

## Modulation of Acute Diarrheal Illness by Persistent Bacterial Infection<sup>∇</sup>

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**Acute diarrheal illness is a global health problem that may be exacerbated by concurrent infection. Using *Citrobacter rodentium*, a murine model of attaching and effacing diarrheagenic *Escherichia coli*, we demonstrate that persistent *Helicobacter hepaticus* infection modulates host responses to diarrheal disease, resulting in delayed recovery from weight loss and from tissue damage. Chronic colitis in concurrently infected mice is characterized by macrophage and Foxp3<sup>+</sup> regulatory T-cell accumulation. Prolonged disease is also associated with increased interleukin-17 expression, which may be due to suppression of gamma interferon during the acute phase of diarrheal infection. This new model of polymicrobial infection provides insight into the mechanism by which subclinical infection can exacerbate morbidity due to an unrelated self-limiting infection.**

Acute diarrheal illness is a major health problem worldwide. In the developed world most cases are self-limiting and can be treated with supportive care; however, in the developing world, diarrheal illness is a major cause of morbidity and mortality, particularly in children. Infection with enteropathogenic *Escherichia coli* is estimated to account for approximately 7% of pediatric acute diarrheal illness (24, 29). Malnutrition, immunosuppression, and concurrent disease influence the severity and outcome of diarrheal illness, but the effect of heterologous infection has not been characterized.

*Citrobacter rodentium* infection of laboratory mice has been studied as a model of enteropathogenic *E. coli* infection in children (3). In C57BL/6 mice *C. rodentium* infection causes loose stool progressing to diarrhea in severe cases, poor overall body condition, and weight loss (28, 37). Colonic lesions consist of epithelial hyperplasia, submucosal edema, and mucosal inflammation that ranges from mild to severe with erosions, ulcerations, and transmural serositis (20, 28). Adult C57BL/6 mice clear *C. rodentium* infection and recover from disease approximately 4 weeks postinoculation (wpi), with full resolution of colonic lesions by 6 wpi (20, 28). Young mice and adults of certain inbred strains develop fatal infection with *C. rodentium* (4, 38). Additionally, comorbidity with helminth infection alters disease severity by inducing interleukin-10 (IL-10)-expressing dendritic cells (8).

With approximately 50% of the world's population infected with *Helicobacter pylori*, subclinical infections in humans are common. *Helicobacter hepaticus* infection in laboratory mice, like *H. pylori* in humans, is highly prevalent and subclinical in otherwise healthy (wild-type) animals (35). Both in cultured cell systems and in vivo, *H. hepaticus* elicits a proinflammatory response from innate and adaptive immune cells including IL-23, gamma interferon (IFN- $\gamma$ ), and tumor necrosis factor alpha (TNF- $\alpha$ ). However, persistent infection with this bacte-

rium is balanced by regulatory responses, including IL-10 production by regulatory T (T<sub>reg</sub>) cells that prevent clinical disease (15–18, 22). Subclinical disease develops in susceptible strains, such as male A/J mice (10, 42), yet the role of IL-10 and T<sub>reg</sub> cells in suppressing clinical disease is revealed with infection of IL-10 or T-cell-deficient mice (6, 16–18, 40). Superimposition of a second, unrelated infection on the dynamic homeostasis of proinflammatory and regulatory cell populations could tip the balance and alter the outcome of the subsequent infection. We tested the hypothesis that heterologous infection can enhance morbidity by challenging mice with *C. rodentium* with or without concurrent *H. hepaticus* infection.

### MATERIALS AND METHODS

**Mice.** Male and female 5- to 12-week-old C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) mice were used for all studies. All experiments were approved by the Massachusetts Institute of Technology Committee on Animal Care. Mice were fed a 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, International. Until experimentally inoculated, the mice were maintained specific pathogen free of known murine bacterial, viral, and parasitic infections including all known *Helicobacter* spp. For each experiment, the mice were divided into four treatment groups: uninoculated ( $n = 10$ ), inoculated with *H. hepaticus* ( $n = 10$ ), inoculated with *C. rodentium* ( $n = 10$ ), and inoculated with *H. hepaticus* followed by *C. rodentium* ( $n = 10$ ). Five independent experiments were conducted: two with necropsy at 1 week after *C. rodentium* inoculation, one with necropsy at 2 wpi, one with necropsy at 3 wpi, and one with necropsy at 4 wpi.

**Bacterial infections.** *H. hepaticus* 3B1 (ATCC 51449) was grown on tryptic soy agar supplemented with 5% sheep red blood cells or in tryptic soy broth (TSB) supplemented with 5% fetal calf serum at 37°C in a microaerobic environment (80% N<sub>2</sub>, 10% H<sub>2</sub>, and 10% CO<sub>2</sub>). *H. hepaticus* inocula were prepared from 3-day liquid cultures from which  $\sim 2 \times 10^8$  bacteria (estimated from the optical density at 600 nm) were administered in 200  $\mu$ l of TSB via intragastric gavage to individual mice. Uninoculated mice were gavaged with 200  $\mu$ l of sterile TSB. For *C. rodentium* infections, mice were gavaged with  $\sim 2 \times 10^9$  bacteria from an overnight culture of Kan<sup>r</sup> *C. rodentium* (DBS120) in 100  $\mu$ l of Luria-Bertani broth, 7 to 8 weeks after *H. hepaticus* infection. *Helicobacter* status was confirmed with an all-*Helicobacter* PCR of fecal DNA as previously described (48). *C. rodentium* fecal shedding was determined by plating serial dilutions of fecal slurries (10% [wt/vol] in phosphate-buffered saline) on Luria-Bertani agar with selection for kanamycin.

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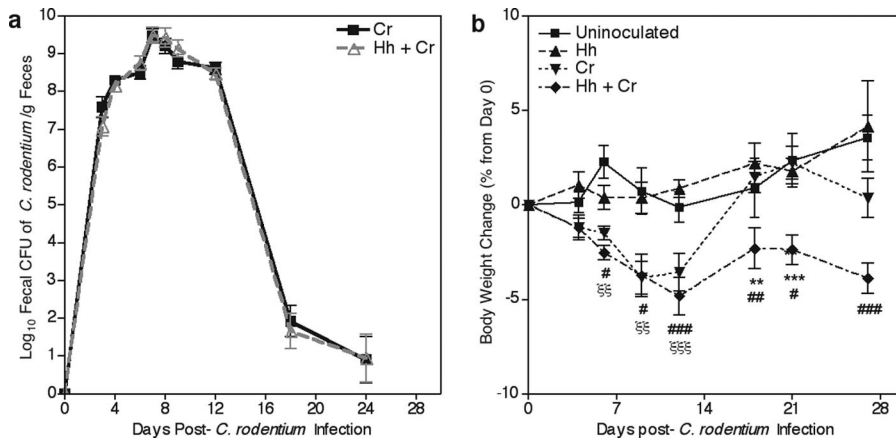


FIG. 1. Concurrent *H. hepaticus* infection impaired recovery of weight loss without altering the fecal shedding of *C. rodentium*. (a) *C. rodentium* fecal shedding at 4 wpi in *Helicobacter*-free mice or mice persistently infected with *H. hepaticus*. (b) Percent change in body weight from day 0 postinoculation with *C. rodentium* through 4 wpi in uninoculated mice and mice inoculated with *H. hepaticus*, *C. rodentium*, or *H. hepaticus* plus *C. rodentium*. Data are represented as means  $\pm$  standard errors of the means. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (for mice inoculated with *C. rodentium* versus *H. hepaticus* plus *C. rodentium*); #,  $P < 0.05$ ; ##,  $P < 0.01$ ; ###,  $P < 0.001$  (for uninoculated mice versus mice inoculated with *H. hepaticus* plus *C. rodentium*);  $\xi\xi$ ,  $P < 0.01$ ;  $\xi\xi\xi$ ,  $P < 0.001$  (for uninoculated mice versus mice inoculated with *C. rodentium*). Two-way ANOVA with Bonferroni posttests was used for statistical analysis. Cr, *C. rodentium*; Hh, *H. hepaticus*.

**Body weight measurements.** Body weights were monitored every 3 to 4 days. Mice were euthanized and excluded from the study if they lost  $>20\%$  of their body weight.

**Tissue collection and histology.** At necropsy fecal and tissue samples were collected. Distal colon ( $\sim 0.5$  cm) was snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until it was used for RNA isolation. The remaining colon was fixed in 10% formalin, paraffin embedded, sectioned at 5  $\mu\text{m}$ , and stained with hematoxylin and eosin for histologic evaluation. Colonic tissue sections were scored on a scale of 0 to 4 (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. Lesion scores are presented as histologic colitis indices that are a sum of all five categorical scores (maximum of 20). Foxp3 immunohistochemistry was performed as previously described (32), using Foxp3 antibody (FJK-16S; eBiosciences, San Diego, CA). Cells expressing Foxp3<sup>+</sup> were counted in the distal to mid-colon at a magnification of  $\times 20$  (1 field is 1.00 mm<sup>2</sup>), excluding gut-associated lymphoid tissue. Ten fields were counted per mouse, and results are presented as the average number of Foxp3<sup>+</sup> cells/mm<sup>2</sup> of colon. F4/80 immunohistochemistry was performed as described for Foxp3 but using F4/80 antibody (CI:A3-1; Abcam, Cambridge, MA). Cells expressing F4/80 were counted in 20 fields of distal colon at  $\times 40$  magnification (1 field is 0.26 mm<sup>2</sup>), also excluding gut-associated lymphoid tissue. Results are presented as the average number of F4/80<sup>+</sup> cells/mm<sup>2</sup> of colon.

**Quantitative real-time PCR.** Total RNA was isolated from distal colon using TRIzol reagent (Invitrogen, Foster City, CA), cleaned up with an RNeasy Kit (Qiagen Sciences, MD), and reverse transcribed (Invitrogen) following the manufacturers' protocols. Quantitative real-time PCR was performed on cDNA using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; assay no. Mm99999915\_g1), IL-6 (Mm00446190\_m1), MCP-1 (Mm00441242\_m1), TNF- $\alpha$  (Mm99999068\_m1), IFN- $\gamma$  (Mm99999071\_m1), IL-10 (Mm00439616\_m1), transforming growth factor  $\beta$  (TGF- $\beta$ ; Mm00498234\_m1), IL-1 $\beta$  (Mm00434228\_m1), IL-12/IL-23p40 (Mm99999067\_m1), IL-12p35 (Mm01208555\_m1), IL-23p19 (Mm00518984\_m1), and IL-17 (Mm00439619\_m1). Each sample was calibrated to internal GAPDH levels and normalized to the average value of control (uninoculated mice) samples at the same time point.

**Statistics.** Statistical significance in bacterial counts, weight change, disease indices, mRNA expression, and F4/80<sup>+</sup> and Foxp3<sup>+</sup> cell numbers was determined by two-way analysis of variance (ANOVA) followed by Bonferroni posttests. A Spearman correlation was used to evaluate the correlation between disease indices and cytokine mRNA expression levels. All analyses were done with GraphPad Prism software, version 4.0.  $P$  values of  $<0.05$  were considered significant.

## RESULTS

### Delayed recovery from acute diarrheal illness as a result of persistent *H. hepaticus* infection.

In agreement with previous reports where the *Helicobacter* spp. status of mice was not specified (21, 43, 44), fecal shedding of *C. rodentium* in *Helicobacter*-free C57BL/6J mice reached a maximum of  $10^9$  CFU/g of feces between 1 and 2 wpi (Fig. 1a). Over the next week *C. rodentium* was cleared, and fecal shedding was undetectable ( $<10^3$  CFU/g of feces) by 3 wpi. Because the severity of *C. rodentium*-induced disease can be decreased by as little as a 1 log reduction in peak bacterial load (19), we carefully compared fecal shedding with and without *H. hepaticus* infection. Persistent infection with *H. hepaticus*, confirmed by PCR on DNA isolated from fecal pellets, did not affect fecal shedding of *C. rodentium* (Fig. 1a). Body weight change in mice infected with *H. hepaticus* alone was not different from uninoculated mice, confirming the subclinical nature of the infection. In contrast, body weight loss was apparent in *Helicobacter*-free *C. rodentium*-inoculated mice by 1 wpi. These mice lost an average of 4% of their initial body weight by 12 days postinoculation (Fig. 1b) ( $P < 0.001$ ). By 3 wpi these mice recovered, and their body weights were not significantly different from those of uninoculated mice. The extent of weight loss in *C. rodentium*-inoculated mice with a persistent subclinical *H. hepaticus* infection was comparable to that of *Helicobacter*-free *C. rodentium*-inoculated mice (Fig. 1b). However, concurrently infected mice did not regain body weight at 3 wpi ( $P < 0.001$  compared to *Helicobacter*-free *C. rodentium*-inoculated mice) and remained underweight compared to control mice through 4 wpi ( $P < 0.001$ ). Given that persistent subclinical *H. hepaticus* infection caused delayed recovery from weight loss, colonic lesions were evaluated to ascertain whether this morbidity was associated with an enhanced chronicity of mucosal disease.

**Prolonged colitis in mice concurrently infected with *H. hepaticus*.** *H. hepaticus* infection alone did not cause intestinal

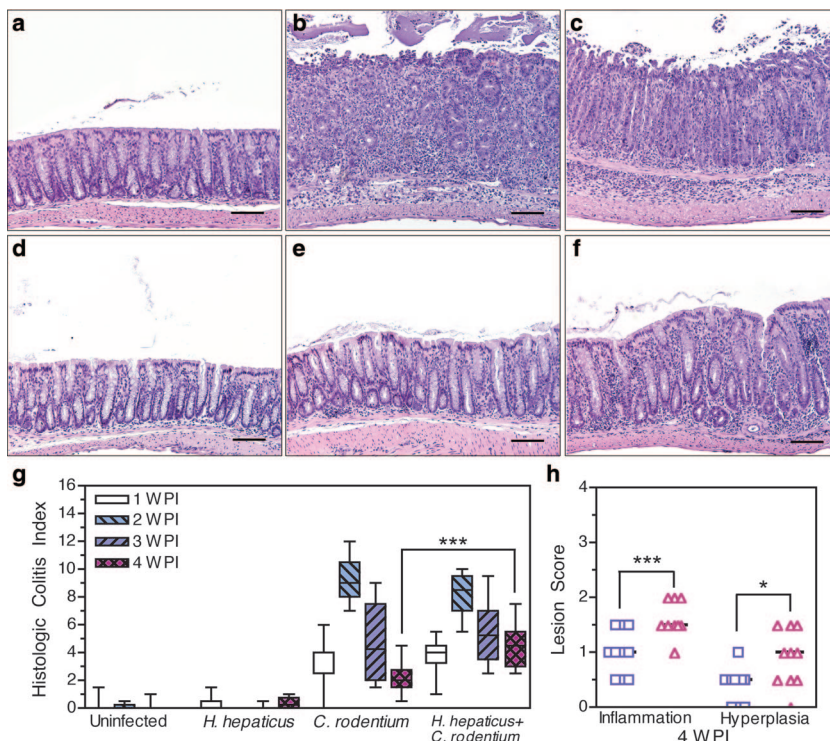


FIG. 2. *C. rodentium* infection causes marked colon disease that is prolonged by persistent subclinical *H. hepaticus* infection. (a to f) Hematoxylin- and eosin-stained colon corresponding to median histologic colitis index of the following treatment groups at the indicated time points: uninoculated (a), *C. rodentium* at 2 wpi (b), *H. hepaticus* and *C. rodentium* at 2 wpi (c), *H. hepaticus* at 3 months post-*H. hepaticus* inoculation (d), *C. rodentium* at 4 wpi (e), and *H. hepaticus* and *C. rodentium* at 4 wpi (f). (g) Histologic colitis index comprised of inflammation, edema, hyperplasia, dysplasia, and epithelial defects, each assessed on a scale of 0 to 4. Boxes represent first to third quartiles, and median values are indicated by a horizontal line. Bars represent ranges. (h) Inflammation and hyperplasia lesion scores for individual mice inoculated with *C. rodentium* (□) and with *H. hepaticus* and *C. rodentium* (△) at 4 wpi. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ . Two-way ANOVA on the histologic colitis index or lesion scores from all groups and time points with Bonferroni posttests was used. Scale bar, 160  $\mu\text{m}$ .

lesions, and histologic colitis in these animals was not significantly different from that of uninoculated mice (Fig. 2a, d, and g). By 1 wpi *Helicobacter*-free *C. rodentium*-inoculated mice developed colonic lesions consisting of inflammation, edema, epithelial destruction, and hyperplasia. Categorical lesion scores for each of these parameters were summed to form a histologic colitis index. At 1 wpi the median histologic colitis index in *Helicobacter*-free *C. rodentium*-inoculated mice was 4.0 (range, 0.0 to 6.0) (Fig. 2g) ( $P < 0.001$  compared to uninoculated mice). Over the following week, histologic colitis reached maximal severity, characterized by gland elongation, goblet cell depletion, mucosal and submucosal inflammation, and epithelial defects, including erosions, crypt atrophy, and mild dysplasia (Fig. 2b). At peak severity, *Helicobacter*-free *C. rodentium*-inoculated mice had an index of 9.0 (range, 7.0 to 12.0) (Fig. 2b and g). Disease resolved in these mice during 3 and 4 wpi (at 4 wpi, index of 2.0; range, 0.5 to 4.5) (Fig. 2e and g). Concurrent infection did not significantly alter the maximal severity of histologic colitis at 2 wpi (index of 8.5; range, 5.5 to 10.0) (Fig. 2c and g), but concurrent *H. hepaticus* infection did delay the resolution of *C. rodentium*-induced lesions. At 4 wpi the index of concurrently infected mice was 4.5 (range, 2.5 to 7.5) compared with 2.0 (range, 0.5 to 4.0) in *Helicobacter*-free *C. rodentium*-inoculated mice (Fig. 2e to g) ( $P < 0.001$ ). Although lesions in both *Helicobacter*-free and concurrently in-

fectected mice were resolving at 4 wpi, inflammation and hyperplasia were more severe in concurrently infected mice (Fig. 2 h) ( $P < 0.001$  and  $P < 0.05$ , respectively). Thus, delayed recovery from weight loss was associated with colitis of a longer duration. Morphometrics were used to determine if the chronicity of disease in concurrently infected mice was associated with an increase in the number of macrophages, which are a hallmark of chronic inflammation (26).

**Chronic colitis is associated with an increased number of F4/80<sup>+</sup> macrophages.** Macrophages expressing F4/80 (a macrophage-specific marker) were present in low numbers in the colon of uninoculated mice (Fig. 3a and b) (average of all time points,  $20 \pm 18$  cells/ $\text{mm}^2$ ). No significant change in macrophage numbers was seen in colons of *H. hepaticus*-infected animals, consistent with a lack of disease (Fig. 3a and c) (average of all time points,  $16 \pm 20$  cells/ $\text{mm}^2$ ). Macrophages did accumulate in the colon during *C. rodentium* infection (Fig. 3a) ( $P < 0.001$ ). Macrophage numbers were maximal in *Helicobacter*-free *C. rodentium*-inoculated mice at 1 wpi ( $175 \pm 36$  cells/ $\text{mm}^2$ ), decreased by 2 wpi ( $72 \pm 23$  cells/ $\text{mm}^2$ ), and remained constant at 3 and 4 wpi ( $98 \pm 34$  and  $125 \pm 28$  cells/ $\text{mm}^2$ , respectively) (Fig. 3a and d). Macrophage numbers in concurrently infected mice were not significantly different from those in mice infected with *C. rodentium* alone at 1, 2, or 3 wpi (Fig. 3a). However, at 4 wpi, instead of staying constant



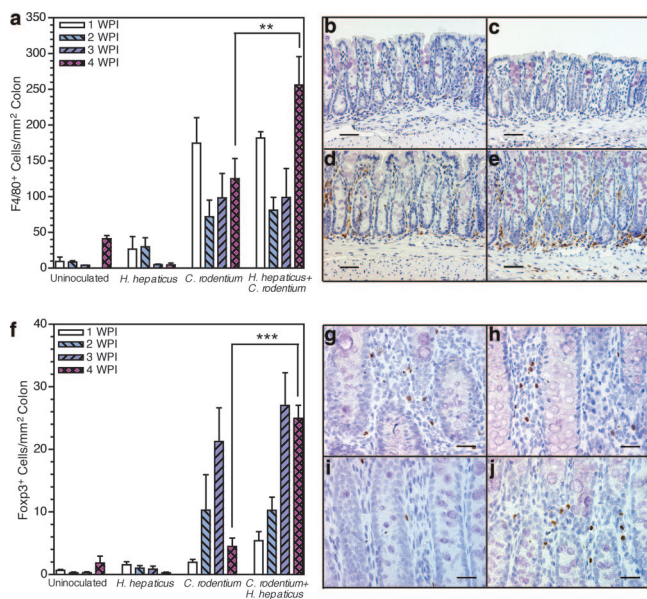


FIG. 3. Concurrent *H. hepaticus* infection increases macrophage infiltration and maintains elevated numbers of natural  $T_{reg}$  cells in colonic tissue 4 weeks after *C. rodentium* inoculation. (a) Numbers of  $F4/80^+$  cells in colons enumerated in 20 fields (magnification,  $\times 400$ ;  $0.26 \text{ mm}^2$ ) per mouse distal colon ( $n = 3$  to 4 per group) at 1, 2, 3, and 4 wpi. (b to e) Representative photomicrographs of  $F4/80^+$  macrophages in the colon of uninoculated mice (b) and mice inoculated with *H. hepaticus* (c) or *C. rodentium* (d) at 4 wpi and with *H. hepaticus* and *C. rodentium* at 4 wpi (e). (f) Numbers of  $Foxp3^+$  cells in the colon assessed from 10 fields (magnification,  $\times 200$ ;  $1.00 \text{ mm}^2$ ) per mouse distal colon ( $n = 3$  to 4 per group) at 1, 2, 3, and 4 wpi.  $***, P < 0.001$  (two-way ANOVA with Bonferroni posttests). (g to j) Representative photomicrographs of  $Foxp3^+$  cells in colon from mice inoculated with *C. rodentium* at 2 wpi (g), *H. hepaticus* and *C. rodentium* at 2 wpi (h), *C. rodentium* at 4 wpi (i), and *H. hepaticus* and *C. rodentium* at 4 wpi (j).  $**$ ,  $P < 0.01$ ;  $***$ ,  $P < 0.001$ . Two-way ANOVA on the numbers of  $F4/80^+$  or  $Foxp3^+$  cells/ $\text{mm}^2$  of colon from all groups and time points with Bonferroni posttests was used. Error bars in panels a and f represent standard errors of the means. Scale bars,  $80 \mu\text{m}$  (b to e) and  $40 \mu\text{m}$  (g to j).

at 2 and 3 wpi, as in *Helicobacter*-free *C. rodentium*-infected mice, the number of colonic macrophages significantly increased to  $256 \pm 40$  cells/ $\text{mm}^2$  in concurrently infected mice (Fig. 3a, d, and e) ( $P < 0.01$ ), indicating chronic mucosal inflammation. Natural  $Foxp3^+$   $T_{reg}$  cells play a key role in limiting immunopathology during infection and chronic inflammatory diseases as well as in contributing to peripheral tolerance (1). Morphometrics were used to enumerate  $Foxp3^+$  cells to determine if greater chronicity of histologic colitis was associated with accumulation of natural  $T_{reg}$  cells.

**$Foxp3^+$  cells accumulate in response to infectious colitis and persist during chronic disease.** As expected in the absence of histologic colitis, *H. hepaticus* infection alone caused no colonic accumulation of  $Foxp3^+$   $T_{reg}$  cells (Fig. 3f) compared to uninoculated mice ( $1 \pm 1$   $Foxp3^+$  cells/ $\text{mm}^2$ ). Early after *C. rodentium* inoculation (1 wpi) there was no significant increase in the number of colonic  $Foxp3^+$  cells in *Helicobacter*-free mice, suggesting that natural  $T_{reg}$  cells accumulate in response to colitis, not infection per se. With the development of histologic colitis at 2 and 3 wpi, there were increased numbers of  $Foxp3^+$   $T_{reg}$  cells in the colon of *Helicobacter*-free *C. rodentium*-inoculated mice ( $10 \pm 6$  and  $21 \pm 5$   $Foxp3^+$  cells/ $\text{mm}^2$ , respectively) (Fig. 3f and g) ( $P < 0.001$ ). By 4 wpi, colonic numbers of  $Foxp3^+$   $T_{reg}$  cells in *Helicobacter*-free *C. rodentium*-inoculated mice had declined and were not significantly different from basal numbers in uninoculated or *H. hepaticus*-infected mice (Fig. 3f and i). Colonic numbers of  $Foxp3^+$   $T_{reg}$  cells in concurrently infected mice began to accumulate earlier (1 wpi) than in mice infected with *C. rodentium* alone. At the peak of histologic colitis at 2 and 3 wpi, accumulation of  $Foxp3^+$   $T_{reg}$  cells in concurrently infected mice was comparable to the level in *Helicobacter*-free *C. rodentium*-inoculated mice (Fig. 3f to h). However, at 4 wpi  $Foxp3^+$   $T_{reg}$ -cell numbers remained elevated in concurrently infected mice, in contrast to the return to basal levels in mice infected with *C. rodentium* alone (Fig. 3f and j) ( $P < 0.001$ ). The continued presence of natural  $T_{reg}$  cells is consistent with a delayed recovery from histologic colitis, resulting in greater chronicity of disease.

***C. rodentium* colitis is associated with a proinflammatory cytokine expression profile dominated by IFN- $\gamma$ .** Colonic cytokine expression levels were measured to further characterize the mechanism by which persistent subclinical infection modulated the host response to acute diarrheal illness. Adaptive immunity is required for the clearance of *C. rodentium* but also contributes to histologic colitis (5, 13). IL-17-producing T cells have also been recently implicated in disease pathogenesis (23). Elevated expression of the Th1 cytokines IFN- $\gamma$ , TNF- $\alpha$ , and IL-12 coincided with increasing histologic colitis, reaching maximal expression at 2 wpi (Fig. 4a and Fig. 5) ( $P < 0.01$  compared with uninoculated or *H. hepaticus*-infected mice). Expression levels of these proinflammatory cytokines declined at 3 and 4 wpi as lesions resolved. The type 2 cytokines IL-4 and IL-13 were not consistently detected in distal colons of control or infected mice. Additionally, the IL-10 expression profile mimicked that of inflammatory cytokines, possibly as a feedback mechanism to limit collateral inflammatory damage (Fig. 5). No significant changes in TGF- $\beta$  expression occurred although expression trended higher at 2 wpi (Fig. 5). IL-23p19 expression was unaltered by *C. rodentium* infection, indicating either constitutive expression or no transcriptional induction of IL-23, a Th17 maintenance (46, 49) and  $Foxp3^+$  T-cell inhibitory factor (14). IL-17 expression increased during *C. rodentium* infection, reaching a plateau at 3 wpi (Fig. 4a). The association between *C. rodentium* disease pathogenesis and Th17 described previously was based on observations made at 8 days postinoculation (23). We also found greater colonic expression of IL-17 than IFN- $\gamma$  at 1 wpi; however, later in the progression of disease IFN- $\gamma$  and MCP-1 were found to predominate. Over the course of 4 weeks of infection, the dominant, IFN- $\gamma$  nature of *C. rodentium* disease was apparent.

Cytokine expression and histologic colitis were correlated for individual mice in all infection groups at each time point. *C. rodentium* colitis correlated with colonic expression of IFN- $\gamma$  but not with IL-17 (Fig. 4b). Analysis revealed a strong positive correlation between histologic colitis severity and levels of the proinflammatory cytokines IL-6, MCP-1, TNF- $\alpha$ , IL-1 $\beta$ , IL-12p35, IL-12/23p40, and in particular IFN- $\gamma$  (Spearman  $r$  for IFN- $\gamma$  of 0.763;  $P = 0.0032$ ). Anti-inflammatory IL-10, TGF- $\beta$ , and transcription factor  $Foxp3$  expression levels had weak (Spearman  $r$  values of 0.557, 0.333, and 0.362, respectively) but

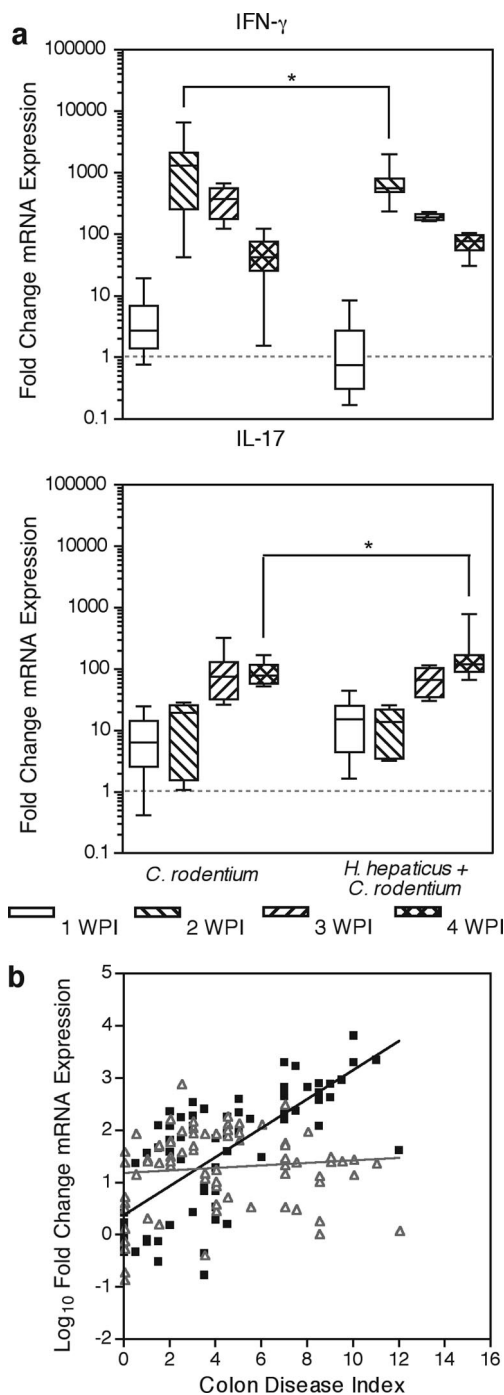


FIG. 4. Persistent *H. hepaticus* infection suppresses IFN- $\gamma$  expression and increases IL-17 expression in *C. rodentium*-infected mice at 4 wpi. (a) Colonic mRNA expression levels of IFN- $\gamma$  and IL-17 were measured by quantitative real-time PCR. mRNA expression normalized to uninoculated mice is presented as box-whisker plots, where boxes represent the first to third quartiles; the median is indicated by a horizontal line. Bars represent ranges. \*,  $P < 0.05$  by two-way ANOVA with Bonferroni post-tests. (b) Spearman correlation of the histologic colitis index and corresponding IFN- $\gamma$  (■) or IL-17 ( $\Delta$ ) mRNA expression demonstrated a positive correlation between colonic disease severity and IFN- $\gamma$  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 regressions of colon disease and cytokine expression, not Spearman correlations, to aid visualization of correlation.

significant ( $P < 0.01$ ) correlation with histologic colitis severity. These weak correlations are likely due to an anti-inflammatory response to counteract the inflammation in the tissue. In contrast to other proinflammatory cytokines, such as IFN- $\gamma$ , neither IL-23p19 nor IL-17 was correlated with histologic colitis (Spearman  $r$  for IL-17 of 0.097;  $P > 0.05$ ).

**Concurrent *H. hepaticus* infection suppresses IFN- $\gamma$  during peak disease and enhances IL-17 expression during chronic colitis.** Persistent concurrent *H. hepaticus* infection did not affect colonic cytokine expression at 1 wpi (Fig. 4a). However, at 2 wpi, concurrently infected mice had significantly lower levels of IFN- $\gamma$  (773-fold over uninoculated controls) and MCP-1 (45-fold) message than *Helicobacter*-free *C. rodentium*-inoculated mice (1,756-fold [ $P < 0.05$ ] and 160-fold [ $P < 0.001$ ], respectively) (Fig. 4a and 5). No significant differences in colonic cytokine expression were noted at 3 wpi between concurrently infected mice and mice inoculated with *C. rodentium* alone. By 4 wpi, when other inflammatory cytokines were returning to basal expression, IL-17 message continued to increase in concurrently infected mice, reaching a 202-fold increase over uninoculated mice. This elevated expression of IL-17 was significantly increased compared with *Helicobacter*-free *C. rodentium*-inoculated mice (92-fold over uninoculated mice) at 4 wpi (Fig. 4a) ( $P < 0.05$ ). Increased IL-17 expression in concurrently infected mice was not accompanied by a change in IL-23p19 or IL-12/23p40. Overall, persistent *H. hepaticus* infection modulated *C. rodentium*-induced cytokine expression by slightly suppressing peak levels of both IFN- $\gamma$  and MCP-1, as well as by modestly enhancing IL-17 expression later in the course of the disease. Decreased IFN- $\gamma$  and MCP-1 expression at 2 wpi was not associated with reduced severity of colitis or body weight loss. However, the increase in IL-17 was associated with delayed recovery from weight loss, chronic colitis, and macrophage and natural T<sub>reg</sub>-cell accumulation in the colon. Given that IL-17 contributes to chronic inflammation (47) and inflammatory bowel disease (11), the association of increased IL-17 expression with increased morbidity suggests that it may prevent resolution of acute disease during self-limiting infection and may promote chronic disease progression.

DISCUSSION

To our knowledge, this is the first demonstration that a persistent subclinical bacterial infection causes delayed recovery from a self-limiting bacterial infection. Elevated IL-17 expression during chronic disease observed in concurrently infected mice is possibly due to lower IFN- $\gamma$  at an earlier stage in disease progression. In this study, eradication of *C. rodentium* infection by the host's immune system was not altered in concurrently infected mice. Rather, *H. hepaticus* altered expression of a key chemokine (MCP-1) and type 1 cytokine (IFN- $\gamma$ ), resulting in delayed resolution of disease and greater chronicity of colitis. Persistent *H. hepaticus* infection did not alter mortality due to *C. rodentium* infection, one indicator of impaired development of adaptive immunity. By enhancing morbidity rather than mortality, as observed during concurrent helminth infection (7), concurrent infection with *H. hepaticus* and *C. rodentium* provides a useful model for evaluating the

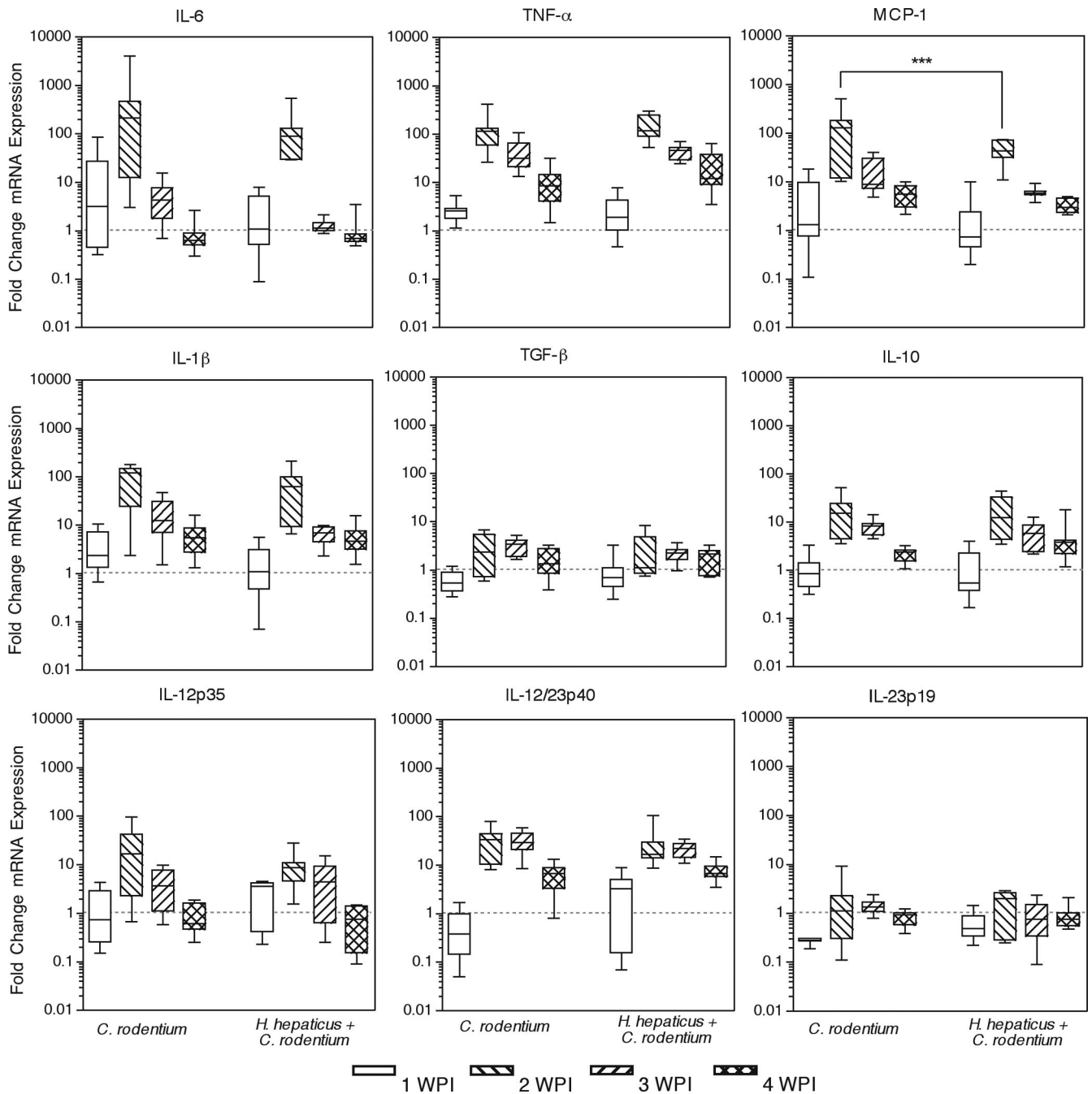


FIG. 5. *C. rodentium* infection induces a Th1 cytokine response that peaks at 2 weeks postinoculation, and MCP-1 expression is suppressed by concurrent *H. hepaticus* infection. Colonic mRNA expression levels of IL-6, TNF- $\alpha$ , MCP-1, IL-1 $\beta$ , TGF- $\beta$ , IL-10, IL-12/23p40, IL-12p35, and IL-23p19 were measured by quantitative real-time PCR. mRNA expression normalized to uninoculated mice is presented in box-whisker plots indicating minimum and maximum, first and third quartiles, and median expression levels. \*\*\*,  $P < 0.001$  by two-way ANOVA with Bonferroni posttests.

effects of subclinical infections on the outcome of a self-limiting infection.

Th1 cytokines, particularly IFN- $\gamma$ , are essential for a proper host response to *C. rodentium* (5, 13, 34); however, the importance of IL-17-producing cells is not well defined in this disease. Our data indicate that expression of many proinflammatory cytokines is highly induced in response to *C. rodentium*

infection and that the pattern of transient expression is not affected by persistent infection with *H. hepaticus*. However, IL-17 expression does not decline when mice have delayed disease resolution due to concurrent *H. hepaticus* infection. IL-17 has been associated with T-cell-mediated colitis (47) as well as with inflammatory bowel disease (11) although the role of this cytokine in disease is not well understood. IL-17 has



been shown to recruit neutrophils to mucosal infection sites (25, 45). However, during chronic disease in concurrently infected mice, few neutrophils were observed (data not shown). The increased IL-17 expression during chronic disease may be a consequence of the decreased IFN- $\gamma$  at the peak of disease, as IFN- $\gamma$  has been shown to inhibit generation of IL-17-producing cells (12). Additionally, MCP-1 is an important chemokine for monocyte recruitment to mucosal tissue (33). Decreased MCP-1 in concert with IFN- $\gamma$  changes cell recruitment and subsequent cytokine production by immune cells in the colon, including epithelial cells. These alterations in cellular responses may have downstream effects on disease resolution, perhaps requiring a longer recovery period or exposing the host's immune response to intestinal microbiota for an extended period of time.

The source of IL-17 in chronic colitis is unknown; however, Th17 cells are the most likely candidates. Th17 cells have been implicated in many inflammatory diseases as well as in protection from infection by extracellular pathogens, yet the role of IL-17 in disease pathogenesis versus control of infectious agents or a balance between the two has not been fully clarified (31). Recently, an increased presence of IL-17-producing T cells in the colon due to the presence of commensal microbiota was demonstrated. This increase was particularly evident during T-cell-mediated colitis (30). Therefore, suppression of IFN- $\gamma$  and MCP-1 during peak colitis leading to a delayed period of recovery could act, at least in part, through enhanced exposure to intestinal microbiota. Mucosal damage and subsequent microbial exposure could directly increase the amount of IL-17 in the colon. Additional studies will be needed to evaluate the contribution of specific cell populations and cytokines to the outcome of concurrent infection.

Although the mechanism by which concurrent *H. hepaticus* infection causes chronic *C. rodentium*-induced colitis is not fully understood, we have demonstrated alterations in mucosal cytokine production and immune cell recruitment to the colon. The paucity of neutrophils at 4 wpi (data not shown) is consistent with this being a phase of chronicity or resolution. Increased numbers of macrophages were observed in the colon throughout the course of *C. rodentium* disease and were still present in *Helicobacter*-free *C. rodentium*-infected mice at 4 wpi. However, the number of macrophages in the colon at 4 wpi was significantly greater in mice with concurrent *H. hepaticus* infection, consistent with chronic mucosal inflammation.

Beyond their essential role in controlling autoimmunity (2, 39), the role of natural T<sub>reg</sub> cells in the gastrointestinal tract remains somewhat unclear (1, 27). Accumulation of natural T<sub>reg</sub> cells has been demonstrated at sites of active disease in tuberculosis, hepatitis C virus, and colitis (9, 36, 41), indicating the importance of these cells in controlling collateral damage during pathogen-directed immune and inflammatory responses. Here, we demonstrate that infection with *C. rodentium* causes a significant increase in the number of natural T<sub>reg</sub> cells in the colon during active disease. These natural T<sub>reg</sub> cells are likely recruited to limit host damage since they accumulate coincident with and serve as a marker for active inflammation. Indeed, the kinetics of natural T<sub>reg</sub>-cell accumulation following initial infiltration of macrophages at 1 wpi, as well as the significant increase in natural T<sub>reg</sub>-cell abundance in concur-

rently infected mice at 4 wpi, confirms their association with active disease in this model.

In humans, delayed disease resolution from a self-limiting infection with chronic inflammation as a consequence of persistent subclinical infection is likely to lead to increased morbidity and mortality. Multiple concurrent infections could act in concert to produce more deleterious outcomes of disease. The true prevalence of persistent subclinical infection and the sequelae of concurrent infection in people remain to be determined. Persistent subclinical infections may at least in part account for different responses to acute infections, comparable to the contribution of genetic polymorphisms. The high prevalence of persistent subclinical infection with *H. pylori* or *Mycobacterium tuberculosis* worldwide suggests that persistent infections may impact disease pathogenesis or treatment outcome of acute infections, particularly in the developing world.

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