IL-23 plays a key role in *Helicobacter hepaticus*–induced T cell–dependent colitis

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Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract that is caused in part by a dysregulated immune response to the intestinal flora. The common interleukin (IL)-12/IL-23p40 subunit is thought to be critical for the pathogenesis of IBD. We have analyzed the role of IL-12 versus IL-23 in two models of *Helicobacter hepaticus*–triggered T cell–dependent colitis, one involving anti–IL-10R monoclonal antibody treatment of infected T cell–sufficient hosts, and the other involving CD4+ T cell transfer into infected Rag−/− recipients. Our data demonstrate that IL-23 and not IL-12 is essential for the development of maximal intestinal disease. Although IL-23 has been implicated in the differentiation of IL-17–producing CD4+ T cells that alone are sufficient to induce autoimmune tissue reactivity, our results instead support a model in which IL-23 drives both interferon γ and IL-17 responses that together synergize to trigger severe intestinal inflammation.

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Abbreviations used: CIA, collagen-induced arthritis; EAE, experimental autoimmune encephalomyelitis; *Hh*+, *H. hepaticus*–infected; IBD, inflammatory bowel disease; MLN, mesenteric lymph node; SHelAg, soluble *H. hepaticus* antigen.

The heterodimeric cytokine IL-12 plays a key role in host defense against intracellular microbes by inducing NK cell IFN-γ production and by favoring the differentiation of IFN-γ–secreting CD4+ Th1 cells (1). IL-12 has also been implicated in chronic inflammatory disorders characterized by excessive Th1 responses. Recently, IL-23 was identified as a new member of the IL-12 cytokine family secreted by activated DCs and macrophages (2). Because of the shared p40 subunit between IL-12 (p40/p35) and IL-23 (p40/p19), early experiments that used anti-p40 mAb to neutralize IL-12 in vivo have had to be reevaluated. With the use of new tools, including p19-deficient mice, several autoimmune and chronic inflammatory diseases previously believed to be caused by IL-12 and Th1 responses have now indeed been shown to depend on IL-23, and not IL-12. Among such diseases are experimental autoimmune encephalomyelitis

(EAE) that affects the central nervous system (3, 4) and collagen-induced arthritis (CIA) of the joints (5).

The precise mechanism(s) by which IL-23 contributes to inflammation is currently unknown. The IL-23 receptor, which is composed of IL-12Rβ1 combined with the IL-23R subunit (6), is expressed on activated and memory T cells, NK cells, DCs, and inflammatory macrophages and microglia (2, 3, 6). IL-23 was originally described to induce the proliferation and secretion of IL-17 (IL-17A)/IL-17F by CD4+ T cells with an activated or memory phenotype (2, 7), and such IL-23–driven IL-17+ CD4+ T cells were subsequently shown to be highly potent inducers of immune pathology in EAE (4). Recent studies have established that IL-17+ CD4+ T cells represent a novel subset of Th cells (Th17 cells) that in addition to IL-17/IL-17F also produces IL-6 and TNF-α (4, 8, 9). Binding of IL-17 and/or IL-17F to receptors on myeloid and endothelial cells promotes the expression of IL-1, IL-6, IL-8, TNF,

K.J. Maloy and A. Sher contributed equally to this work.
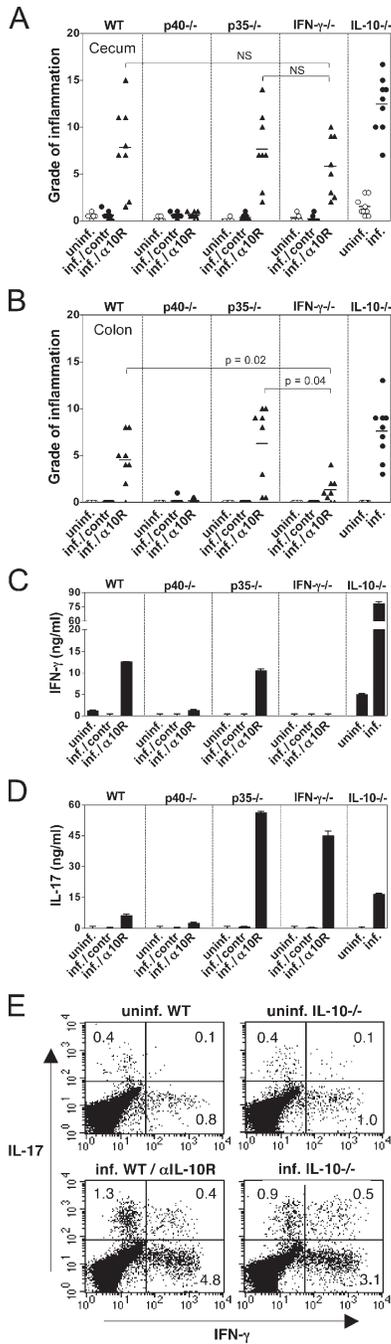
Figure 1. *H. hepaticus* infection plus anti–IL-10R mAb treatment leads to severe typhlocolitis in WT and p35⁻/⁻ mice but reduced inflammation in the ascending colon of IFN–γ⁻/⁻ animals. B6 WT, p40⁻/⁻, p35⁻/⁻, IFN–γ⁻/⁻, and B10 IL-10⁻/⁻ mice were infected with *H. hepaticus* and treated with control or anti–IL-10R mAb once a week for 4 wk. Uninfected mice were included as controls. 1 wk after the last mAb administration, pathology was analyzed in the cecum (A) and ascending colon (B). MLN cells were cultured with 5 μg/ml SHelAg, and IFN–γ (C) and IL-17 (D) were measured in 72-h supernatants. No IFN–γ or IL-17 was detected from cells cultured in medium alone. Data in A and B are pooled from two separate experiments and show histology scores from individual mice, with the horizontal line indicating the average for each group. Bars in C and D represent means ± SEM of culture duplicates of three to five

and chemokines that are involved in mobilizing neutrophils and in driving chronic inflammation (3, 10).

Inflammatory bowel disease (IBD), comprising Crohn's disease and ulcerative colitis, is a chronic inflammatory disorder of the gastrointestinal tract that is caused in part by a dysregulated immune response to the intestinal flora. Levels of IL-12 and IL-23 have been shown to be increased in inflamed intestinal tissues (11–14), and anti-p40 mAb treatment has proven beneficial in Crohn's disease as well as in experimental models of intestinal inflammation (15–19). However, little information is currently available on the relative contribution of IL-12 versus IL-23 in IBD pathogenesis.

We previously established a new model of bacterial-induced colitis involving infection of specific pathogen-free IL-10⁻/⁻ mice with *Helicobacter hepaticus* (20, 21). The disease that develops in *H. hepaticus*–infected (*Hh⁺*) IL-10⁻/⁻ animals is associated with a Th1 response to the bacterium, and treatment from the start of the infection with anti-p40 mAb abrogates the development of intestinal inflammation (20). Furthermore, mice rendered double deficient for both IL-10 and p40 are resistant to *H. hepaticus*–induced colitis, together demonstrating a crucial role for p40 in disease pathogenesis (21).

In this study, we have investigated the role of IL-12 versus IL-23 in two bacterially driven T cell–dependent colitis models. Using a novel model of colitis involving anti–IL-10R treatment of *Hh⁺* WT mice, as well as our previously established *H. hepaticus* CD4 T cell adoptive transfer model (22), we demonstrate that IL-23 and not IL-12 is essential to trigger intestinal inflammation. Unexpectedly, although IL-23 has been implicated in the induction of pathogenic IL-17–producing T cells in EAE (4), in our model IL-17 in the absence of IFN-γ does not appear to be sufficient to induce maximal colitis. Instead, our results support a model in which maximal intestinal inflammation depends on the function of both IFN-γ and IL-17.

RESULTS

Anti–IL-10R treatment leads to severe intestinal inflammation in *Hh⁺* WT and IL-12p35⁻/⁻ mice

Using a T cell transfer model of colitis triggered by adoptive transfer of CD4⁺ T cells into *Hh⁺* Rag⁻/⁻ recipients, we previously described the appearance of disease-protective CD4⁺ IL-10–secreting T regulatory cells after *H. hepaticus* infection of WT animals (22). Here we demonstrate that the CD4-mediated T regulatory cell mechanism revealed in the

mice/group from one representative experiment of the two shown in A and B. (E) Single cell analysis of IL-17 and IFN-γ–producing CD4⁺ T cells in anti–IL-10R–treated *Hh⁺* WT and *Hh⁺* IL-10⁻/⁻ mice. MLN cells from uninfected (top) or 6-wk-infected anti–IL-10R–treated WT (bottom left) or 6-wk-infected IL-10⁻/⁻ mice (bottom right) were cultured with SHelAg for 72 h. After an additional incubation for 18 h in medium, cells were stimulated with PMA and ionomycin in the presence of brefeldin A, and three-color staining for CD4, IL-17, and IFN-γ was performed. The FACS dot plots shown are gated on CD4⁺ cells and are representative of more than three experiments performed.
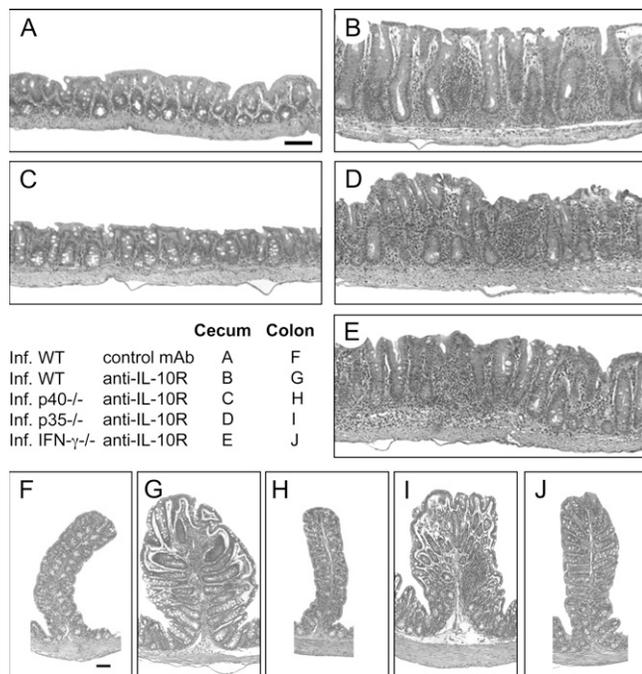
Figure 2. Intestinal pathology in anti–IL-10R–treated *Hh*+ T cell–sufficient mice. B6 WT, p40-/-, p35-/-, and IFN-γ-/- mice were infected with *H. hepaticus* and treated with control or anti–IL-10R mAb once a week for 4 wk. 1 wk after the last mAb administration, pathology was analyzed in the cecum (A–E) and ascending colon (F–J). Shown is the histology of representative cecal and colonic sections from the mice in Fig. 1 (A and B). (A and F) *Hh*+ WT plus control mAb, (B and G) *Hh*+ WT plus anti–IL-10R, (C and H) *Hh*+ p40-/- plus anti–IL-10R, (D and I) *Hh*+ p35-/- plus anti–IL-10R, and (E and J) *Hh*+ IFN-γ-/- plus anti–IL-10R. The tissue photomicrographs shown were scored as follows: A, 0; B, 5; C, 0; D, 6; E, 7; F, 0; G, 3; H, 0; I, 2; J, 0. Note that the scores in Fig. 1 represent a total score for the whole section and include cells infiltrating the serosa as well as crypt abscesses and ulcers, all of which are absent in the above photomicrographs. Hematoxylin and eosin staining. Bars, 50 μm.

Rag-/- transfer model also functions in intact *Hh*+ WT hosts, as treatment of these animals with anti–IL-10R, but not a control mAb, led to the development of typhlocolitis similar to that seen in *Hh*+ IL-10-/- mice (Figs. 1, A and B, and 2, A, B, F, and G). Uninfected WT mice given anti–IL-10R did not develop intestinal inflammation (not depicted), demonstrating that the endogenous flora by itself was not sufficient to induce colitis, but that *H. hepaticus* was required for disease initiation.

In agreement with our earlier findings of a critical role for p40 in colitis induction (20, 21), *Hh*+ p40-/- mice treated with anti–IL-10R failed to develop typhlocolitis (Figs. 1, A and B, and 2, C and H). Importantly however, anti–IL-10R–treated *Hh*+ p35-/- mice, which lack the ability to produce IL-12, developed intestinal inflammation comparable to that seen in anti–IL-10R–treated *Hh*+ WT mice (Figs. 1, A and B, and 2, D and I). Collectively, these findings demonstrate that although the IL-12p40 subunit is essential for colitis development, IL-12 in itself is dispensable for disease pathogenesis.

To investigate the role of IFN-γ, mice deficient in this cytokine were infected with *H. hepaticus* and treated with anti–IL-10R. Although the degree of cecal inflammation was comparable to that observed in colitic *Hh*+ WT and p35-/- animals (Figs. 1 A and 2 E), IL-10R blockade led to only a mild inflammatory response in the colon of *Hh*+ IFN-γ-/- animals (Figs. 1 B and 2 J), suggesting a role for IFN-γ in exacerbating disease at this site. This conclusion was supported by similar findings in an adoptive transfer model (see below), emphasizing a role for IFN-γ in promoting colonic inflammation.

H. hepaticus–specific IFN-γ and IL-17 responses are elevated in colitic mice

To examine *H. hepaticus*–specific T cell cytokine responses, mesenteric lymph node (MLN) cells were stimulated in vitro with soluble *H. hepaticus* antigen (SHelAg) and levels of IFN-γ and IL-17 were measured. After bacterial antigen stimulation, MLN cells from *Hh*+ IL-10-/- mice mounted a strong IFN-γ response and secreted substantial amounts of IL-17, whereas MLN cells from *Hh*+ WT mice given control mAb failed to produce these cytokines (Fig. 1, C and D). Similarly, no IFN-γ or IL-17 was detected in SHelAg-stimulated MLN cultures from control mAb–treated *Hh*+ p40-/-, p35-/-, or IFN-γ-/- mice. In contrast, MLN cells from anti–IL-10R–treated *Hh*+ WT mice secreted elevated amounts of both IFN-γ and IL-17 in response to SHelAg (Fig. 1, C and D). Interestingly, a similar picture was seen for anti–IL-10R–treated *Hh*+ p35-/- mice, with a significant IFN-γ response and markedly enhanced IL-17 levels after SHelAg stimulation (Fig. 1, C and D). Importantly, although displaying attenuated colonic inflammation, MLN cells from anti–IL-10R–treated *Hh*+ IFN-γ-/- mice exhibited a strong SHelAg-induced IL-17 response (Fig. 1 D). Intracellular cytokine staining of MLN cells from anti–IL-10R–treated *Hh*+ WT and *Hh*+ IL-10-/- mice demonstrated that SHelAg-induced IL-17 and IFN-γ were mainly produced by distinct populations of CD4+ cells, with a smaller fraction of cells staining positive for both cytokines (Fig. 1 E). In preliminary
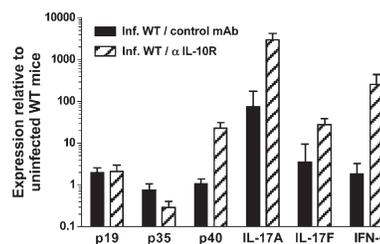
Figure 3. Cytokine mRNA levels in the colons of anti–IL-10R–treated *Hh*+ WT mice. Colonic tissues from uninfected or 4-wk-infected WT mice treated with control mAb or anti–IL-10R were collected and processed for real-time RT-PCR analysis of p19, p35, p40, IL-17A, IL-17F, and IFN-γ. Bars represent data from individual mice (two uninfected and four *Hh*+ mice per group) in one of the experiments shown in Fig. 1 (A and B). Error bars represent the range of fold difference in gene expression.

experiments, anti–IL-10R–treated Hh^+ IFN-γ$^{-/-}$ mice demonstrated an increased frequency of IL-17$^+$ CD4$^+$ cells after SHelAg stimulation (1.8–4.7-fold increase compared with similarly treated WT mice; data from two independent experiments). Collectively, these results indicate that although Th17 responses may be sufficient to induce severe cecal inflammation, maximal inflammation in the colon also depends on IFN-γ.

To examine cytokine responses at the site of inflammation, mRNA levels for p19, p35, p40, IL-17A, IL-17F, and IFN-γ were measured in colon samples from Hh^+ WT mice given control or anti–IL-10R mAb. As shown in Fig. 3, both Hh^+ groups showed an approximately twofold increase in p19 levels compared with uninfected controls. In contrast, p35 levels were slightly reduced in both Hh^+ groups, whereas p40 levels were unchanged in control mAb–treated Hh^+ WT mice but increased >20-fold in anti–IL-10R–treated Hh^+ WT mice. Interestingly, mRNA levels for IL-17A, IL-17F, and IFN-γ were approximately 40-fold, ninefold, and 150-fold higher in anti–IL-10R–treated compared with control mAb–treated Hh^+ WT mice (Fig. 3).

Anti–p40 treatment prevents intestinal pathology in anti–IL-10R–treated Hh^+ mice

To examine if the IL-12p40 subunit is also required for disease development in the absence of IL-12, p35$^{-/-}$ mice were infected with *H. hepaticus* and treated with anti–IL-10R alone or anti–IL-10R plus anti-p40 mAb. As shown in Fig. 4 A, anti-p40 treatment completely abrogated both cecal and colonic inflammation in anti–IL-10R–treated Hh^+ WT or p35$^{-/-}$ mice. In addition, anti-p40 treatment blocked SHelAg-induced IFN-γ and IL-17 secretion observed in MLN cultures from the same groups of Hh^+ mice (Fig. 4, B and C). Importantly, anti-p40 treatment also prevented the cecal inflammation observed in Hh^+ IFN-γ$^{-/-}$ mice given anti–IL-10R (Fig. 4 A), and this correlated with a complete loss of SHelAg-induced IL-17 production (Fig. 4 C). Collectively, these findings confirmed a crucial role for p40 in the development of *H. hepaticus*–induced intestinal inflammation even in the absence of IL-12, and again indicated a correlation between IL-17, IFN-γ, and pathology.

IL-12 is not required for the development of T cell–dependent colitis in Hh^+ Rag$^{-/-}$ mice

In light of the shared p40 subunit between IL-12 and IL-23, we reasoned that the p40-dependent, but IL-12–independent induction of *H. hepaticus*–triggered colitis was likely to be mediated by IL-23. As various cytokine-deficient Rag$^{-/-}$ animals were available to us, we next turned to an adoptive transfer model of colitis using Rag$^{-/-}$ mice deficient in IL-12 (p35Rag$^{-/-}$), IL-23 (p19Rag$^{-/-}$), or both IL-12 and IL-23 (p40Rag$^{-/-}$) to further elucidate the role of IL-23 in disease development. In this model, colitis is induced in an IL-10–sufficient setting in Hh^+ B6 or B10 Rag$^{-/-}$ recipients by transfer of CD4$^+$ T cells (22). We first confirmed a critical role for p40 in the adoptive transfer model, as Hh^+ p40Rag$^{-/-}$ mice
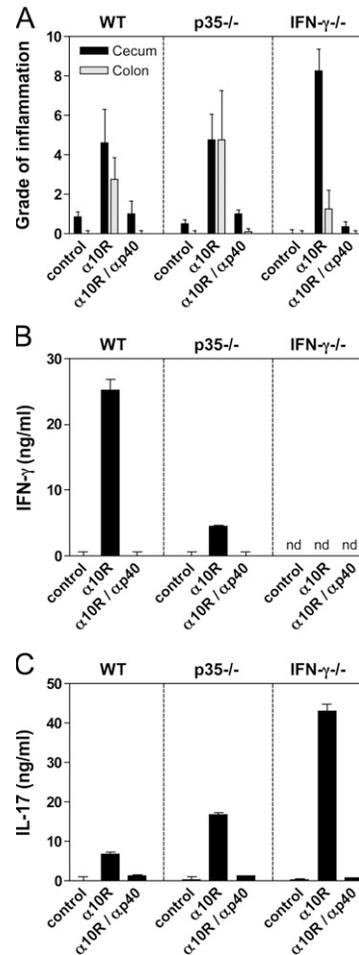
Figure 4. Anti–p40 mAb treatment prevents intestinal pathology in anti–IL-10R–treated Hh^+ mice. B6 WT, p35$^{-/-}$, and IFN-γ$^{-/-}$ mice were infected with *H. hepaticus* and treated with control, anti–IL-10R, or anti–IL-10R plus anti-p40 mAb once a week for 4 wk. 1 wk after the last mAb administration, pathology was analyzed in the cecum (A, black bars) and ascending colon (A, gray bars). Despite significant cecal pathology, anti–IL-10R–treated Hh^+ IFN-γ$^{-/-}$ mice failed to develop inflammation in the colon. MLN cells were cultured with SHelAg, and IFN-γ (B) and IL-17 (C) were measured in 72-h supernatants. No IFN-γ or IL-17 was detected from cells cultured in medium alone. Bars in A represent mean histology scores ± SEM of four mice/group. Bars in B and C represent means ± SEM of culture duplicates of MLN cells pooled from the mice in A. nd, not detected.

failed to develop typhlocolitis after transfer of WT CD4$^+$ CD45RB^{hi} cells (Fig. 5, A and B) or IL-10$^{-/-}$ CD4$^+$ cells (not depicted). In contrast, Hh^+ Rag$^{-/-}$ and p35Rag$^{-/-}$ mice developed severe typhlocolitis after reconstitution with the same T cell populations (Fig. 5, A and B, and not depicted). Importantly, anti-p40 treatment inhibited intestinal inflammation in T cell–reconstituted Hh^+ Rag$^{-/-}$ and p35Rag$^{-/-}$ animals (Fig. 5, A and B), demonstrating a requirement for p40 but not IL-12 in disease induction also in this model. In contrast, anti–IFN-γ treatment had no effect on cecal pathology (Fig. 5 A) but significantly reduced the inflammation in the
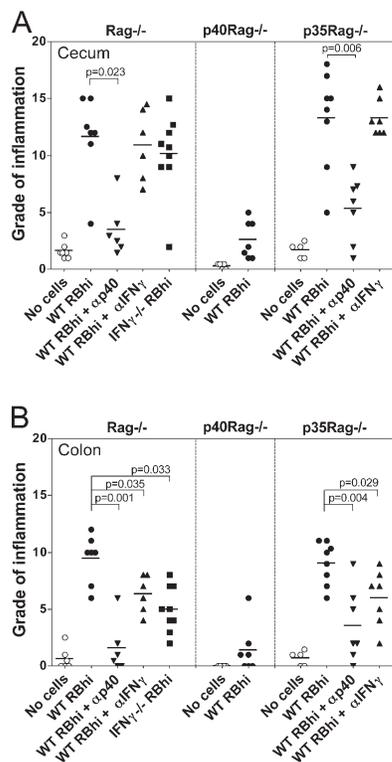
Figure 5. *Hh*$^+$ Rag$^{-/-}$ and p35Rag$^{-/-}$, but not p40Rag$^{-/-}$, mice develop intestinal inflammation after T cell reconstitution. *Hh*$^+$ B6/B10 Rag$^{-/-}$, p40Rag$^{-/-}$, and p35Rag$^{-/-}$ mice were inoculated i.v. with 3 × 10^5 CD4$^+$ CD45RB^{hi} cells from uninfected WT or IFN-γ$^{-/-}$ animals as indicated. Separate groups of *Hh*$^+$ Rag$^{-/-}$ and p35Rag$^{-/-}$ recipients of WT CD4$^+$ CD45RB^{hi} cells were also treated with anti-p40 or anti–IFN-γ on days 0, 7, 14, and 21 of cell transfer. Pathology in the cecum (A) and ascending colon (B) was analyzed 4 wk after cell transfer. Infected mice receiving no cells were included as controls (○). Data are pooled from two separate experiments and show histology scores from individual mice, with the horizontal line indicating the average for each group.

colon of the *Hh*$^+$ Rag$^{-/-}$ and p35Rag$^{-/-}$ recipients (Fig. 5 B). Similarly, although WT and IFN-γ$^{-/-}$ CD4$^+$ CD45RB^{hi} cells induced a similar degree of cecal inflammation in *Hh*$^+$ Rag$^{-/-}$ mice (Fig. 5 A), recipients of IFN-γ$^{-/-}$ CD4$^+$ cells showed reduced inflammation in the colon (Fig. 5 B). These findings are consistent with the previously observed difference in disease severity in the cecum versus colon of *Hh*$^+$ IFN-γ$^{-/-}$ mice treated with anti–IL-10R (Figs. 1, A and B, and 2, E and J), and further support an important role for T cell–derived IFN-γ in disease pathogenesis in the colon.

IL-23 is essential for the development of maximal colitis and intestinal proinflammatory cytokine production

To directly assess the contribution of IL-23 in the development of colitis in the *H. hepaticus*/Rag$^{-/-}$ transfer model, an additional series of experiments was performed that included p19Rag$^{-/-}$ recipients, which lack the ability to produce IL-23. As shown in Fig. 6 (A and B), CD4$^+$ T cell–reconstituted *Hh*$^+$ p19Rag$^{-/-}$ mice displayed attenuated intestinal inflam-

mation compared with Rag$^{-/-}$ recipients, demonstrating a crucial role for IL-23 in disease pathogenesis. However, compared with disease-free *Hh*$^+$ p40Rag$^{-/-}$ recipients, some residual intestinal inflammation was observed in T cell–reconstituted *Hh*$^+$ p19Rag$^{-/-}$ mice (Fig. 6, A and B). These data suggest that, although not absolutely required for intestinal inflammation, IL-12 contributes to the mild intestinal inflammation observed in the absence of IL-23. Nevertheless, of the two cytokines, IL-23 appears to be more important in the disease process, as the inflammation present in *Hh*$^+$ p19Rag$^{-/-}$ recipients was significantly reduced compared with that observed in p35Rag$^{-/-}$ hosts (Fig. 6, A and B). Importantly, flow cytometric analysis revealed no significant difference in CD4$^+$ cell numbers in the spleens of *Hh*$^+$ p19Rag$^{-/-}$ and p35Rag$^{-/-}$ recipients (not depicted), demonstrating that the attenuated pathology in p19Rag$^{-/-}$ mice was not due to lower T cell reconstitution. Moreover, there was no correlation between degree of inflammation and *H. hepaticus* colonization levels, as all groups harbored similar concentrations of *H. hepaticus* in their cecal contents (Fig. 6 C).

We next quantitated cytokines in colon homogenates from the different groups of T cell–reconstituted *Hh*$^+$ Rag$^{-/-}$ mice. As shown in Fig. 6 (D–G), the absence of both IL-12 and IL-23 led to abolished TNF-α, IFN-γ, MCP-1, and IL-6 levels in colon homogenates of T cell–reconstituted *Hh*$^+$ p40Rag$^{-/-}$ animals. IL-12–deficient *Hh*$^+$ p35Rag$^{-/-}$ recipients showed similar or marginally decreased levels of proinflammatory cytokines compared with *Hh*$^+$ Rag$^{-/-}$ recipients. Finally, the absence of IL-23 in *Hh*$^+$ p19Rag$^{-/-}$ mice led to a further reduction in proinflammatory cytokine production (Fig. 6, D–G), emphasizing the crucial role for IL-23 in mediating this response.

DISCUSSION
Before the discovery of IL-23 and its recently documented role in autoimmune disorders (2–5), the common view in the field of Th1-mediated intestinal inflammation has been that IL-12, by initiating and maintaining Th1 responses, is crucial for disease pathogenesis. We here demonstrate that IL-23 and not IL-12 is essential for the induction of maximal bacterial-induced T cell–dependent colitis. This conclusion is based on results from two complementary models of intestinal inflammation triggered by *H. hepaticus*. Using an established CD4$^+$ T cell adoptive transfer model of colitis (22), we observed that although severe intestinal inflammation was still induced in IL-12–deficient (p35Rag$^{-/-}$) recipients, selective ablation of IL-23 (p19Rag$^{-/-}$) resulted in attenuation of disease. In addition, using a novel model of colitis involving anti–IL-10R treatment of *Hh*$^+$ WT animals that circumvents the lymphopenic environment of Rag$^{-/-}$ mice, we confirm an essential role for p40 and not IL-12 in intestinal inflammation also in immune competent hosts.

IL-23, which is produced by DCs and macrophages in response to Toll-like receptor stimuli (23), may contribute to intestinal pathology in multiple ways. By inducing the production of IL-1β, TNF, IL-12, and IFN-γ by cells of the

innate immune system (3, 24), IL-23 can directly participate in triggering a proinflammatory cytokine cascade. A key role for IL-23 in driving innate intestinal pathology was indeed observed in a complementary study by Hue et al. (25), in which depletion of IL-23p19 attenuated the T cell–independent typhlocolitis that develops in Hh^+ 129SvEvRag$^{-/-}$ mice. IL-23 is also known to amplify and/or stabilize Th17 cells (26), which through their secretion of IL-17 induce myeloid and endothelial cell expression of proinflammatory cytokines (IL-1, IL-6, IL-8, TNF-α, and GM-CSF) and chemokines (3, 10, 27). In addition to our findings of increased IL-17 responses in colitic mice, several of the effects we observed in our T cell–dependent model of colitis may be interpreted in terms of Th17 function and subsequent recruitment of inflammatory cells to the intestine. Thus, both cecal inflammation and SHelAg-specific IL-17 responses observed in anti–IL-10R–treated Hh^+ IFN-γ$^{-/-}$ mice were inhibited by co-administration of anti-p40 mAb. Moreover, IFN-γ$^{-/-}$ CD4$^+$ CD45RB^{hi} cells were equivalent to their WT counterparts in terms of inducing cecal inflammation (Fig. 5 A), and these cells were also pathogenic in an IL-12–deficient setting in p35Rag$^{-/-}$

hosts (not depicted). Collectively, these findings argue for an IL-23–driven IFN-γ–independent component in disease pathogenesis, a conclusion in agreement with the pathogenic role of Th17 cells in EAE and CIA (3–5, 28).

Th17 cells represent a unique linage distinct from the Th1 and Th2 subsets, as their development is inhibited by IFN-γ and IL-4 and is independent of STAT4, STAT6, STAT1, and T-bet (8, 9). Three recent reports have now established that TGF-β, in the presence of IL-6, is critical for the development of Th17 cells, whereas IL-23 appears dispensable for Th17 commitment and initial IL-17 production (29–31). IL-23 is, however, clearly essential for a fully effective Th17 response in vivo, as exemplified in EAE and CIA as well as in various infections with extracellular bacteria (e.g., *Klebsiella pneumoniae* and *Citrobacter rodentium*; references 3–5, 28, 30, and 32). Our findings of up-regulated IL-17A (and to a lesser extent IL-17F) mRNA levels in the absence of significant induction of IL-23p19 or p40 in colonic tissues of Hh^+ control mAb–treated WT mice that show no colitis (Fig. 3) support these conclusions. The mechanism behind the IL-23 dependency for effective Th17 responses is still
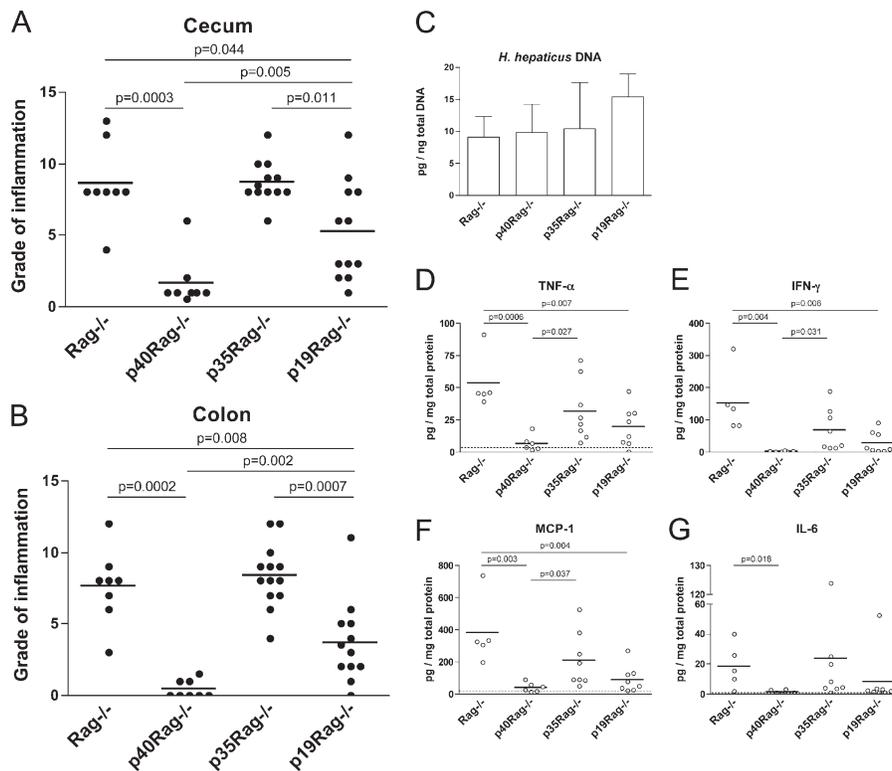
Figure 6. IL-23 is essential for the development of maximal colitis and intestinal proinflammatory cytokine production. Hh^+ B6 Rag$^{-/-}$, p40Rag$^{-/-}$, p35Rag$^{-/-}$, and p19Rag$^{-/-}$ mice were inoculated i.p. with 4×10^5 CD4$^+$ CD45RB^{hi} cells from uninfected WT animals as indicated. Pathology in the cecum (A) and ascending colon (B) was analyzed 4 wk later. Data are pooled from two separate experiments and show histology scores from individual mice, with the horizontal line indicating the average for each group. (C) $H.$ $hepaticus$ colonization levels were quantified using real-time PCR. Bars represent means ± SEM of $H.$ $hepaticus$ DNA levels from the individual mice shown in A and B. (D–G) Cytokine levels in colon homogenates were determined by cytometric bead assay and normalized to the total amount of protein in each sample. Shown are TNF-α (D), IFN-γ (E), MCP-1 (F), and IL-6 (G). Data are pooled from the two experiments shown in A and B assayed as individual mice in experiment 1 and as two pools of three mice/group in experiment 2. The dotted line represents cytokine levels in unreconstituted Hh^+ Rag$^{-/-}$ mice.

unknown. As IL-23R expression is up-regulated by TGF-β, thus downstream of signals that initiate Th17 differentiation (8, 30), one possibility is that IL-23 stabilizes and/or maintains Th17 cells, analogous to the role of IL-12 in stabilizing Th1 cells after their expression of IL-12Rβ2 (26). So far, the role of IL-10 in Th17 development has not been carefully examined. To the best of our knowledge, the results presented here are the first to demonstrate an inhibitory role for IL-10 in the development of IL-17–producing CD4$^+$ T cells in vivo, as anti–IL-10R treatment of Hh^+ WT mice led to markedly increased $H.$ $hepaticus$ antigen-specific IL-17 responses. The mechanism by which IL-10 exerts its down-regulatory effect on IL-17 production is currently unknown but was shown to be independent of IL-12 and IFN-γ, as anti–IL-10R–treated Hh^+ p35$^{-/-}$ and IFN-γ$^{-/-}$ mice also displayed increased IL-17 responses. One possibility is that IL-10 inhibits IL-23 secretion by cells of the innate immune system, thereby limiting the amount of IL-23 available to maintain Th17 cells. Alternatively, IL-10 might have a down-regulatory effect on IL-23R expression on CD4$^+$ cells, as has been described for bone marrow–derived macrophages (6), thereby rendering these cells less responsive to IL-23. In the study by Veldhoen et al. (29), IL-17–secreting T cells were generated in vitro in cocultures of naive CD4$^+$ cells with naturally occurring CD25$^+$ CD4$^+$ T regulatory cells in the presence of Toll-like receptor stimuli. In contrast to our findings, blocking IL-10 in this model did not result in an increased frequency of IL-17–producing T cells (29). However, as the percentage of IFN-γ–secreting CD4$^+$ cells increased in the same cultures (29), a possible inhibitory effect of IFN-γ on the development of IL-17–producing cells cannot be excluded.

Although, as shown in this study, IL-23 and not IL-12 is essential for the development of maximal pathology in T cell–dependent bacterial-induced intestinal inflammation, there are some differences between our model and the EAE and CIA models in regard to the contribution of IL-12 and IFN-γ to disease pathogenesis. Thus, in the sole absence of IL-12 (p35$^{-/-}$ mice), disease severity was enhanced in EAE and CIA (3, 5), whereas for colitis induced by anti–IL-10R treatment of Hh^+ p35$^{-/-}$ mice or by T cell transfer into Hh^+ p35Rag$^{-/-}$ recipients, this was not the case. One explanation for this difference could be that IL-17–secreting CD4$^+$ T cells are the major pathogenic population in the former two models, with IL-12–driven IFN-γ suppressing the generation of IL-17$^+$ Th cells (8, 9, 30), whereas in the colitis setting, both IL-17 and IFN-γ contribute to disease pathogenesis. Indeed, loss of IFN-γ or the IFN-γ receptor was associated with an increased disease severity in both EAE and CIA (33–37), whereas as shown here, IFN-γ deficiency led to largely unaffected cecal pathology but reduced colonic inflammation. These findings indicate that IFN-γ enhances rather than suppresses inflammation in the intestinal tract, a conclusion supported by previous studies demonstrating that anti–IFN-γ treatment prevents the development of colitis (20, 38, 39). Yen et al. (40) have recently reported that IL-23 is required

for the development of spontaneous enterocolitis in IL-10$^{-/-}$ animals, a finding attributed to IL-17 and IL-6 based on mAb neutralization studies in an adoptive transfer model. Importantly, neutralization of IL-17 or IL-6 alone did not significantly reduce pathology, and although combined treatment attenuated the colitis, it did not completely inhibit the inflammation (40). Based on our findings, we hypothesize that additional pathogenic effector mechanisms synergize with IL-17/IL-6 to induce severe intestinal inflammation. In particular, our results highlight that although not absolutely required for cecal disease, IFN-γ plays a synergistic role in the induction of colonic inflammation. The reason for the difference in cytokine requirements to induce maximal pathology in the cecum versus the colon is currently unclear, but it may reflect distinct levels of bacterial colonization (41) leading to different induction of cytokines and/or chemokines.

Interestingly, this study revealed a role for $H.$ $hepaticus$–induced IL-12–independent, but p40-dependent IFN-γ in intestinal pathology. Whether the molecular mechanism behind this finding involves p40 acting alone, as a homodimer, or in association with other chains remains unknown. However, it is possible that these observations reflect the involvement of IL-23 in triggering $H.$ $hepaticus$–induced CD4$^+$ IFN-γ production in IL-12–deficient mice. This conclusion is supported by recent findings from the $Mycobacterium tuberculosis$ model in which antigen-specific IFN-γ$^+$ CD4$^+$ cells were shown to develop in infected p35$^{-/-}$ mice via a p19-dependent mechanism, contributing to the prolonged survival of these hosts compared with infected p40$^{-/-}$ animals (42, 43). The co-appearance of antigen-reactive IFN-γ$^+$ CD4$^+$ and IL-17$^+$ CD4$^+$ cells in p35$^{-/-}$ mice in both the $M.$ $tuberculosis$ (43) and $H.$ $hepaticus$ models suggests that the IL-23–induced IFN-γ detected in these animals may not be sufficient to block the generation of IL-17–secreting CD4$^+$ cells. Based on the data presented here, we instead propose a modified model in which IFN-γ may have either inhibitory or synergistic effects on Th17 development and/or effector function. Thus, in settings with large amounts of the cytokine, IL-12–induced IFN-γ exerts negative effects on Th17 development, as described by Harrington et al. (8), Park et al. (9), and Mangan et al. (30), and as exemplified by Hh^+ IL-10$^{-/-}$ mice that mount markedly enhanced IFN-γ but reduced IL-17 levels compared with anti–IL-10R–treated Hh^+ p35$^{-/-}$ mice (Fig. 1, C and D). However, in settings with no IL-12, lower levels of IL-23–dependent IFN-γ (or IFN-γ induced by another p40-dependent mechanism) act together with IL-17 to enhance intestinal inflammation (Fig. 1, B–D, compare p35$^{-/-}$ with IFN-γ$^{-/-}$ mice). It is important to point out that although IL-12 is dispensable for colitis induction, this cytokine may still contribute to intestinal pathology, as shown in the T cell transfer experiments in which p19Rag$^{-/-}$ recipients displayed a significantly higher level of intestinal inflammation compared with p40Rag$^{-/-}$ recipients (Fig. 6, A and B). Besides the role of IL-12 in Th1 development, the IL-12p40 subunit by itself (possibly as a homodimer) may also contribute to disease pathogenesis through its effects on myeloid cells

(44–46) and its recently described role in DC migration and subsequent T cell priming (47).

Collectively, the data presented in this study demonstrate that IL-23 and not IL-12 is essential for the pathogenesis of T cell–dependent bacterial–induced intestinal inflammation. Although IL-23 may constitute a potential therapeutic target for IBD, our findings further support a model in which multiple factors downstream of IL-23 (e.g., IL-17, IL-6, and IFN-γ) may contribute to intestinal inflammation and together synergize to induce maximal pathology. Further identification and characterization of these downstream components and mechanisms should help dissect the immunological pathways involved in IBD, and should aid in the development of new therapeutic treatments for patients with these chronic inflammatory diseases of the intestinal tract.

MATERIALS AND METHODS

Experimental animals and infections. Female or male specific pathogen-free C57BL/6 (B6) WT, B6 p40$^{-/-}$, B6 IFN-γ$^{-/-}$, C57BL/10 (B10) IL-10$^{-/-}$, B10 Rag-2$^{-/-}$ (Taconic Farms), and B6 p35$^{-/-}$ mice (The Jackson Laboratory) were used at 6–10 wk of age. p40Rag$^{-/-}$ and p35Rag$^{-/-}$ animals were generated by crossing the above mentioned single cytokine-deficient mice, and the progeny were intercrossed to generate double-deficient offspring. For Fig. 6, Rag$^{-/-}$, p40Rag$^{-/-}$, p35Rag$^{-/-}$, and p19Rag$^{-/-}$ animals on the B6 background were used (25). The animals used tested negative for antibodies to specific murine viruses and were free of *Helicobacter* species as assessed by PCR. Animals were housed in sterile microisolator cages at the National Institute of Allergy and Infectious Disease (NIAID) animal facility under a study proposal approved by the NIAID Animal Care and Use Committee or in accredited animal facilities at the University of Oxford according to the UK Scientific Procedures Act 1986.

Mice were inoculated intragastrically with 0.5 ml of an *H. hepaticus* suspension (NCI-Frederick isolate 1A [reference 48] isolated from the same mouse colony as isolate Hh-1; American Type Culture Collection 51449 [reference 49]) prepared to a McFarland turbidity standard of 1.0 in PBS representing 2.45×10^9 CFU/ml. For the experiments shown in Fig. 6, three doses of 5×10^7–2×10^8 CFU were given to mice on days 0, 2, and 4. Age- and sex-matched uninfected animals were included as controls.

In vivo mAb treatment. To analyze the effect of in vivo neutralization of IL-10R, IL-12/IL-23 p40, or IFN-γ, mice were treated i.p. with 1 mg per injection of mAb 1B1.3a (anti–IL-10R), C17.8 (anti-p40), XMG-6 (anti–IFN-γ), or a control mAb, GL113 (anti–β-galactosidase), in 0.5 ml PBS on days 0, 7, 14, and 21 of *H. hepaticus* infection or cell transfer (for T cell–sufficient mice and Rag$^{-/-}$ recipients, respectively). 1 wk after the last mAb injection, mice were killed, MLNs were collected for in vitro culture, and intestinal tissues were collected in Bouin's fixative for histology and in RNA-later (Ambion) for real-time PCR analysis.

Cell preparations and purifications. Single cell suspensions were prepared from MLNs, and cells were resuspended in complete tissue culture medium (22). For purification of CD4$^+$ CD45RB^{hi} cells, MLN cells from naive WT or IFN-γ$^{-/-}$ mice were stained with anti–CD4-PE (clone RM4-5) or anti–CD4-CyChrome (RM4-5) and anti–CD45RB-FITC (16A; all from BD Biosciences) and sorted on a FACSVantage or a FACStarPlus (Becton Dickinson), gating on ∼50% of the brightest CD4$^+$ cells. For the experiment shown in Fig. 6, splenic CD4$^+$ CD45RB^{hi} cells from WT mice were used. The sorted CD4$^+$ CD45RB^{hi} cells were ≥99% pure.

Cell cultures and cytokine assays. MLN cells (5×10^6/ml) pooled from three to five mice/group were cultured in medium alone or with 5 μg/ml of SHelAg in 96-well flat-bottomed plates (0.2 ml/well) at 37°C and 5%

CO$_2$, and supernatants were collected after 72 h. SHelAg was prepared as described previously (20), boiled for 5 min (22), and stored at −40°C until use. IFN-γ and IL-17 were measured by ELISA using mAb from BD Biosciences and R&D Systems, respectively.

Intracellular cytokine staining. Analyses of intracellular cytokine expression were performed on cells from the same SHelAg-stimulated MLN cultures used for cytokine secretion assays as described previously (22, 50). In brief, cells were incubated for an additional 18 h in fresh medium added to replace the culture supernatant collected at 72 h. Thereafter, cells were stimulated with 10 ng/ml PMA and 1 μg/ml ionomycin (both from Sigma-Aldrich) for 4.5 h with the addition of 10 μg/ml brefeldin A (Sigma-Aldrich) during the last 2 h. Intracellular staining was then performed as described previously (22, 50) using anti–IL-17–PE (TC11-18H10.1) and anti–IFN-γ–FITC (XMG1.2; both from BD Biosciences). Cell fluorescence was measured using a FACSCalibur (Becton Dickinson), and data were analyzed using CELLQuest software (Becton Dickinson).

Cell transfers to Rag$^{-/-}$ mice. Unless otherwise indicated, CD4$^+$ CD45RB^{hi} cells (3×10^5/mouse) were transferred i.v. to naive or 2–4-d-infected Rag$^{-/-}$ mice. Mice were killed 4 wk after cell transfer, and intestinal tissues were collected for histologic analysis and measurements of cytokines.

Pathology. Intestinal tissues were processed and inflammation in the cecum (typhlitis) and ascending colon was scored in a blinded fashion by the same pathologist (A.W. Cheever) on a scale from 0 to 20 as described previously (22). In brief, a longitudinal section of the entire cecum was made together with a cross section of the ascending colon ∼1 cm from the cecum, and a total score for the whole section was assigned based on a 0–4+ scoring system for different features with emphasis on the number of infiltrating cells for the lamina propria, the submucosa, and the serosa. In addition, crypt abscesses and ulcers were each given a score from 0 to 4. A total score was calculated by adding the individual scores.

Real-time RT–PCR for detection of cytokine mRNA. Because, in general, the degree of inflammation in the colon proportionally reflects that observed in the cecum, the colon was chosen for real-time RT-PCR analyses (and cytokine protein determinations; see below) to avoid the lymphocyte patches present irregularly throughout the cecum while preserving the intact cecum for histological examination. Total RNA was isolated from polytron–homogenized colonic tissue (∼3 mm of ascending colon distal of the piece used for histology) using the RNeasy mini kit, including a DNase treatment step as recommended by the manufacturer (QIAGEN). 6–9 μg RNA was reverse transcribed using Superscript III reverse transcriptase and oligo-dT primers (both from Invitrogen). cDNA was amplified using TaqMan reagents and a Chromo4 detection system (MJ Research). Cytokine expression levels for each individual sample (run in triplicates) were normalized to HPRT using ΔCt calculations. Mean relative cytokine expression levels between control and experimental groups (n = 2–4 mice/group) were determined using the 2$^{-\Delta\Delta Ct}$ formula. Specific primer pairs and FAM/TAMARA- or VIC/TAMARA-labeled probes were as follows: HPRT: primers 5′-GACCGGTCCCGTCATGC-3′, 5′-TCATAACCTG-GTTCATCATCGC-3′, probe 5′-ACCCGCAGTCCCAGCGTCGTC-3′; IL-23p19: primers 5′-AGCGGGACATATGAATCTACTAAGAGA-3′, 5′-GTCCTAGTAGGGAGGTGTGAAGTTG-3′, probe 5′-CCAGTTCT-GCTTGCAAAGGATCCGC-3′; IL-12p35: primers 5′-TACTAGAGA-GACTTCTTCCACAACAAGAG-3′, 5′-TCTGGTACATCTTCAAGT-CCTCATAGA-3′, probe 5′-AGACGTCTTTGATGATGACCCTGTG-CCT-3′; IL-12p40: primers 5′-GACCATCACTGTCAAAGAGTTTCT-AGAT-3′, 5′-AGGAAAGTCTTGTTTTTGAAATTTTTTAA-3′, probe 5′-CCACTCACATCTGCTGCTCCACAAGAAG-3′; IL-17A: primers 5′-GCTCCAGAAGGCCCTCAG-3′, 5′-CTTTCCCTCCGCATTGA-CA-3′, probe 5′-ACCTCAACCGTTCCACGTCACCCTG-3′; IL-17F:

primers 5′-AGGGCATTTCTGTCCCACGTGAAT-3′, 5′-GCATTGA-TGCAGCCTGAGTGTCT-3′, probe 5′-CATGGGATTACAACATC-ACTCGAGACCC-3′; and IFN-γ: primers 5′-GGATGCATTCATGA-GTATTGC-3′, 5′-GCTTCCTGAGGCTGGATTC-3′; probe 5′-TTT-GAGGTCAACAACCCACAGGTCCA-3′.

Cytokine protein determinations in colon homogenates. To measure cytokines at the protein level in the intestine, colonic tissues were snap frozen and then homogenized in PBS containing a cocktail of protease inhibitors (Protease Inhibitor Cocktail Tablets; Roche) using a tissue polytron. After centrifugation at 10,000 g to pellet debris, levels of TNF-α, IFN-γ, MCP-1, and IL-6 were measured in supernatants using the cytometric bead assay (BD Biosciences). Cytokine amounts were normalized to the total amount of protein in each sample, as measured by Bradford assay (Bio-Rad Laboratories).

Quantitation of _H. hepaticus_ using real-time PCR. DNA was purified from cecal contents of Hh^+ mice using the DNA Stool kit (QIAGEN). _H. hepaticus_ DNA was quantified as described previously (41, 51) using a real-time quantitative PCR method based on the _cdtB_ gene, performed with a Chromo4 detection system. Standard curves were constructed using _H. hepaticus_ DNA that was purified from bacterial cultures using the DNeasy kit (QIAGEN).

Statistical analysis. Colitis scores were compared using the nonparametric Mann–Whitney U test. Cytokine data and _H. hepaticus_ colonization levels were analyzed by Student's unpaired two-tailed _t_ test. Differences were considered statistically significant with $P < 0.05$.

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