 Foxp3$^+$ cells per mm^2 cecum at all time points, infection with *C. rodentium* did not result in an accumulation of Foxp3$^+$ cells even when disease was present at 1 and 2 WPI, $P > 0.05$ (Figure 5-9). Additionally, mice also persistently infected with *H. hepaticus* had no change in Foxp3$^+$ cells in the cecum (Figure 5-9). Given the strong induction of colonic cytokines during peak colon disease, the cecal cytokines were measured to determine whether similar expression occurred during cecal disease.

Cecal disease is associated with a shift in the balance of anti-inflammatory and proinflammatory cytokines

Regardless of *H. hepaticus* infection status, expression levels of proinflammatory IL-17 in cecal tissue was increased in *C. rodentium* inoculated compared to uninoculated mice at 1 WPI, $P < 0.05$ (Figure 5-10). Other proinflammatory mediators (TNF-α, IFN-γ, MCP-1) trended higher for *C. rodentium* alone, whereas anti-inflammatory mediators (TGF-β and IL-10) and transcription factor Foxp3 trended towards lower expression, but were not significant (Figure 5-10). In comparison with mice persistently infected with *H. hepaticus* that were challenged with *C. rodentium*, TNF-α expression was higher at 1 WPI and IL-10 was lower at 3 WPI in

Helicobacter-free mice. Cytokine expression was also measured in the mesenteric lymph nodes (MLN) to determine whether changes were occurring in the draining lymph nodes. Again, expression levels did not change more than 10-fold in the MLN, however several differences were apparent between *H. hepaticus* infected and *Helicobacter*-free mice inoculated with *C. rodentium*, including decreased levels of IL-17 (at 1 and 3 WPI) as well as MCP-1 at 3 WPI and IFN-γ at 1 WPI (Figure 5-10). Spearman correlation of cecal disease indices with cytokine expression levels revealed that cecal disease was positively correlated with increased proinflammatory cytokines (of innate, T_h1 and T_h17 lineages) and negatively correlated with anti-inflammatory cytokines (Figure 5-11).

Discussion

The role of naturally-occurring regulatory T cells in the normal colon as well as in disease has yet to be clarified (1, 58, 77). In the normal gastrointestinal tract, in the absence of inflammation, nT_{reg} are thought to limit innate and adaptive immune activation by dietary components and commensal microbiota (14, 58, 80). In situations where inflammation develops, nT_{reg} cells may serve to limit collateral damage to adjacent tissue. This hypothesis is supported by recent studies in tuberculosis patients and colitis patients where nT_{reg} cells have been shown to accumulate at the site of disease (11, 66, 67, 79). Here we demonstrate that infection with *C. rodentium* causes a significant increase in the number of nT_{reg} in the colon during established, active disease. These nT_{reg} could be recruited because they react to *C. rodentium* antigens, self-antigens, or even the commensal microflora. However, the temporal distribution of nT_{reg} suggests they play a role in suppressing inflammation generated in response to *C. rodentium.* These findings confirm and extend the findings of Uhlig et al. that showed increased numbers

Foxp3$^+$ cells in colon tissue from active infectious colitis and IBD colitis, and validates *C. rodentium* as a model for studying human infectious colitis and IBD (79).

In addition to nT_{reg} accumulation during active disease, *C. rodentium* lesions were strongly associated with T_h1 cytokines in the colon.. Previous studies have also shown a requirement for T_h1 cytokines in *C. rodentium* disease development (6, 34, 75). A recent study however, highlighted a potential role of T_h17 cells in host protection from mortality and disease development, as mice lacking IL-23 (IL-23p19-deficient mice) died within 2 WPI. (55) Although exhibiting high mortality, the mice lacking IL-23, a cytokine required for maintenance of T_h17 cells, did not develop disease comparable to wild type C57BL/6 mice by 8 DPI. Mangan et al. also found increased numbers of IL-17 producing T cells (T_h17) compared with IFN-γ producing T cells (T_h1) in the colonic lamina propria at 8 DPI. The finding of a strong correlation between T_h1 cytokines and disease severity along with a lack of correlation with T_h17 cytokine expression is not in contradiction to the results of Mangan et al. In the current study, IL-17 expression was strongly induced by 1 WPI indicating a role for IL-17 early during infection. IL-17 has been shown to recruit neutrophils to mucosal infection sites (57, 92). Mice deficient in IL-23 would not maintain a population of IL-17 producing T_h17 cells resulting in decreased IL-17 production and neutrophil recruitment. Since neutrophil produce signals to enhance T_h1 responses, this lack of recruitment could be detrimental to the host, if T_h1 cells are needed for proper host response to *C. rodentium.*

In this mouse model of colitis, the presence of a persistent *H. hepaticus* infection resulted in slight suppression of *C. rodentium*-induced T_h1 cytokines during maximal disease. Appreciably prolonged disease (clinically and histologically) was associated with a modest increase in IL-17 expression, as well as sustained marked elevation in nT_{reg} cells. Increased IL-

17 expression in mice concurrently infected with *H. hepaticus* could be due to increased IL-17 on a per cell basis or non-helper T cell sources. However, an expanded T_h17 cell population as a result of the lower IFN-γ is more likely because IFN-γ has been shown to suppress T_h17 cell generation. Lower T_h1 cytokine production would allow for an increase in the T_h17 cell population, in concordance with what is observed with *H. hepaticus* infected mice during *C. rodentium* infection.

The suppression of T_h1 cytokines associated with concurrent *H. hepaticus* infection may be attributable to inducible regulatory T cells (IL-10 producing T_R1 cells) rather than nT_{reg}, as increases in nT_{reg} cells occur too late to explain the reduced T_h1 host response that has been shown to be associated with active disease. Several $CD4^+$ regulatory T cell populations are thought to exist *in vivo*, including T_h3, thymus-derived naturally-occurring regulatory T cells (nT_{reg}) that are $CD25^+Foxp3^+$, and T regulatory-1 (T_R1) that are induced upon antigen exposure (1, 77). *H. hepaticus* infection has been shown to induce a population of $CD4^+CD25^-$ T cells with a regulatory phenotype dependent on IL-10 that are likely T_R1, however to-date there are no markers exclusive to this regulatory T cell subset (41). IL-10 has been shown to inhibit T_h1 cells, therefore suppression of the *C. rodentium*-induced T_h1 response may be due to IL-10 secretion from T_R1 cells as a result of persistent *H. hepaticus* infection (41). Although this study did not find elevated IL-10 mRNA expression in *H. hepaticus* infected mice, other studies have shown induction of IL-10 mRNA upon *H. hepaticus* infection, as well as demonstrating the functional importance of IL-10 during *H. hepaticus* infection (8, 19, 41-43). A detailed analysis of the lamina propria lymphocyte populations throughout the duration of *C. rodentium* infection with and without *H. hepaticus* and correlation with disease state would help determine the roles of different T cell subsets and their cytokine secretion in disease progression.

This study demonstrates that concurrent infections not only modulate the T_h1-T_h2 balance, but the T_h1-T_h17 balance as well, causing a different disease outcome (10, 25). A previous study showed helminth infection (T_h2-biased) during *C. rodentium* infection resulted in sustained bacterial burden with increased mortality (10). This altered outcome may be due to a shift in T_h1 (or even T_h17) responses to *C. rodentium* to inappropriate T_h2 responses.. Similarly *H. hepaticus* alters the balance of IL-17-producing and IFN-γ-producing T cell populations in response to *C. rodentium* infection. This shift in T_h1-T_h17 balance occurs through modest suppression of T_h1 responses that allows for enhanced expansion of T_h17 cells. Associated with the shift in T_h1-T_h17 balance is prolonged disease exhibited by a delay in weight re-gain and lesion resolution. Additionally, modulation is caused by a persistent subclinical pathogen that would not be detected unless specifically tested for its presence. These findings highlight the impact that undetected infections can have on host response to clinical infections and disease outcome. Persistent subclinical infections may partially explain the diversity of responses people have to acute infections. However, additional studies are required to assess the potential spectrum of roles persistent infections, such as *H. pylori*, *Mycobacterium tuberculosis,* etc. in humans, play in altering host responses to unrelated pathogens. In this model system, host-pathogen interactions during polymicrobial infections can be investigated, as well as the role of subclinical bacterial infection in modulation of inflammatory bowel disease and irritable bowel syndrome.

Figures

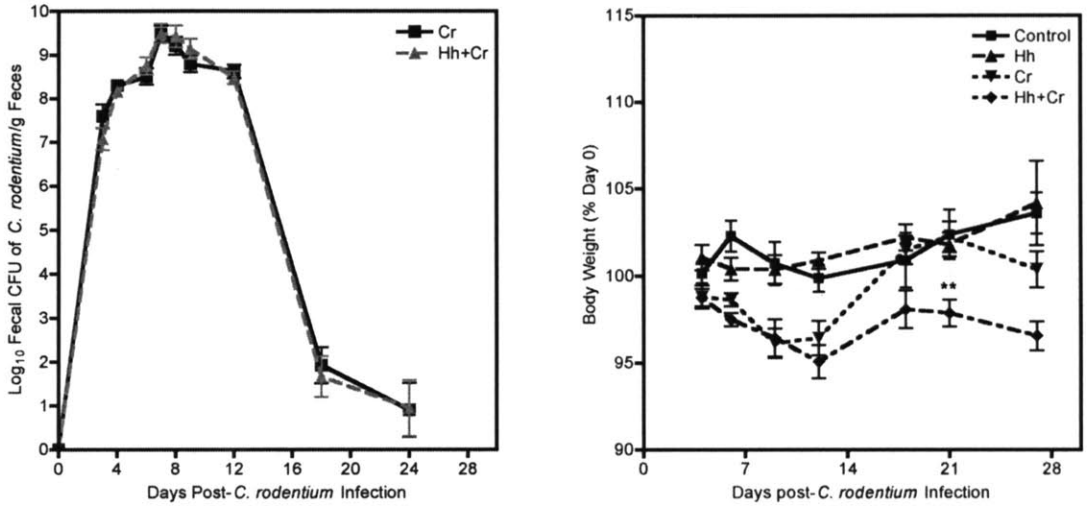

Figure 5-1. Persistent *H. hepaticus* infection impairs recovery of weight loss without changing kinetics of *C. rodentium* bacterial shedding. *C. rodentium* bacterial burden **(A)** reached a maximum by 2 WPI followed by clearance during weeks 3 and 4. Persistent *H. hepaticus* infection did not affect *C. rodentium* bacterial burden. Weight loss **(B)** did not occur in uninoculated or *H. hepaticus* infected mice, where as mice inoculated with *C. rodentium* lost up to 5% of their initial body weight by 2 WPI followed by rapid weight regain, becoming comparable to uninoculated mice by 3 WPI. Weight loss in mice with a persistent *H. hepaticus* infection inoculated with *C. rodentium* was comparable to *Helicobacter*-free mice with *C. rodentium*. However, persistently infected mice did not regain their weight by 4 WPI. Data are represented as mean ± SEM. Two-way ANOVA with Bonferroni post-test, ** $P < 0.01$.

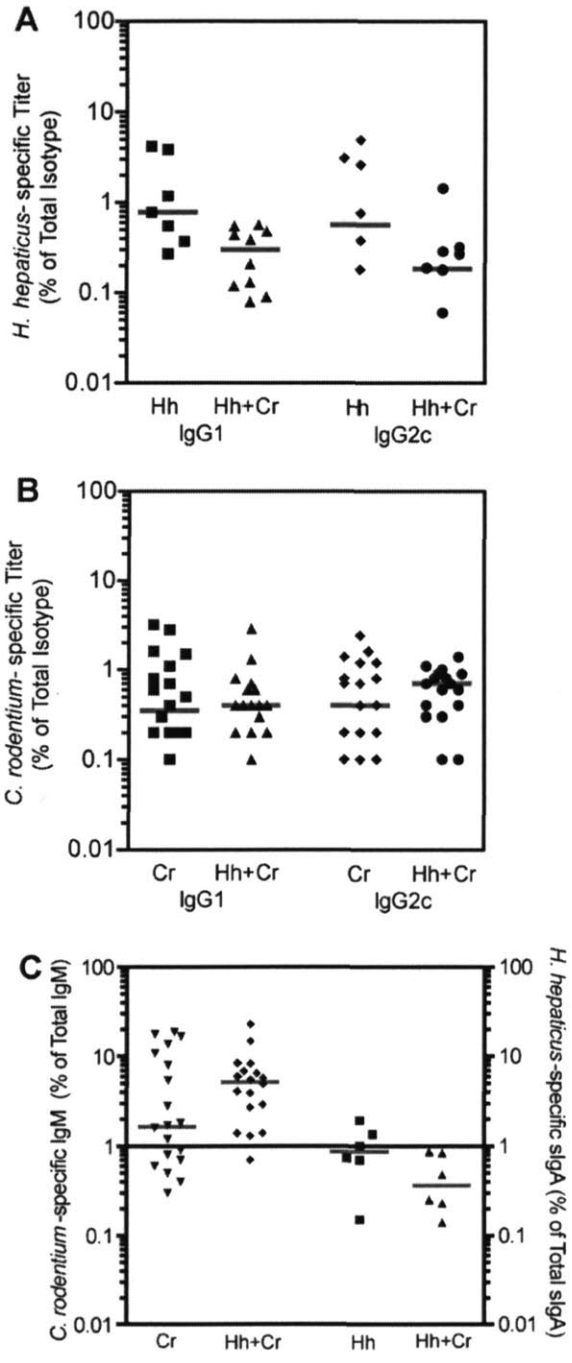

Figure 5-2. Pathogen-specific immunoglobulin titers are not affected by persistent *H. hepaticus* infection. Infection with *H. hepaticus* leads to development of humoral immune response with *H. hepaticus*-specific IgG1 and IgG2c titers that were not different between *H. hepaticus* singly-infected and mice also infected with *C. rodentium* for one week (**A**). *C. rodentium* infected mice, regardless of *Helicobacter* status, develop comparable specific humoral immune responses (IgG2c and IgG1) against *C. rodentium* by 3 WPI (**B**). Development of *C. rodentium*-specific IgM after three weeks of infection was not significantly modulated by an ongoing *H. hepaticus* infection (**C, left Y-axis**). Secretion of IgA, specific to *H. hepaticus,* into the cecal lumen in mice also infected with *C. rodentium* for 1 week was equivalent to *H. hepaticus* singly-infected mice (**C, right Y-axis**). Bars indicate group mean value.

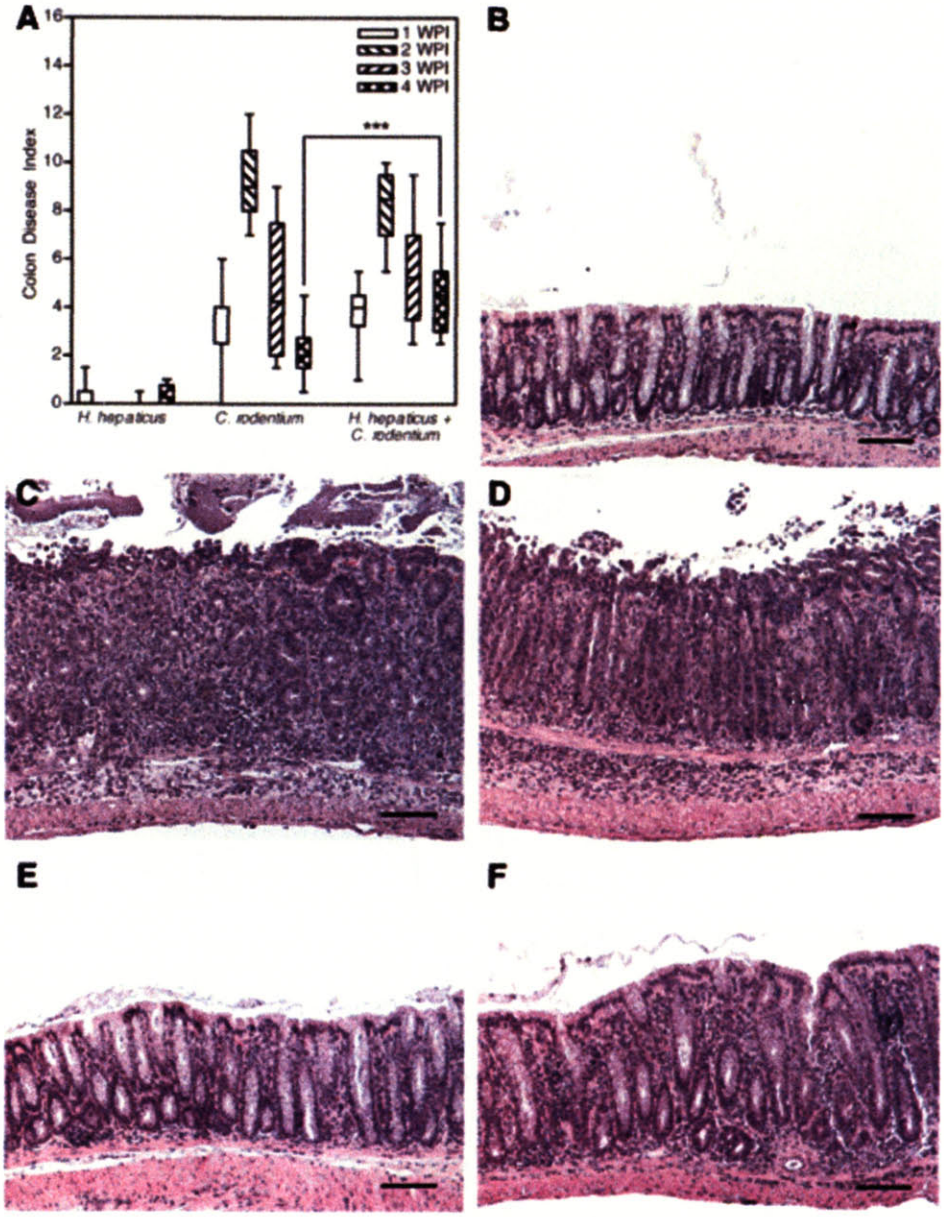

Figure 5-3. *C. rodentium* infection causes marked colon disease that is prolonged by persistent *H. hepaticus* infection. (**A**) Box-whisker plot of disease index attained by summing colonic inflammation, edema, hyperplasia, dysplasia, and epithelial defects each assessed on a scale of 0 to 4. ***$P < 0.001$ (two-way ANOVA with Bonferroni post-test). (**B-F**) Representative (median disease index) Hemoxylin and Eosin stained colon from *H. hepaticus* 3 months post-*H. hepaticus* inoculation (B), *C. rodentium* 2 WPI (C), *H. hepaticus* and *C. rodentium* 2 WPI (D), *C. rodentium* 4 WPI (E), and *H. hepaticus* and *C. rodentium* 4 WPI (F). Scale bar = 160 μm (B-F).

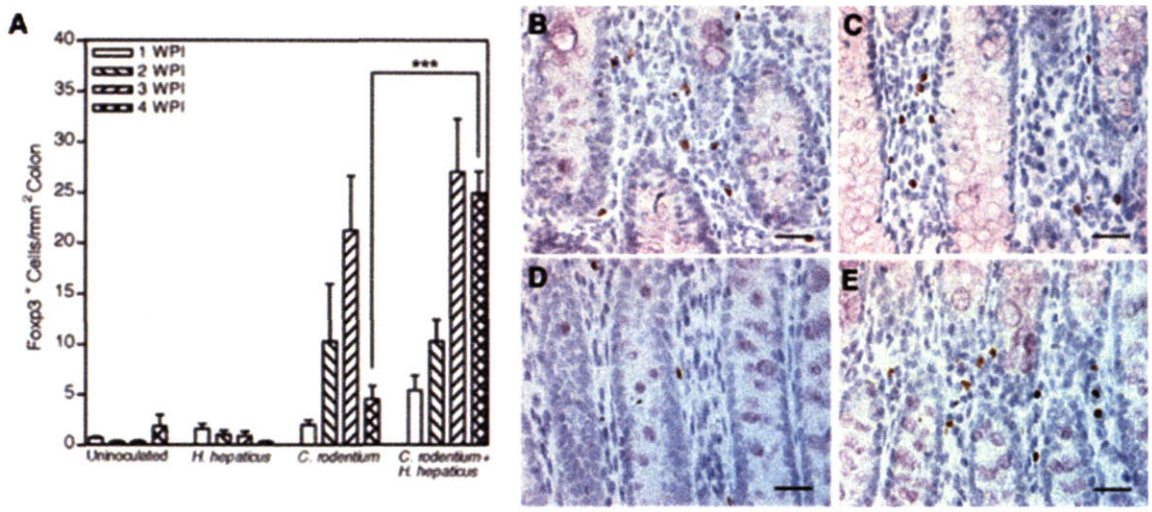

Figure 5-4. Persistent *H. hepaticus* infection maintains elevated numbers of Foxp3 expressing cells in colonic tissue as a result of *C. rodentium* infection. (**A**) Numbers of Foxp3$^+$ cells in colon over 4 WPI. ***$P < 0.001$ (two-way ANOVA with Bonferroni post-test). (**B-E**) Representative photomicrographs of Foxp3 stained colon from *C. rodentium* 3 WPI (B), *H. hepaticus* and *C. rodentium* 3 WPI (C), *C. rodentium* 4 WPI (D), and *H. hepaticus* and *C. rodentium* 4 WPI (E). Error bars in (A) represent SEM. Scale bar = 40 μm (B-E).

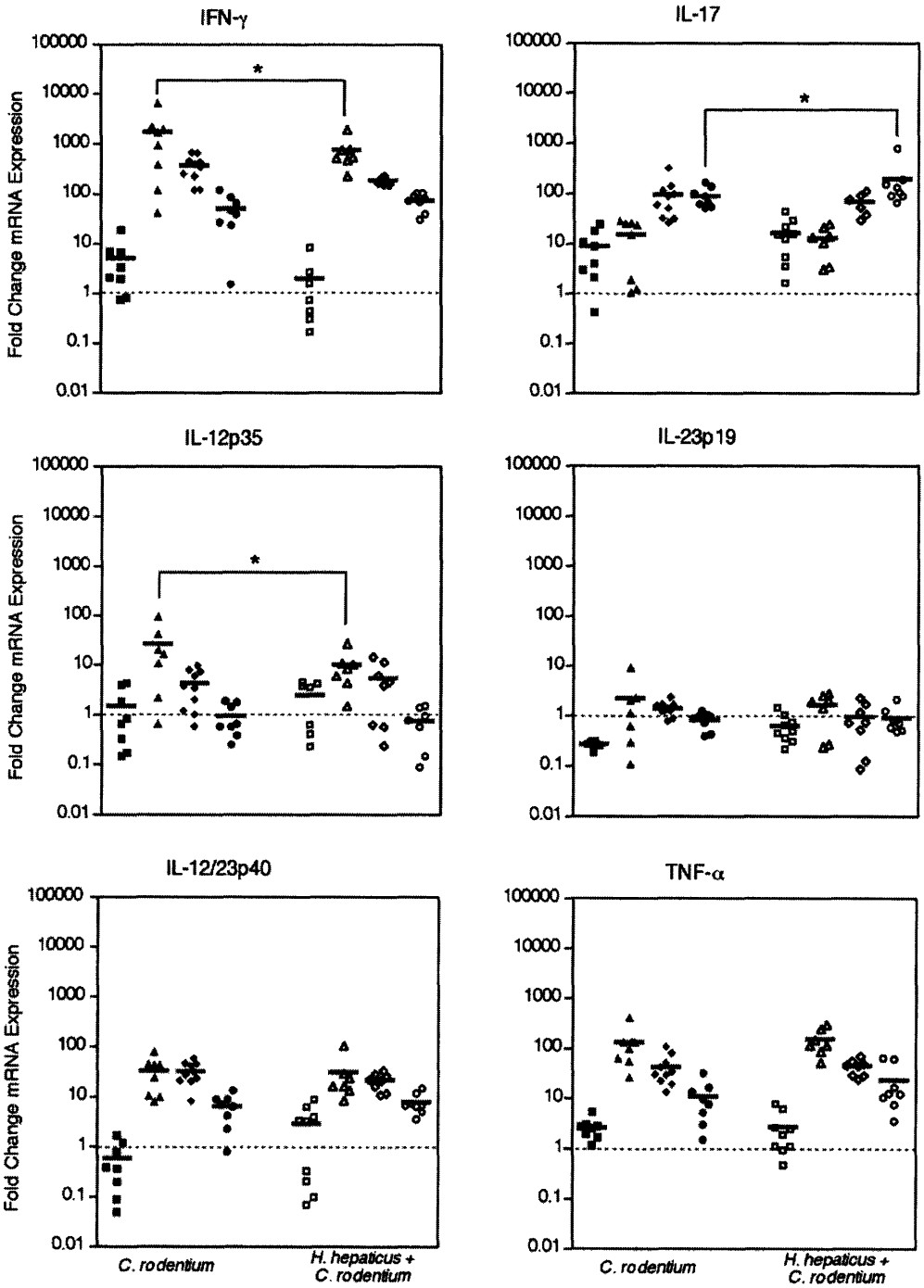

Figure 5-5. Persistent *H. hepaticus* infection suppresses the T_h1 pro-inflammatory cytokine profile induced after *C. rodentium* infection resulting in higher IL-17 (T_h17) expression at 4 WPI. Colonic mRNA expression levels of IFN-γ, IL-17, IL-12p35, IL-23p19, IL-12/23p40, and TNF-α were measured by qRT-PCR. Squares (■, □) are expression levels at 1 WPI; triangles (▲, △) are 2 WPI; diamonds (◆, ◇) are 3 WPI; and circles (●, ○) are 4 WPI. $*P < 0.05$ (two-way ANOVA with Bonferroni post-tests).

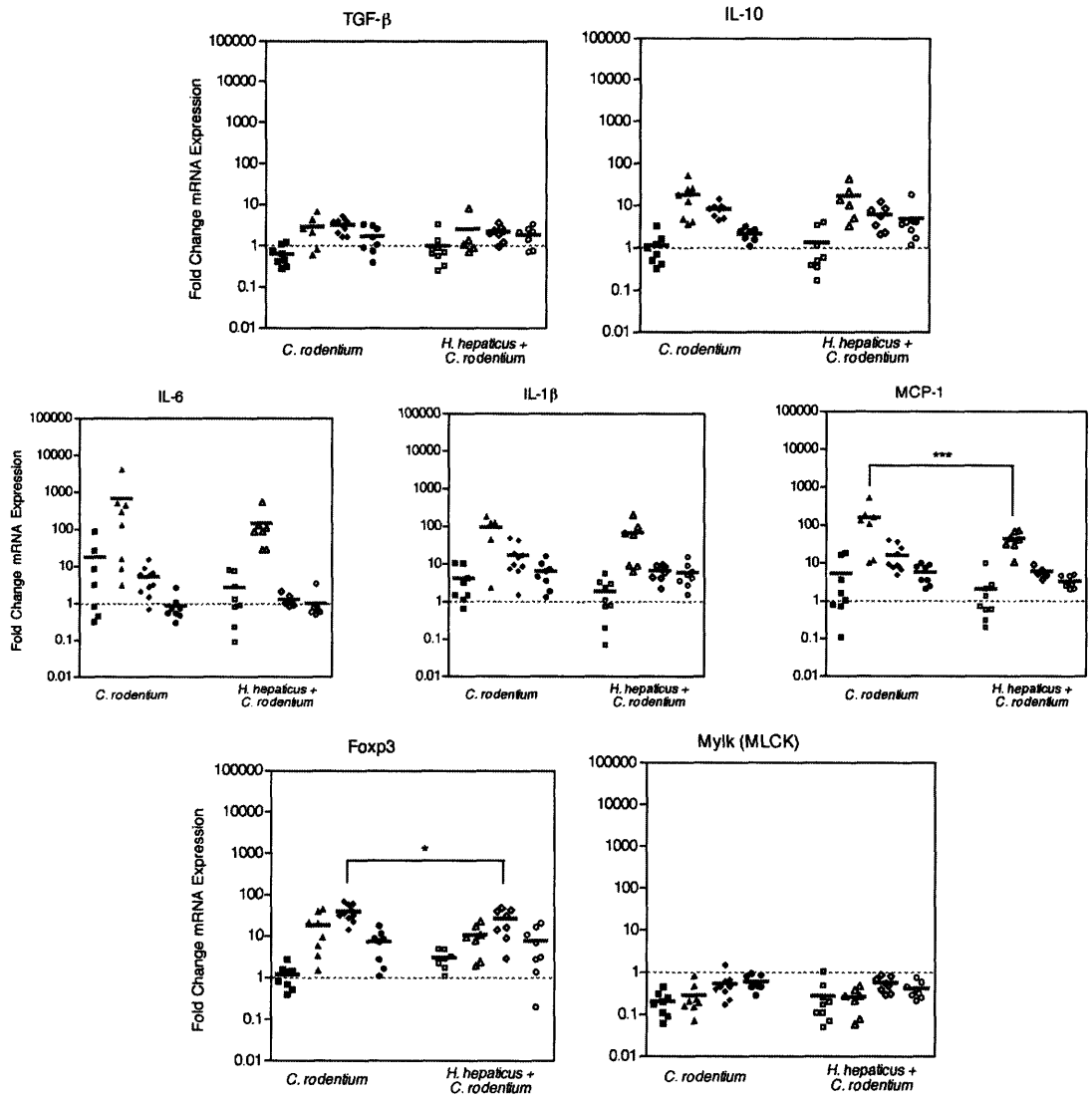

Figure 5-6. Changes in colonic mRNA expression levels of other colitis-associated factors were either not affected by *C. rodentium* infection or concurrent persistent *H. hepaticus* infection. Colonic expression of TGF-β, IL-10, IL-6, IL-1β, MCP-1, Foxp3, and Mylk (MLCK) were measured by qRT-PCR. Squares (■, □) are expression levels at 1 WPI; triangles (▲, △) are 2 WPI; diamonds (◆, ◇) are 3 WPI; and circles (●, ○) are 4 WPI. $*P < 0.05$ (two-way ANOVA with Bonferroni post-test).

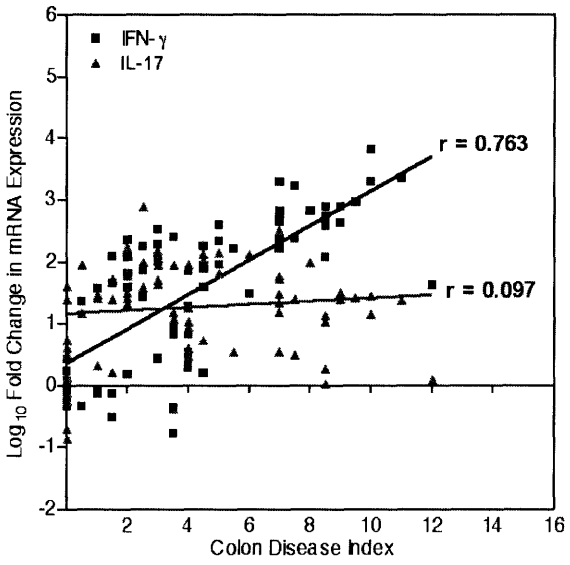

Figure 5-7. Colonic disease severity is positively correlated with IFN-γ expression, not IL-17 expression. Spearman correlation of the colon disease index and corresponding IFN-γ or IL-17 mRNA expression demonstrated a positive correlation between colonic disease severity and IFN- γ expression (Spearman r = 0.763, $P < 0.0032$), but no correlation between colonic disease severity and IL-17 (Spearman r = 0.097, $P > 0.05$). Solid lines are linear regression of colon disease and cytokine expression, not spearman correlation, to aid visualization of correlation.

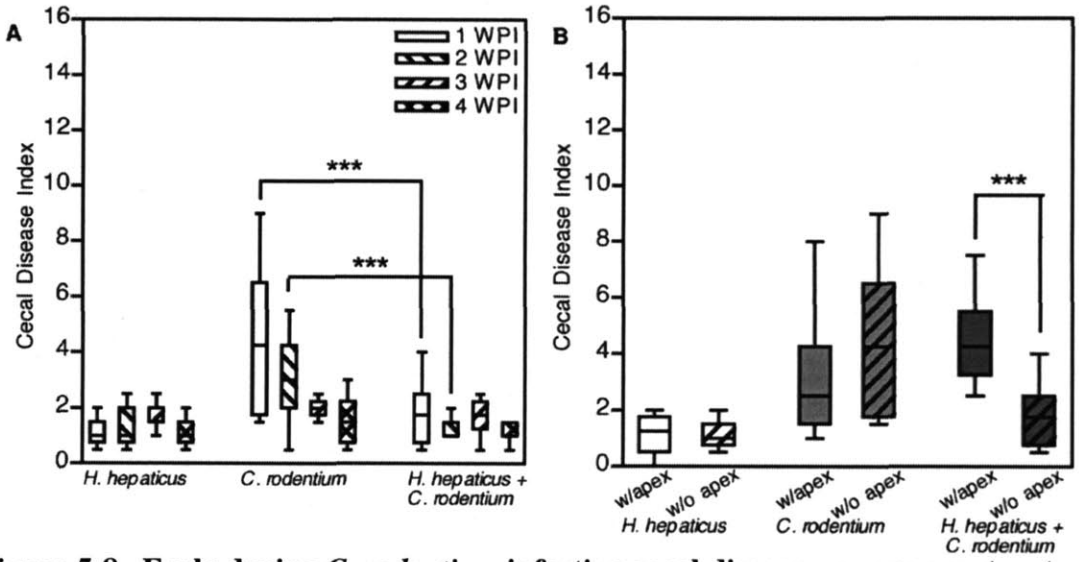

Figure 5-8. Early during *C. rodentium* infection cecal disease was apparent, yet when the cecal apex was not included in evaluation persistent *H. hepaticus* infection prevented disease. When cecal apex was excluded from evaluation, cecal disease **(A)** was present in *C. rodentium* inoculated mice compared with *H. hepaticus* infected, and mice concurrently infected with *H. hepaticus* at 1 week and 2 weeks. Cecal disease in *C. rodentium* inoculated mice was equivalent to *C. rodentium* uninoculated mice by 3 WPI. When the apex of the cecum was evaluated (only at 1 WPI) **(B)** *C. rodentium*-induced disease was observed in mice regardless of *Helicobacter* status. *** $P < 0.001$, two-way ANOVA with Bonferroni post-test.

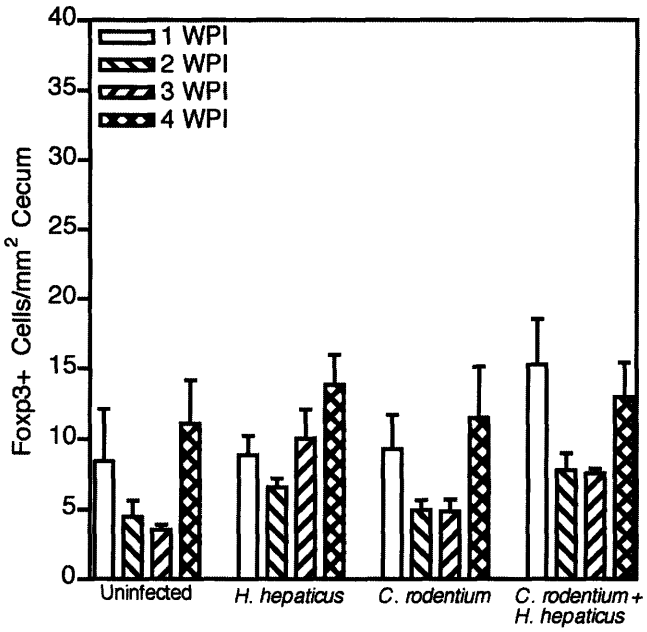

Figure 5-9. Foxp3$^+$ cell numbers in the cecum did not change with *H. hepaticus*, *C. rodentium*, or concurrent infection. Compared with the basal number of Foxp3$^+$ cells in the cecum, *H. hepaticus* infection did not cause a change in number. Between 1 WPI and 4 WPI *C. rodentium* infection did not affect cecal numbers of Foxp3$^+$ cells. No synergistic effects of persistent *H. hepaticus* and *C. rodentium* infection were observed with Foxp3$^+$ cell numbers. $P < 0.05$ was considered significant, two-way ANOVA with Bonferroni post-test.

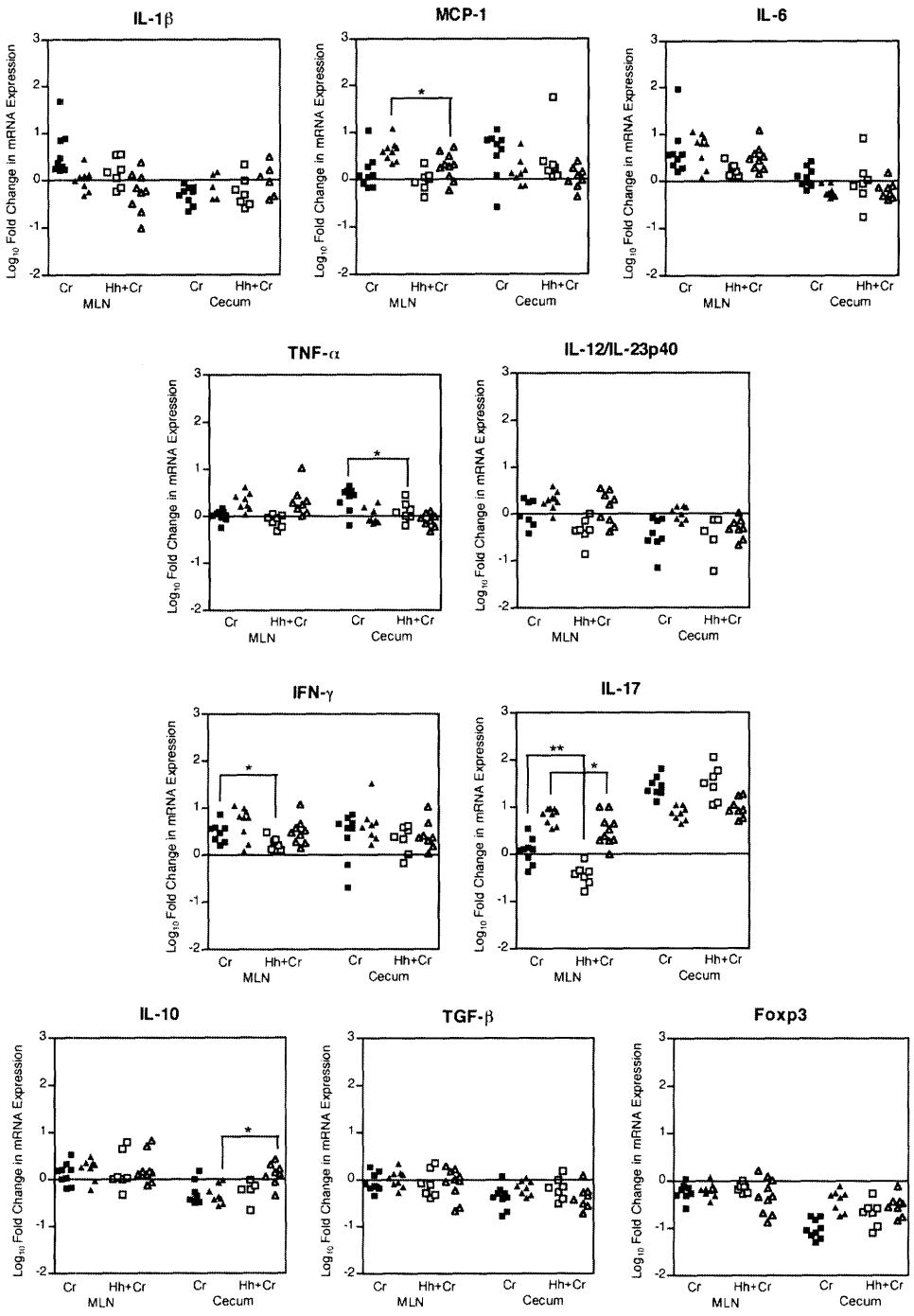

Figure 5-10. ***C. rodentium*** **infection caused modest changes of cytokine expression in the MLN and the cecum with slight suppression of pro-inflammatory cytokines seen with persistent** ***H. hepaticus*** **infection.** MLN mRNA expression levels of MCP-1, IL-17, and IFN-γ were decreased when mice were persistently infected with *H. hepaticus*, along with cecal TNF-α levels. IL-10 expression in the cecum increased with persistent *H. hepaticus* infection. However, in comparison to colonic expression, MLN and cecal mRNA expression levels were modest. Squares (■, □) are expression levels at 1 WPI; triangles (▲, △) are 3 WPI. $*P < 0.05$, $** P < 0.01$ (two-way ANOVA with Bonferroni post-test).

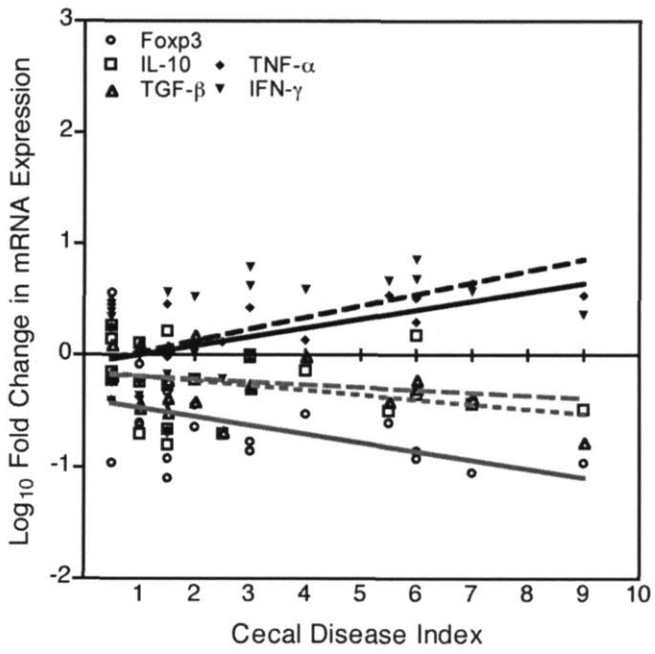

Figure 5-11. Cecal disease severity at 1 WPI is positively correlated with proinflammatory cytokine expression, and negatively correlated with anti-inflammatory cytokine expression. Spearman correlation of the cecal disease index and corresponding IFN-γ or TNF-α mRNA expression demonstrated a positive correlation between cecal disease severity expression of each proinflammatory cytokine (Spearman r = 0.624, $P < 0.01$ and r = 0.516 $P < 0.05$, respectively). The anti-inflammatory cytokines TGF-β and IL-10 had negative correlations with cecal disease severity at 1 WPI, although they were not significant (Spearman r = -0.363, $P > 0.05$ and r = -0.240, $P > 0.05$, respectively). However, Foxp3 expression was significantly negatively correlated with cecal disease severity (Spearman r = -0.519, $P < 0.05$). Lines are linear regression of cecal disease and cytokine expression, not spearman correlation, to aid visualization of correlation.

Chapter 6

Conclusions

Persistent, subclinical infections are prevalent throughout the world, often unbeknownst to the host unless clinical disease develops. However, the host's immune system keeps the infections under control through careful regulation, likely via regulatory T cells, which evolved to minimize damage and benefit both host and pathogen. The resulting regulation may cause subtle changes to the hosts' response to other stimuli by suppressing immune responses. *Helicobacter pylori* is one such organism, residing in the stomachs of approximately 50% of the world population yet causing clinical gastritis in only a small fraction of infected individuals. *H. pylori* elicits a host response that includes immunomodulatory regulatory T cells, which have the ability to suppress the host response. The subclincal persistent murine intestinal pathogen, *Helicobacter hepaticus*, closely related to *H. pylori*, is enzootic in mouse research colonies throughout the world. When regulatory functions of the immune system are absent from the host, IBD-like disease develops in the lower bowel of *H. hepaticus* infected mice. This *H. hepaticus*-dependent disease has been characterized, and develops with or without effector T cells, implicating both innate and adaptive immunity in the disease process. Additionally, in the susceptible A/J strain of mouse, *H. hepaticus* causes hepatitis, hepatocellular carcinoma, and mild lesions in the lower bowel. Differences in host immune responses between disease-resistant C57BL/6 mice and disease-susceptible A/J mice have not been evaluated. The immune response (tissue cytokines and specific antibodies) to *H. hepaticus* in the disease-resistant C57BL/6 mice

was measured early and late during infection, then compared with the disease-susceptible A/J mice at the late time point when disease is present.

To test the hypothesis that host-pathogen interactions during *H. hepaticus* infection of C57BL/6 mice prevents the tolerogenic host responses to soluble oral antigen, disrupts immunogenic protection from acute enteric infection, and host response to enteric infection, the immunomodulatory potential of *H. hepaticus* was evaluated using the model food antigen ovalbumin (OVA), and the murine pathogen *Citrobacter rodentium*, a mouse model of enteropathogenic *Escherichia coli* (EPEC) infection.

Infection of C57BL/6 mice with *H. hepaticus* for up to 6 months results in no clinical signs or disease development. To confirm that *H. hepaticus* infection remained subclinical due to minimal immune activation at the site of infection, histological evaluation of cecal and colonic tissue, cytokines expression levels and antibody titers were compared at 2 months and 18 months post-inoculation. Minimal histological changes in the cecum and no lesions in colon or liver were observed in C57BL/6 mice infected with *H. hepaticus* after 18 months. At 2-3 MPI colonic, MLN, and cecal cytokine expression levels were equivalent to uninoculated C57BL/6 control mice, except for a significant increase in cecal IL-17 mRNA. Cecal tissue cytokines at 18 MPI remained equivalent to uninoculated age-matched C57BL/6 mice, as well as the levels measured at 2-3 MPI. Additionally, comparison of cytokine levels of C57BL/6 mice 18 MPI with matched samples from susceptible A/J mice revealed significant strain differences with increases of both pro-inflammatory as well as anti-inflammatory cytokines in the *Helicobacter*-free A/J mice. Infection of A/J mice for 18 months resulted in a broad decrease in cecal cytokine expression compared with *Helicobacter*-free A/J mice. Overall, C57BL/6 mice have low

background levels of cytokines compared with A/J mice throughout their infection with *H. hepaticus* that correlate with the lack of tissue damage.

Oral vaccines are attractive to manufacture because mucosal immunity is the first line of defense against most pathogens, and they have better patient compliance due to the ease of delivery. Complicating the design and development of oral vaccines, is natural immunologic non-responsiveness to exposure of antigen at the mucosal surface either through tolerance or ignorance. Oral delivery of soluble antigen not in the context of an adjuvant leads to tolerance, rather than immunity. Using a model of oral tolerance to investigate the immunomodulatory effects of a concurrent infection by acting as an adjuvant in the mucosa, the ability of subclinical *H. hepaticus* infection to influence tolerance to the soluble antigen OVA was investigated. Previous studies demonstrated that enteric helminth infection acts as an adjuvant, priming for a T_h2-biased response to soluble OVA (71, 72). A comparable T_h1 adjuvant effect was mediated by *H. hepaticus* when feeding OVA to infected mice, as persistent infection with *H. hepaticus* primed for a systemic IFN-γ response to a single high-dose of intragastrically administered OVA. In the same study, *in vivo* assessment of tolerance, OVA-specific IgG2c antibody titers, were also not tolerized in mice with a subclinical *H. hepaticus* infection. When OVA was administered at a high-dose continuously, robust oral tolerance was induced as evident by a complete shutdown of OVA-specific recall responses *ex-vivo*. *H. hepaticus* infection was not able alter the tolerogenic host responses elicited by continuous high-dose feeding of OVA, indicating continuous feeding may induce oral tolerance outside the gastrointestinal tract. Additionally, IL-10 was not required for tolerogenic host responses, as OVA-specific responses in IL-10-deficient mice were comparable to wild-type mice. Thus, the ability of enteric pathogens to influence immunologic responses to a model dietary antigen is not limited to T_h2

polarizing infections, and does not depend on IL-10 signaling or the establishment of an inflammatory cell infiltrate at the site of antigen uptake, as *H. hepaticus* infection in wild type C57BL/6 mice is subclinical. These findings have implications for understanding the basic mechanism of oral tolerance as well as for possible immunomodulatory consequences of persistent subclinical infections with *Helicobacter* species. In addition, these studies suggest continuous feeding of antigen at a high dose would be the best option when using oral tolerance therapy for autoimmune diseases in populations that may have subclinical gastrointestinal infections.

In addition to priming, instead of tolerance, persistent infections may interfere with development of immunogenic responses, which are essential for successful vaccination. Acute diarrheal disease is a major world health problem with successful vaccines for only a few of infectious agents. Enteropathogenic *Escherichia coli* (EPEC) infection is a common cause of diarrheal disease for which effective vaccines have yet to be developed. The influence of a persistent infection on development of protective immunity to EPEC was determined using *C. rodentium* as a model of EPEC infection and *H. hepaticus* as a model of persistent infection. Mice developed antibodies specific to *C. rodentium* after immunization with *C. rodentium* proteins, however the mice were not consistently protected from infection or disease. Convalescent mice do have sterilizing immunity, as judged by the lack of infection, clinical weight loss, and disease development upon re-challenge. Persistent *H. hepaticus* infection did not interfere with development of protective immunity from *C. rodentium* infection as *H. hepaticus* infected mice had comparable bacterial burdens, weight, and disease as *Helicobacter*-free mice. It is possible that immunity to *C. rodentium* is too strong to be blocked or altered by another infection. In the current model persistent underlying infections do not appear to interfere

with immunity acquired through infection, although their ability to alter immunogenic responses to vaccines remains undetermined.

Modulation of host response to acute diarrheal infection by a persistent underlying infection was evaluated using *H. hepaticus* and *C. rodentium* as model of persistent subclinical infection during an acute diarrheal infection. Persistent subclinical *H. hepaticus* infection resulted in prolonged *C. rodentium*-induced disease. During acute disease expression of T_h1 pro-inflammatory cytokines were slightly suppressed in mice with an underlying infection. These persistently infected mice had appreciably delayed disease resolution. Associated with delayed resolution was a lack of weight re-gain and a markedly sustained presence of colonic $Foxp3^+$ regulatory T cells. A modest increase in colonic IL-17 expression was also observed. Both T_h17 cells and $Foxp3^+$ regulatory T cells have been associated with chronic inflammation,[*ref, Uhlig] indicating persistent subclinical infections may contribute to intestinal inflammation commonly observed in developing countries.

The consequences of persistent infection are broad, including possible prevention of oral tolerance and prolongation of acute disease. As many subclinical infections go undetected, variations in host responses to vaccines or other infections could be the result of a persistent infection with an immunomodulatory subclinical pathogen, such as *H. hepaticus* in mice or *H. pylori* in humans. The host-pathogen interactions that have evolved to dampen overt pathology to these organisms are able to modulate host responses to other antigens via the cytokine environment, which may result in different helper T cells populations, or suppression of host response through regulatory T cells. These studies establish mouse model systems suitable for further evaluation of the immunologic and clinical consequences of persistent subclinical infections.

Chapter 7

Materials and Methods

Bacterial cultures and inoculations

H. hepaticus 3B1 (ATCC 51449) were grown on tryptic soy agar supplemented with 5% sheep red blood cells or tryptic soy broth (TSB) supplemented with 5% fetal calf serum at 37°C in a microaerobic environment (80% N_2, 10% H_2 and 10% CO_2). Bacteria used for inoculation were visualized by phase contrast and dark field microscopy to confirm the purity and the viability of the cultures. Inocula were prepared from 2- to 3-day liquid cultures that were centrifuged (10 minutes, 8,000 rpm) and re-suspended in TSB to an optical density of 1.0 at 600 nm (10^8 CFU). Mice were inoculated with 200µl of inocula intragastrically using a 24-gauge ball-tipped gavage needle. Control mice were inoculated with sterile TSB. Infection status was confirmed with *H. hepaticus*-specific PCR on fecal DNA from individual mice as previously described (94). For *C. rodentium* infections, mice were gavaged with ~$2x10^9$ of an o/n culture of *C. rodentium* (DBS120 kan^r, DBS129 $strep^r$ or DBS135 tet^r) in 100µl LB broth 7 to 8 weeks post-*H. hepaticus* infection. *C. rodentium* fecal shedding was determined by serial dilution plating of fecal slurries (10µl/mg feces) on LB agar with selection for antibiotic marked strain.

Animals and study designs

Male and female C57BL/6J mice (5-12 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were provided with rodent diet and water *ad libitum* and housed in microisolator cages that were maintained in facilities approved by the Association for

Assessment and Accreditation of Laboratory Animal Care. Until experimentally infected, the mice were maintain specific-pathogen-free including all known *Helicobacter* spp. At indicated time points mice were euthanized by CO_2 asphyxiation for tissue collection.

Oral tolerance studies. Female C57BL/6J mice age 6-8 weeks were used. Female and male C57BL/6 IL10$^{-/-}$ mice (4-6 weeks of age) were a gracious gift from M. Whary (MIT). Either four weeks or 7 to 8 weeks post-inoculation (WPI), mice were fed ovalbumin (OVA) followed by immunization with OVA in Complete Freud's Adjuvant (CFA) one week later. Three weeks after immunization, serum was collected and mice were euthanized.

Vaccine study. Male and female C57BL/6J mice age 5 weeks at study onset. The study included five groups each consisting of 5 female and 5 male mice. The treatment groups were as follows: vehicle+LB; vehicle+*C. rodentium*; s.c. prime/oral boost+LB; s.c. prime/oral boost+*C. rodentium*; and s.c. prime/s.c. boost+*C. rodentium*. S.c. prime/oral boost groups received s.c. immunization on day 0 followed by oral boosting on days 14, 16, and 18. S.c prime/s.c. boost received s.c. prime on day 4 and s.c. boost on day 11. Pre-challenge serum was collected the day prior to challenge. Mice were inoculated with DBS120 or LB broth 4 weeks (Day 28) after first immunization. At 14 days post-inoculation (DPI), serum was collected and mice were euthanized for tissue collection.

Protective immunity pilot study. Male and female, 10 each, C57BL/6J mice age 6 weeks at study onset were used. Five males and five females were inoculated with LB broth and the remainder with DBS120. At 43 DPI, all mice were challenged with DBS135. All 20 mice were euthanized 11 days later (54 DPI) for tissue collection. Serum was collected at 14, 28, and 54 DPI.

Protective immunity with *H. hepaticus* study. Study consisted of 50 female C57BL/6J mice C57BL/6 mice age 6 weeks at study onset divided in the six groups: TSB+*C. rodentium*+LB broth (n = 5); *H. hepaticus*+*C. rodentium*+LB broth (n = 5); TSB+LB broth+*C. rodentium* (n = 10); *H. hepaticus*+LB broth+*C. rodentium* (n = 10); TSB+*C. rodentium*+*C. rodentium* (n = 10); and *H. hepaticus*+*C. rodentium*+*C. rodentium* (n = 10). Four weeks after inoculation with TSB or *H. hepaticus*, mice were inoculated with LB broth or DBS120. At 28 DPI, mice were challenged with either LB broth or DBS129. Serum was collected at 21, 27 and 49 DPI (21 DP-2nd inoculation) when all mice were euthanized for tissue collection.

Concurrent *H. hepaticus* and *C. rodentium* infections studies. Male and female C57BL/6J mice age 5-12 weeks were used. For each study the mice were divided into four infection groups: uninoculated (n = 10); *H. hepaticus* (n = 10); *C. rodentium* (n = 10); and *H. hepaticus*+*C. rodentium* (n = 10). Five studies were conducted: two with euthanasia at 6 DPI (1 WPI); one with euthanasia at 11 DPI (2 WPI); one with euthanasia 21 DPI (3 WPI); and one with euthanasia at 27 DPI (4 WPI). Serum was collected at time of euthanasia.

Survival and body weight measurements

Body weights were monitored on indicated days post-*C. rodentium* infection to evaluate systemic effects of the bacterial infections. Mice were euthanized by CO_2 asphyxiation as necessary (20% body weight loss).

Tissue collection and Histopathology

Draining popliteal and inguinal lymph nodes (PLN), and mesenteric lymph nodes (MLN) were harvested under sterile conditions for re-stimulation assays. Cecal contents and colon fecal

pellets were collected for fecal sIgA or infection confirmation. MLN, cecal apex (~0.5cm) and distal colon (~0.5cm) were snap frozen in liquid nitrogen and stored at –80°C until RNA isolation. Remaining colon and cecum were fixed in 10% formalin, paraffin embedded, and stained with hemoxilyn and eosin for histological evaluation. Cecal and colonic tissue sections were scored on a scale of 0-4 (where 0 = no lesion, 1 = minimal, 2 = mild, 3 = moderate, and 4 = severe) for inflammation, edema, hyperplasia, dysplasia, and epithelial defects by a board-certified blinded pathologist (A. Rogers or B. Rickman). Lesion scores are, in places, presented as tissue disease indices that are a sum of all five categorical scores. Foxp3 immunohistochemistry was performed with Foxp3 primary antibody (FJK-16S, eBiosciences) by DCM Histology Laboratory staff. Numbers of mucosal tissue Foxp3 expressing cells were counted in the cecum (starting from ileocecal junction) and distal colon (starting closest to rectum), excluding gut-associated lymphoid tissue when possible. Ten fields at 20X magnification were counted per tissue per mouse with 3 to 4 mice per group.

C. rodentium immunizations

C. rodentium protein homogenate was prepared from a 500mL o/n culture that was pelleted, washed, resuspended in 10mL PBS, and homogenized twice at >10,000psi. Homogenate was spun down to remove cell membranes and insoluble proteins followed by syringe filtering (0.2μm filter). Protein concentration of the homogenate was measured by Lowry assay according to manufacturer's protocol (Pierce). Each subcutaneous injection consisted of 200μg *C. rodentium* protein homogenate in PBS:Imject Alum (1:1) with a total volume of 100μl total volume. Vehicle/adjuvant control mice received 100μl of PBS:Imject Alum (Pierce). For oral boosting, each mouse received 225μg *C. rodentium* protein homogenate and 5μg Cholera Toxin

in PBS with a total volume of 100μl via intragastric gavage. Vehicle/adjuvant control mice received 5μg Cholera Toxin in 100μl PBS.

Oral antigen administration and peripheral immunization

For the single high-dose experiments, 4 to 7 weeks post-*H. hepaticus* infection, 0.25 ml of either PBS or ovalbumin (OVA) grade V (25 mg in PBS; Sigma-Aldrich) was administered intragastrically. Control mice received PBS or OVA without concurrent *H. hepaticus* infection. For the continuous antigen feeding experiments, 4 to 7 WPI, animals were offered, ad libitum, a 1% OVA grade II (Sigma-Aldrich) solution dissolved in drinking water for 5 consecutive days or standard drinking water as described previously (59). Control mice received 1% OVA grade II solution or standard drinking water in the absence of *H, hepaticus* infection. For both single and continuous high-dose feeding experiments, one week after the first feeding, mice were immunized in the hind footpads with 40μl of 100 μg of OVA in Complete Freund's Adjuvant (CFA) (Sigma-Aldrich).

In vitro re-stimulation

Pooled PLN cells from each group of four or five mice were cultured in 48-well flat bottom plates at $2.5x10^6$ cells/well in 1ml of complete DMEM (Invitrogen; complete DMEM contains 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 100 U penicillin/ml, 100 mg streptomycin/ml, 50 mM b-mercaptoethanol, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate) with 0, 10, 100μg/ml of OVA or purified anti-CD3 (5μg/ml R&D Systems).

Measurement of cytokine production

After 72 hours of culture, supernatants were collected and frozen at -20°C until analyzed for levels of IFN-γ and IL-13 by ELISA as previously described (70, 72). For IFN-γ assays, Immulon II plates (Dynatech Labs) were coated with anti-IFN-γ capture antibody (R4-6A2, BD PharMingen) overnight at 4°C, followed by blocking in PBS with 3% FCS at 37°C for 1 hour. After washing with PBS-Tween (0.05% Tween 20), the culture supernatants or recombinant murine IFN-γ (BD PharMingen), were incubated in triplicate for 1 hour at 37°C. Plates were then washed and incubated with biotinylated anti-IFN-γ secondary antibodies (XMG1.2, BD PharMingen), followed by peroxidase-conjugated streptavidin (Invitrogen) and developed with *O*-phenylenediamine (Invitrogen) or TMB (Pierce). Reactions were stopped with 2N H_2SO_4 and the plates were read at 490nm (*O*-phenylenediamine) or 450nm (TMB) using an Emax microplate reader (Molecular Devices). IL-13 was measured with a DuoSet ELISA development kit for the detection of murine IL-13 according to the manufacturer's instructions (R&D Systems). The concentrations of cytokine in each sample were calculated from standard curves using SOFTmax PRO software (Molecular Devices).

Antibody titers

OVA. OVA-specific IgG1 and IgG2c titers in serum collected at sacrifice from individual mice were measured with ELISAs. OVA-coated Immulon II plates were blocked with PBS/3% FCS for 1 hours at room temperature (RT) and washed in PBS/Tween 20 before addition of diluted serum samples, in triplicate. After 2 hours at RT, isotype specific antibody responses were detected using horseradish peroxidase conjugated goat anti-mouse IgG1 and IgG2c (Southern Biotech). The reaction was developed with TMB and read at 450 nm. OD values were converted to mg/ml of OVA-specific IgG1 or IgG2c.

H. hepaticus, C. rodentium, and total immunoglobulin. Total and bacteria-specific fecal sIgA, and serum IgM, IgG1, and IgG2c were measured by ELISA. Total antibody titers were measured according to manufacturers' protocol (BD PharMingen). Unlabeled and biotin-labeled matched antibody pairs for IgA (C10-1 and C10-3, BD PharMingen), IgM (II/41 and R6 60.2, BD PharMingen), IgG1 (A85-3 and A85-1, BD PharMingen) and IgG2c (Southern Biotech) were used for capture and detection. For bacteria-specific titers, sample wells were coated with either *C. rodentium* (1:20 in PBS) or 100μg *H. hepaticus* outer membrane preps in coating buffer prepared as described previously (87). Titers in diluted serum were quantified by OD value comparison to standard curves of purified mouse IgA (S107, Southern Biotech), IgM (11E10, Southern Biotech), IgG1 (MOPC-31C, BD PharMingen) and IgG2c quantitated serum (550μg/ml, Bethyl Laboratories).

Q-PCR

Total RNA was isolated from distal colon, cecal tip, or MLN using TRIzol reagent (Invitrogen), cleaned with RNeasy Kit (Qiagen), and reverse transcribed (Invitrogen) following manufacturers' protocols. Quantitative real-time PCR was performed on cDNA using TaqMan® Gene Expression Assays (Applied Biosystems) specific for murine GAPDH (Mm99999915_g1), IL-4 (Mm99999154_m1), IL-13 (Mm00434204_m1), IL-10 (Mm00439616_m1, Mm99999062_m1), TGF-β (Mm00441724_m1), TNF-α (Mm99999068_m1), IFN-γ (Mm99999071_m1), IL-12/IL-23p40 (Mm99999067_m1), IL-12p35 (Mm01208555_m1), IL-23p19 (Mm00518984_m1), IL-6 (Mm00446190_m1, Mm99999064_m1), IL-17 (Mm00439619_m1), MCP-1 (Mm00441242_m1), IL-1β (Mm00434228_m1), Foxp3 (Mm00475156_m1), and Mylk (MLCK, Mm00653039_m1). Each sample was normalized to

internal GAPDH levels and to the average of control (uninoculated mice) samples at the same time point.

Statistics

Statistical significance in bacterial counts, weight change, and disease indices were determined by two-way ANOVA, Kruskal-Wallis followed by Dunn's post-test, Mann-Whitney, or unpaired two-tailed Student's t test where appropriate. Cytokine production in re-stimulation assays and antibody titers were evaluated for statistical significance by one-way ANOVA with Bonferroni post-tests. Statistical differences in mRNA expression were evaluated by two-way ANOVA for all time points and infection statuses followed by Bonferroni post-tests. Spearman correlation was used to evaluate correlation between disease indices and cytokine mRNA expression levels. All analyses were done with GraphPad Prizm Software version 4.0. P values of < 0.05 were considered significant.

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