Infections with intestinal helminth and bacterial pathogens, such as enteropathogenic Escherichia coli, continue to be a major global health threat for children. To test the hypothesis that intestinal helminth infection may be a risk factor for enteric bacterial infection, a murine model was established by using the intestinal helminth Heliomosomoides polygyrus. To analyze the modulatory effect of a Th2-inducing helminth on the outcome of enteric bacterium Citrobacter rodentium infection, BALB/c and STAT 6 knockout (KO) mice were infected with H. polygyrus, C. rodentium, or both. We found that only BALB/c mice coinfected with H. polygyrus and C. rodentium displayed a marked morbidity and mortality. The enhanced susceptibility to C. rodentium and intestinal injury of coinfected BALB/c mice were shown to be associated with a significant increase in helminth-driven Th2 responses, mucosally and systemically, and correlated with a significant downregulation of protective gamma interferon and with a dramatic upregulation of the proinflammatory tumor necrosis factor alpha response. In addition, C. rodentium-associated colonic pathology in coinfected BALB/c mice was significantly enhanced, whereas bacterial burden was increased and clearance was delayed. In contrast, coinfection in STAT 6 KO mice failed to promote C. rodentium infection or to induce a more severe intestinal inflammation and tissue injury, demonstrating a mechanism by which helminth influences the development of host protective immunity and susceptibility to bacterial infections. We conclude that H. polygyrus coinfection can promote C. rodentium-associated disease and colitis through a STAT 6-mediated immune mechanism.
Citrobacter rodentium murine model, which selectively stimulates a TH1-type immune response (16, 17). C. rodentium, a gram-negative rod that colonizes the distal colon, is the causative agent of transmissible colonic hyperplasia and induces lesions at the epithelial surface similar to those caused by EPEC (3), including colonic crypt hyperplasia, goblet cell depletion, and mucosal erosion. These intestinal pathological changes are similar to those seen in many mouse models of colitis. In the present study, we investigate whether infection with the helminth H. polygyrus influences the development of C. rodentium-induced TH1 responses and immunopathology in mice.

MATERIALS AND METHODS

Mice. Six- to eight-week-old female BALB/cByJ and STAT 6 knockout (KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), fed autoclaved food and water, and maintained in a specific-pathogen-free (including helminths such as pinworm) facility at Massachusetts General Hospital.

H. polygyrus infections and parasite preparation. H. polygyrus were propagated as previously described and stored at 4°C until used (37). Mice were inoculated orally with 200 third-stage larvae. Seven days after parasite infection, some of the H. polygyrus-infected mice were fed C. rodentium. Parasite antigens were prepared as previously described (39). Briefly, adult worms were recovered from intestinal lumen of infected mice, washed in phosphate-buffered saline (PBS) containing 100 U penicillin/ml and 100 µg of streptomycin/ml and then rinsed in PBS. The parasites were next homogenized on ice in PBS. The homogenate was centrifuged at 10,000 rpm at 4°C for 1 h, and the supernatant recovered and sterilized by using a 0.2-mm Acrodisc (Gelman Science, Ann Arbor, MI). The parasite antigen was stored in aliquots at −20°C.

C. rodentium infection and antigen preparation. Mice were orally inoculated with C. rodentium (strain DBS100; ATCC number 51459). Bacteria were grown overnight in Luria broth (LB) and resuspended in PBS before the mice were infected (0.5 ml/mouse, ca. 5 × 10^10 CFU of C. rodentium). To assess the clearance of C. rodentium, fecal pellets were collected from each mouse weekly over the course of the experiment. Fecal pellets were weighed, homogenized, and serially diluted and plated on selective MacConkey agar. After overnight incubation at 37°C, bacterial colonies were counted as described by Vallance et al. (44). C. rodentium antigen was prepared by collecting an overnight culture of C. rodentium in LB. The bacterial culture was washed with PBS (three times) and sonicated on ice. The homogenate was then centrifuged (14,000 rpm) at 4°C for 30 min. Supernatants were collected, and protein concentrations were determined. Aliquots were stored at −20°C until use.

Sample collection and body weight measurement. To assess the antibody and serum cytokine response during infection, serum samples were collected weekly, and the total and antigen-specific immunoglobulin G1 (IgG1) and IgG2a was measured as described above. To assess the systemic effect of H. polygyrus infection, C. rodentium infection, and concurrent infection on the host, the body weight and the survival of the infected mice were measured throughout the experimental period. Body weight changes are represented as a percentage of each individual mouse’s initial weight. The data presented are pooled data from three independent experiments.

Lymphocyte isolation. Mice were sacrificed 2 or 4 weeks after C. rodentium infection. Lymphocyte suspensions were prepared from mesenteric lymph nodes (MLN) and spleens by pressing the cells through a 70-µm-pore-size nylon cell strainer (Falcon; BD Labware, Franklin Lakes, NJ) in complete Dulbecco modified Eagle medium (10% fetal calf serum, 10 m M HEPES, 2 m M L-glutamine, 100 U of penicillin/ml, 100 µg of streptomycin/ml, 50 m M 2-mercaptoethanol, 0.1 m M nonessential amino acids, and 1 m M sodium pyruvate [Life Technologies, Grand Island, NY]). Cells (5 × 10^7 cells/ml) were cultured in 24-well plates in the presence or absence of C. rodentium antigen (50 µg/ml), H. polygyrus antigen (10 µg/ml), or plate-bound anti-CD3 monoclonal antibody (MAb; 10 µg/ml), and culture supernatants were collected 48 h later and stored at −20°C until assayed for cytokine production. The concentration of antigens used in the in vitro stimulation assays was chosen based on preliminary experiments that optimized the amount of antigen required to reproducibly detect an antigen-specific response with minimal background levels (data not shown).

Measurement of cytokine production. Culture supernatants were collected for the assessment of both TH1 (IFN-γ and Th2 ( interleukin-4 [IL-4], IL-5, and IL-10) cytokine production. Samples were prepared for tumor necrosis factor alpha (TNF-α) determination by enzyme-linked immunosorbent assay (ELISA). ELISA capture antibodies (BVD4-1D11, IL-4; R4-6A2, IFN-γ, TRFK-5, IL-5, JESS-2A5, IL-10, and MP6-XT22 for TNF-α) and biotinylated secondary antibodies (BVD6-2G4, IL-4; XM121, IFN-γ, TRFK4, IL-5, SXC-1, IL-10, and CI150-14 for TNF-α) were purchased from Pharmingen (San Diego, CA). The biotinylated secondary antibodies were used as second layer, and the reactions were visualized with o-phenylenediamine (ODP; Zymed Labs). Standard curves were obtained by using recombinant murine IFN-γ, IL-4 (Genzyme Corp.), IL-10 (R&D Systems), IL-5 (Pharmingen), and TNF-α (Pharmin- gen). Optical density values were converted to pg/ml for each cytokine by linear regression with Delta Soft II (Biometalliks, Princeton, NJ).

Measurement of serum antibody levels. Each mouse was bled weekly over the course of experiments and at sacrifice. Individual sera were assayed for antigen-specific IgG1 and IgG2a by ELISA. Immuno II plates were coated with bacterial (10 µg/ml) or parasite antigen (10 µg/ml) and incubated overnight at 4°C. There were two rows in each plate coated with a goat anti-mouse IgG1 or anti-mouse IgG2a (Southern Biotechnology Associates) and used to generate a standard curve. After being blocked and washed, the plates were incubated with diluted serum samples and standard mouse IgG1 or IgG2a (Southern Biotechnology Associates). Antigen-specific IgG1 and IgG2a were detected by using horseradish peroxidase-conjugated anti-IgG1 and IgG2a as described above. The reaction was developed with OPD and read at 492 nm. Optical density values were converted to µg of C. rodentium or H. polygyrus-specific IgG1 or IgG2a/ml compared to a standard curve developed with either anti-mouse Ig1 or anti- mouse Ig2a.

Histopathological examinations. At necropsy, colonic tissues were collected, frozen in Tissue Tek OCT compound (Miles, Inc., Elkhart, IN) and then stored at −80°C. Then, 5-µm sections were cut on a 2800 Frigocut cryostat (Reichert-Jung, Germany) and were stained with hematoxylin and eosin. Colon sections were cut into the lamina propria; 3, confluence of inflammatory cells extending into the submucosa; and 4, transmural extension of the inflammatory infiltrate cells). Part 2 is the evaluation of colony tissue damage, with scores that also range from 0 to 4 (0, normal tissue pattern; 1, minimal inflammation and colonic crypt hyperplasia; 2, mild colonic crypt hyperplasia with or without focal invasion of epithelium; 3, obvious colonic crypt hyperplasia, invasion of epithelium, and goblet cell depletion; and 4, extensive mucosal damage and extension through deeper structures of the bowel wall). The total colon pathology score equals the inflammatory cell score plus the tissue damage score.

Detection of colonic cytokine expression. Cytokine mRNA expression in colon tissues was determined by real-time PCR. Total cellular RNA was isolated from frozen colonic tissue (distal part of colon) by using TRIzol (Gibco Life Tech- nologies, Grand Island, NY) according to the manufacturer’s instructions. cDNA was prepared from total RNA using 2 µg of target RNA (Ready-to-Go Kit; Pharmacia Biotech). The cDNA samples were then subjected to real-time PCR (SYBR Green dye assay). Mouse IL-4, IL-12, IFN-γ, and TNF-α real-time PCR probes and primer pairs were purchased from Biosource International, Inc. (Camarillo, Ca.). Amplification of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was included for each experimental sample as an endogenous control to account for the differences in the amount of total RNA loaded in each reaction. Target cytokine gene expres- sion was normalized between different samples based on the values of the expression of the GAPDH gene. All experimental samples were amplified in triplicates.

Determination of C. rodentium translocation. To examine whether co-infection with helminth parasites result in an enhanced C. rodentium translocation into both the mucosal and the systemic compartments, mice were infected with H. polygyrus, and 7 days later they were infected with C. rodentium. Separate mice were infected with C. rodentium only or not infected. The mice were sacrificed at 7 and 14 days after bacterial infection. At necropsy, the MLN and spleens were removed and homogenized in sterilized 1% Triton 100. An aliquot of the ho- mogenate was plated on selective MacConkey agar plates. Bacterial colonies were counted after overnight incubation as described above. No commensal bacteria were detected in the MLN or spleen.

Statistical analysis. All of the results are expressed as the mean ± the standard error of the mean (SEM). “N” refers to the number of mice used. Statistical differences were determined by using a two-tailed Student t test with StatView software (Abacus Concepts, Berkeley, CA). A P value of <0.05 was considered significant. Statistical differences of colonic pathology scores between groups.
RESULTS

H. polygyrus infection increases susceptibility of BALB/c mice to C. rodentium infection and results in weight loss and death in the coinfected host. C. rodentium infection in most adult mice is self-limiting, with little morbidity and mortality. To examine whether an intestinal helminth infection can be a risk factor for concurrent infection with bacterial pathogens, thereby enhancing the susceptibility of the host to C. rodentium infection, BALB/c mice were infected with H. polygyrus and inoculated with C. rodentium 7 days later. As expected, mice infected only with C. rodentium showed signs of citrobacter-associated disease, such as soft stool, a hunched posture, disturbed body hair, and body weight loss early during the infection, but recovered by 2 to 3 weeks postinfection. In contrast, mice with coinfection of H. polygyrus and C. rodentium developed more severe disease, resulting in a more significant and prolonged body weight loss during the course of the experiment (Fig. 1, top panel), gastrointestinal tract bleeding, and ca. 40% of the coinfected mice developed anal prolapse 10 days after bacterial inoculation. Such pathology was not observed with mice infected with H. polygyrus or C. rodentium alone. The significant impact of H. polygyrus on mice with C. rodentium infection was further demonstrated by a 20 to 33% mortality in coinfected animals that occurred 2 to 3 weeks after C. rodentium infection (Fig. 1, bottom panel). No mortality was observed in mice infected with C. rodentium alone.

FIG. 1. Coinfection with H. polygyrus and C. rodentium induces significant body weight loss (top panel) and mortality (bottom panel). BALB/c mice were infected with H. polygyrus (200L3) and inoculated with C. rodentium (5 x 10⁶ CFU) 7 days later. (Top panel) Body weight changes of mice that were infected with C. rodentium (Cr; ○), H. polygyrus (Hp; ■), and both H. polygyrus and C. rodentium (Hp + Cr; ●) and of normal controls (□) during the course of the experiment (4 weeks). The body weights of C. rodentium-infected mice were significantly lower than those of noninfected controls. Mice with coinfection weighed significantly less than mice with C. rodentium infection alone, whereas H. polygyrus infection had no effect on host body weight gain. The data shown are pooled from three independent experiments and are expressed as the body weight change as a percentage of the individual mouse’s initial body weight ± the standard error (SE). n = 12 to 15 for C. rodentium-infected mice, n = 9 to 12 for control mice, n = 9 to 12 for H. polygyrus-infected mice, and n = 18 for coinfected mice. (Bottom panel) Survival curve. There was no mortality in C. rodentium-infected mice.
*H. polygyrus* infection results in enhanced citrobacter-associated colonic pathology in BALB/c mice. *C. rodentium* causes colonic hyperplasia and induces similar intestinal pathological changes to that seen in many murine models of colitis, including colonic crypt hyperplasia, goblet cell depletion, and mucosal erosion. During the course of infection, BALB/c mice of different groups (*C. rodentium* infection alone, *H. polygyrus* infection, coinfection, and noninfected controls) were sacrificed, and the colons were examined both macroscopically and microscopically. Thicker colons were observed in mice with *C. rodentium* infection 1 and 2 weeks postinfection, in agreement with previous reports (44). In addition, the thickness of the colon in coinfeated mice was also more pronounced at 1 and 2 weeks postinfection. By week 4 postinfection, the colons of *C. rodentium*-infected mice began to return to normal. However, a thicker colon remained in the coinfeated mice (data not shown). No visible changes were observed in the small intestine and cecum in any of the mice.

Microscopic examination showed that colonic inflammation developed in both *C. rodentium* only and coinfeated groups at 2 and 4 weeks after *C. rodentium* infection (Fig. 2C, 2D, 3C, and 3D). Mice infected with *C. rodentium* showed typical pathological changes associated with this bacterial infection in the intestine, including thickening of the wall of the colon, colonic epithelial cell hyperplasia, crypt elongation, and goblet cell depletion (Fig. 2C and 3C). Microscopic analysis of colonic tissues of coinfeated mice at both 2 and 4 weeks postinfection showed a more severe pathology compared to citrobacter-infected mice, including colonic crypt elongation, massive cellular infiltration of the colonic lamina propria, epithelial erosions, and edema of the gut wall (Fig. 2C, 2D, 3C, and 3D). The pathology scores for inflammation and intestinal damage were significantly higher in coinfeated mice than in mice with *C. rodentium* infection only (Fig. 2E and 3E). These results demonstrate that concurrent intestinal helminth infection promotes *C. rodentium* induced colonic injury.

**Impact of intestinal helminth coinfection on fecal *C. rodentium* output in BALB/c mice.** We next examined whether *H. polygyrus* infection affected the dynamics of the bacterial infection by collecting the fecal pellets during the experimental periods, homogenizing and plating them onto selective MacConkey agar plates. Our results show that the bacterial output was significantly higher in coinfeated mice at an early stage of infection (1 week postinfection), indicating that helminth infection may enhance the colonization of *C. rodentium* on the colonic epithelial surface. The significantly higher level of bacteria shed in feces was detected in coinfeated mice throughout the experimental period (up to 3 weeks postinfection) (Fig. 4). By the third week after bacterial infection, mice infected with *C. rodentium* showed a significantly diminished bacterial output, whereas coinfeated mice remained heavily colonized with *C. rodentium*, suggesting that intestinal helminth infection increased bacterial output and decreased bacterial clearance in coinfeated mice.

**Helminth infection results in enhanced translocation of *C. rodentium* into mucosal and systemic immune compartments in BALB/c mice.** Since mice coinfeated with *H. polygyrus* and *C. rodentium* harbored higher bacterial loads and developed more severe colitis in BALB/c mice, we questioned whether the severely inflamed, disrupted colonic epithelium in the host could become the entry point for luminal bacteria, which in turn may contribute to a more exacerbated inflammation. Therefore, 1 and 2 weeks after *C. rodentium* infection, at the peak of inflammation, we sacrificed mice and collected the MLN and spleens. The tissues were then homogenized and plated on selective MacConkey agar plates. There were significantly more viable *C. rodentium* bacteria recovered from the MLN of coinfeated mice than from mice infected with *C. rodentium* alone (Fig. 5A). At this stage of infection, similar low levels of bacteria were detected in the spleens of both *C. rodentium*-infected and coinfeated mice (Fig. 5C). At 2 weeks after bacterial infection, the number of *C. rodentium* recovered in the MLN of mice infected with *C. rodentium* alone decreased, whereas the number of bacteria in coinfeated mice remained high (Fig. 5B). Also, at this stage of infection, similar to what has been found in the MLN, the numbers of bacteria recovered in spleen were very low in *C. rodentium*-infected mice. In contrast, there were significantly more bacteria recovered from the spleens of coinfeated mice (Fig. 5D). The results suggest that an intestinal nematode parasite infection may impair the colonic epithelial barrier function, allowing an enhanced entry of luminal bacteria into the mucosal immune system.

**Helminth coinfection results in alterations of host anti-*C. rodentium* specific immune responses in BALB/c mice.** Evidence from murine experimental models indicates that in addition to an increase in the Th2 cytokine production, helminth infection also causes a downregulation of the Th1 response. To examine the impact of this intestinal nematode parasite infection on the host antigen-specific immune response to bacterial infection, we monitored antigen-specific antibody responses of mice with either bacterial or parasitic infection and coinfection during the course of the experiment. As expected, mice infected with *H. polygyrus* generated a strong anti-parasite antigen-specific IgG1 response (Fig. 6A) and produced low levels of the anti-parasite IgG2a response (Fig. 6B), showing the development of the Th2 polarized response. The parasite-driven IgG1 response was also detected in coinfeated hosts (Fig. 6A). However, the level of IgG1 was lower in coinfeated mice than in mice infected with *H. polygyrus* only. This finding suggests that *C. rodentium* infection also influences the development of a parasite-induced immune response. In the present study, the serum level of antibacterial antigen specific antibody and the impact of helminth infection on these responses were also examined. Our results show that *C. rodentium* infection alone induces an IgG2a response, e.g., a Th1 response (Fig. 6D). Interestingly, however, this response is found to be enhanced by coinfection with *H. polygyrus* (Fig. 6D). More significantly, our results reveal that coinfection with *H. polygyrus* drives the generation of a high level of the anti-*C. rodentium* specific IgG1 response (Fig. 6C), which is absent in mice infected with *C. rodentium* alone (Fig. 6C), demonstrating that prior infection with helminth parasite promotes a Th2 biased antibody response in the host.
specific cytokine production of MLN by collecting lymphocytes from MLN and restimulating them in vitro with anti-CD3 MAb, bacterial or parasite antigen. As anticipated, a highly Th2 polarized response was detected in the MLN cells from mice with *H. polygyrus* infection with an increased production of IL-4, IL-5, and IL-10 (Fig. 7A and B). MLN cells from mice with *H. polygyrus* infection, as well as those with coinfection, produced higher amounts of IL-4, IL-5, and IL-10 when stim-

FIG. 2. Coinfection with the intestinal helminth parasite, *H. polygyrus*, promotes *C. rodentium*-mediated colonic pathological alterations. Colon tissue was removed from uninfected mice (A) or from mice infected with *H. polygyrus* (B), *C. rodentium* (C), or both (D) at 2 weeks after bacterial infection, frozen in Tissue Tek OCT compound, and stained with hematoxylin and eosin. (A) Uninfected colon. (B) Colon from *H. polygyrus*-infected mice. There were no significant changes observed. (C) *C. rodentium*-induced colon inflammatory response with epithelial hyperplasia, inflammatory cell infiltration, and thickening of the gut wall. (D) Mice infected with both pathogens developed disrupted epithelial surfaces and induced massive cellular infiltration into colon lamina propria, and the submucosal became edematous. Magnification, ×100. (E) Disease score of colonic inflammation in uninfected mice and in mice infected with *H. polygyrus*, *C. rodentium*, or both. The scores were assessed by determination of infiltration of inflammatory cells (score range, 0 to 4), together with the evaluation of colon tissue damage (score range, 0 to 4), with 0 as normal and 4 as the most diseased. The data shown are pooled from three independent experiments with total (n = 9 to 12 per group).
ulated with a surface-bound anti-CD3 MAb or with a parasite antigen over that of C. rodentium-infected or uninfected control mice (Fig. 7A and B). The data also suggest that parasite antigen-specific IL-4 production was significantly inhibited in mice coinfected with H. polygyrus and C. rodentium, showing a cross-regulation of the C. rodentium-induced Th1 response in the parasite antigen-specific Th2 response that correlated with the reduced serum IgG1 response described above. Interestingly, however, coinfection of H. polygyrus and C. rodentium resulted in an enhanced parasite-specific IL-10 production in both an antigen-specific and nonspecific fashion (Fig. 7A and B). In contrast to the helminth infection, the C. rodentium infection induced a strong Th1 polarized cytokine response in the intestinal mucosa, as shown by an increased production of IFN-γ in vitro in response to anti-CD3 and bacterial antigen restimulation (Fig. 7A and C). Bacterial antigen-specific IFN-γ production in the MLN was significantly lower in coinfected mice than in the group infected with only C. rodentium, suggesting that a helminth infection can significantly inhibit the bacterium-specific Th1 response. This may result in an im-

FIG. 3. H. polygyrus coinfection prolonged C. rodentium-mediated colonic inflammation. Colon tissues were removed from uninfected mice (A) or from mice infected with H. polygyrus (B), C. rodentium (C), or both (D) at 4 weeks after bacterial infection; frozen in Tissue Tek OCT compound; and stained with hematoxylin and eosin. For a detailed description, see Fig. 2. Magnification, ×100.
paired host protective immunity to intestinal bacterial infection. A significant reduction of IFN-γ production was also detected in spleen cells from coinfected mice (data not shown).

Analysis of the serum TNF-α level showed that there was a similar low level of TNF-α detected in noninfected mice (60 ± 20 pg/ml) or in mice in infected with C. rodentium (102 ± 35 pg/ml) or H. polygyrus (98 ± 38 pg/ml). In contrast, a significantly enhanced serum TNF-α level was found in mice with coinfection (297 ± 75 pg/ml).

H. polygyrus coinfection differentially regulates C. rodentium-induced colonic proinflammatory Th1 cytokine responses in the colon in BALB/c mice. It has been suggested that C. rodentium infection is associated with a Th1 response in the colon (17) and that the Th1 type cytokines (IFN-γ, TNF-α, and IL-12) may play a role in host resistance to C. rodentium infection (14, 41). To examine whether H. polygyrus, which inhabits the mouse duodenum, affects the cytokine response in the colon and to further explore the contribution of a Th2 immune response induced by an intestinal parasite in the cytokine response during coinfection, we examined the cytokine expression in the colon by real-time PCR. The results presented in Fig. 8 show that C. rodentium infection significantly upregulated the colonic IFN-γ expression that is significantly reduced in the colons of coinfected mice (Fig. 8A). Real-time PCR analysis of Th2 cytokine expression (IL-4) in the colon revealed elevated levels of IL-4 in tissues from mice infected with H. polygyrus alone and from coinfected mice (Fig. 8B), demonstrating that H. polygyrus infection upregulates colonic IL-4 expression. This parasite-driven Th2 cytokine response may contribute to the inhibited colonic IFN-γ response observed in coinfected mice.

Analysis of the colonic TNF-α response showed that this proinflammatory cytokine was moderately increased by C. rodentium infection (Fig. 8C). However, coinfection with H. polygyrus significantly enhanced TNF-α expression in the colon.

![FIG. 4. Coinfection with H. polygyrus results in an enhanced C. rodentium output in the fecal pellets. The data shown are the numbers of bacteria recovered from fecal samples of C. rodentium-infected and coinfected mice at 1, 2, and 3 weeks postinfection. The data shown are log transformed and are represented as the mean ± the SEM (n = 5 to 10 mice at each time point). *P < 0.05 for a comparison of coinfected versus C. rodentium-infected mice.](image)

![FIG. 5. Viable C. rodentium recovered in the MLN (A and B) and spleen (C and D). Mice were infected with H. polygyrus or uninfected. At 1 week after H. polygyrus infection, some of the infected as well as uninfected mice were inoculated with C. rodentium (5 × 10⁸ CFU/mouse). At 1 and 2 weeks after bacterial infection, mice were killed, and the MLN and spleens were removed and homogenized. Aliquots of diluted homogenized tissue were plated on selective MacConkey agar plates and incubated overnight. Viable bacteria were counted. (A and C) Recovery of bacteria in MLN and spleen at 1 week after bacterial infection. (B and D) Bacterial recovery at 2 weeks after bacterial infection. The data presented are mean ± the SEM (n = 5 per group). *P < 0.05 for a comparison of coinfected versus C. rodentium-infected mice.](image)
Our results also show that there is a significant increase in the expression level of Th1 cytokine, IL-12, in *C. rodentium*-infected mice. Moreover, colonic IL-12 expression was also enhanced in coinfected mice (Fig. 8D), suggesting that *H. polygyrus* infection had no effect on the *C. rodentium*-induced IL-12 response in the intestine. Taken together, our data show that coinfection with a helminth parasite and *C. rodentium* results in a significant alteration in intestinal cytokine responses, which are associated with the highest susceptibility to *C. rodentium* infection and the most severe colitis.

Coinfection with *H. polygyrus* in Th2-deficient (STAT 6 KO) mice fails to result in an enhanced *C. rodentium*-induced colitis. The results presented above suggest that the Th2 response driven by helminth infection may play a role in altering host’s protective immune responses and rendering the host more susceptible to enteric bacterial infection, resulting in more severe intestinal injury. To test this hypothesis and to explore the potential mechanism responsible for the observed alterations in coinfected BALB/c mice, we used STAT 6 KO mice. Mice that lack the STAT 6 signaling pathway are deficient in the functional differentiation of Th2 cells (19) and thus are an ideal in vivo model system in which to explore the role of Th2 cytokine regulation in host protective immunity to concurrent infected enteric bacterial pathogens.

To examine the systemic impact of *C. rodentium* and coinfection with helminth in the STAT 6 KO Th2-deficient mice, we monitored the body weight change of these mice. As shown in Fig. 9A, infection with *C. rodentium* in STAT 6 KO mice had no impact on body weight gain. For example, at 2 weeks after *C. rodentium* infection, *C. rodentium*-infected STAT 6 KO mice gained a similar amount of weight compared to noninfected or *H. polygyrus*-infected STAT 6 KO mice (Fig. 9A). However, coinfection with both pathogens significantly inhibited the growth of the infected host; consequently, the body weights of these coinfected STAT 6-deficient mice remained unchanged at 2 weeks after coinfection with *C. rodentium* (Fig. 9A). In contrast, coinfected BALB/c mice (the control) showed a significant reduction in body weight at 2 weeks after *C. rodentium* infection (Fig. 9A), further substantiating our results in Fig. 1.

We also examined whether helminth coinfection in STAT 6 KO mice might affect the fecal bacterial output, as we observed in coinfected BALB/c mice (Fig. 4). We determined that, unlike coinfected BALB/c mice, coinfected STAT 6 KO mice produced numbers of viable bacteria similar to those produced by STAT 6KO mice infected with *C. rodentium* at 1 (coinfected = 3,294 ± 312 versus *C. rodentium* only = 2,500 ± 780 [× 10^8 CFU/g of fecal pellets]) and 2 (coinfected = 1,403 ± 261 versus *C. rodentium* only = 1,152 ± 400 [× 10^8 CFU/g of fecal pellets]) weeks after *C. rodentium* infection.
To determine the impact of coinfection with H. polygyrus on the MLN cytokine responses of STAT 6 KO mice, we examined antigen-specific and nonspecific cytokine production of the MLN cells. In response to anti-CD3 stimulation, the MLN cells from STAT 6 KO mice with a single inoculum of C. rodentium or H. polygyrus or coinfection secreted elevated levels of Th1 cytokine (IFN-γ) (Fig. 9B). H. polygyrus infection also induced an elevated level of IL-10 production in the MLN from STAT 6 KO mice (Fig. 9B). As expected, the MLN cells produced a diminished amount of the Th2 cytokine (IL-4) (Fig. 9B and C). C. rodentium infection induces antigen-specific IFN-γ production in the MLN of STAT 6 KO mice, and this response was partially inhibited by helminth coinfection (Fig. 9C).

To demonstrate the contribution of Th2 response induced by helminth to the induction of an enhanced intestinal injury in C. rodentium-infected mice, we examined and compared colonic pathological alterations at 2 weeks after C. rodentium infection, when the peak intestinal inflammation has developed. Our histopathological analysis determined that C. rodentium infection induced colitis in STAT 6 KO mice (Fig. 10C). Unlike the results in BALB/c mice (Fig. 10F), coinfection with the Th2-inducing helminth in STAT 6 KO mice failed to induce the development of more severe C. rodentium-mediated intestinal injury (Fig. 10D and F). Although there were no significant differences in colonic scores of inflammation and injury between STAT 6 KO with C. rodentium infection versus coinfection, we observed that the C. rodentium-induced colitis, which developed in some of C. rodentium-infected STAT 6 KO mice appeared to be milder than that seen in coinfected STAT 6 KO mice (Fig. 10G). Coinfection in BALB/c mice (Fig. 10F) again demonstrated a more significant intestinal injury compared to that in C. rodentium-infected BALB/c mice (Fig. 10E), further confirming the results described above (Fig. 2C and D). Therefore, these observations demonstrate a role of the Th2 response induced by helminth infection in the regulation and modulation of host response to concurrently infected enteric bacterial pathogens, as well as the outcome of the disease they produce.

FIG. 7. Th1 and Th2 cytokine profile of mice infected with C. rodentium, H. polygyrus, or both at 2 weeks after bacterial infection. MLN cells were cultured with surface-bound anti-CD3 MAb (10 μg/ml) (A), parasite antigen (B), or C. rodentium antigen (C). Culture supernatants were collected 48 h later. Cytokine secretion into the culture supernatants was determined by ELISA. The data show that H. polygyrus infection drove a Th2 response, whereas C. rodentium infection induced a Th1-type response. Coinfection with H. polygyrus significantly inhibited C. rodentium induced IFN-γ production. C. rodentium infection negatively influenced the parasite-induced IL-4 response in the MLN of coinfection hosts. The results are displayed as means ± the SEM and are representative of three independent experiments. Different letters represent significant difference (P < 0.05; n = 5 mice per group).
DISCUSSION

*C. rodentium* is an attaching and effacing pathogen in mice and has been used as the animal model for studying the pathogenesis of EPEC and enterohemorrhagic *E. coli* and for investigating the host response to enteric pathogens and the resulting immunopathology (3, 17, 23, 36). Recent studies with various gene KO mice deficient in T and B cells have indicated an essential and protective role of T (including the CD4 subset) and B cells in host defense (6, 24, 40, 44). Colonization of *C. rodentium* on the epithelial surface induced a significant CD3 and CD4+ T-cell infiltration into the colonic lamina propria and resulted in a Th1-type immune response in the intestine, characterized by upregulation of IFN-γ, TNF-α, and IL-12 (14, 44). The role of these Th1 cytokines in host protection was demonstrated by subsequent studies using mice lacking either IFN-γ, IL-12, or the receptor for TNF, all of which showed reduced resistance to the infection and enhanced intestinal injury (14, 41).

In the present study, we examined the effect of an ongoing Th2 polarizing helminth parasite infection on the pathogenesis of *C. rodentium* infection. Our results show that BALB/c mice, coinfected with *C. rodentium* and the intestinal nematode, *H. polygyrus*, displayed a marked increase in morbidity and mortality compared to mice that were infected with *C. rodentium* alone. The enhanced susceptibility to *C. rodentium* and intestinal injury in coinfected BALB/c mice was found to be associated with helminth-induced Th2 responses and correlated with a dysregulated proinflammatory Th1 response (with a downregulation of protective IFN-γ response and an upregulation of proinflammatory TNF-α) and with an increase in bacterial burden. In contrast, coinfection with helminth failed to enhance colitis or bacterial colonization in STAT 6 KO mice, which are unable to transmit signals initiated by the helminth-induced Th2 cytokines (IL-4 and IL-13). The results from STAT 6 KO mice confirm the mechanistic explanation of the helminth-induced Th2 response in the altered inflammation and bacterial clearance in the coinfected animals. To our knowledge, this is the first study to demonstrate that an ongoing intestinal helminth infection impairs host protective immunity to *C. rodentium* and promotes *C. rodentium*-mediated colitis.

Involvement of the STAT 6 pathway in the regulation of intestinal inflammation has also been suggested in recent studies. Van Kampen et al. showed that overexpression of IL-4 (or IL-12) in the colonic mucosa (by gene transfer) induced fatal colitis in healthy mice, whereas STAT 6 KO mice failed to develop colitis (46). In a different murine model system with trinitrobenzenesulfonic acid (TNBS)-induced colitis, the helminth-activated STAT 6 pathway was shown to be required for attenuating TNBS-induced Th1 colitis (11). Interestingly, our data also reveal that in the absence of functional Th2 responses coinfection with helminth results in a significant inhibition in body weight gain and a suppression of bacterial antigen-specific IFN-γ response in STAT 6 KO mice. These results suggest that helminth infection may also exert its effects on the response of the host through a STAT 6-independent mechanism.

FIG. 8. Cytokine mRNA expression in colon tissue as measured by real-time PCR at 2 weeks after bacterial infection. Values are mean fold increases compared to the baseline obtained from normal control animals. (A) IFN-γ; (B) IL-4; (C) TNF-α; (D) IL-12. The data shown are from one of two experiments performed, showing similar results. Different letters represent significant difference (*P* < 0.05, *n* = 3 to 5 mice per group).
FIG. 9. Impact of H. polygyrus coinfection on STAT 6 KO mice exposed to C. rodentium. (A) Body weight changes of mice that were infected with C. rodentium, H. polygyrus, or both H. polygyrus and C. rodentium and normal controls at 2 weeks after C. rodentium infection. The data shown are pooled from two experiments and are the body weight change as a percentage of an individual mouse’s initial body weight ± the SEM (n = 6 to 8 animals per group). (B and C) Th1 and Th2 cytokine profile of STAT 6 KO mice infected with C. rodentium, H. polygyrus, or both at 2 weeks after C. rodentium infection. MLN cells were cultured with surface-bound anti-CD3 MAb (10 μg/ml) (B) or to C. rodentium antigen (C). The results are displayed as the mean ± the SEM and are pooled from two independent experiments. Different letters represent significant differences within a mouse strain (P < 0.05, n = 6 to 8 mice per group).
In the present study we also observed that helminth infection induced a significant induction of IL-10 production in both BALB/c and STAT 6 KO mice. Although, initially and often classified as a Th2 cytokine (as it is referred in the present study), IL-10 is multifunctional cytokine and is also associated with T regulatory cells. Helminth-induced IL-10 has been suggested to play a role in attenuating allergic responses (45). Our data provide evidence suggesting the possibility that helminth induced immunoregulatory cytokines, such as IL-10, may contribute to the suppression of host protective Th1 response against *C. rodentium* via the induction and/or function of T regulatory cells. An impaired host protection against *C. rodentium* can lead to the development of more severe bacterium-associated colitis in a coinfected host. Thus, our observations highlight the complexity of the immune circuitry coordinating the response of coinfected mice, the details of which are under investigation.

Concomitant with an enhanced Th2 and an impaired protective Th1 IFN-γ response, coinfected BALB/c mice had significantly increased levels of bacterial colonization, showed enhanced proliferation, and displayed a delayed clearance. These results are in line with the observation that mice deficient in IFN-γ displayed a higher bacterial load in the colon and developed a more severe colonic pathology (41). Further-

![Image](https://example.com/image.png)
more, in our experiments coinfect ed BALB/c mice also had a systemic spread of infection, as evidenced by an increase in bacterial translocation, with bacteria infiltrating deep into the MLN and spleen because of a damaged colonic mucosa. An increase in bacterial translocation may further suggest that coinfection with *H. polygyrus* impaired local defense mechanisms that normally restrict the growth of bacteria that manage to penetrate the epithelium (25). This notion is supported by observations from mice harboring defects in their immune functions (i.e., IFN-γ−/−, TNFRp55−/−, and CD4−/− KO animals) which showed a significant burden of *C. rodentium* in the MLN and spleen as well as other sites (liver and blood) (6, 17). Our data are also consistent with the finding that a greater number of bacteria translocated into the MLN in *C. rodentium*-susceptible C3H/HeJ mice (43). Based on these observations, it is likely that impaired host defense promotes increased bacterial loads and delays bacterial clearance, which can be expected to cause more severe and prolonged tissue damage. 

Helminth infection has been known to dampen Th1 reactions to other infections (1, 5, 26, 27) and to attenuate damaging Th1-driven inflammatory responses in the host (11, 13, 20, 29). For example, infection with the helmint *Fasciola hepatica* reduced the protective Th1 response to coinfection *Borrelélla pertussis* and exacerbated bacterial infection (5). Likewise, infection with *Schistosoma mansoni* downregulated antigen-specific Th1 cytokines and cytotoxic-T-lymphocyte responses, resulting in a delay in vaccinia virus clearance (1). Moreover, our results are consistent with the observation that a combined infection of *Trichuris suis* and *Campylobacter jejuni* in immunologically naive, germfree piglets, resulted in an enhanced invasion of the colon by *C. jejuni*, leading to the development of a more severe pathology in the colon (26). Unlike *T. suis*, which resides in the colon, *H. polygyrus* is primarily localized in the duodenum of the small intestine and does not cause direct mechanical damage in the colon on its own. The impact of the *H. polygyrus* on the colonic *C. rodentium* infection is therefore likely to be largely indirect via alterations in the immune response.

Experimentally induced colitis by dinitrobenzenesulfonic acid or TNBS, which is characterized by a Th1-type inflammation, has been shown to be significantly attenuated in mice preinfected with *Trichinella spiralis* or pretreated with *S. mansoni* eggs, both of which induce a Th2 response (11, 20). Concurrent infection with *S. mansoni* was also shown to significantly attenuate the course of TNBS induced colitis in rats (29). Similar to the results presented here, mice coinfect ed with *H. polygyrus* and *Helicobacter felis* were found to respond with an increased Th2 response (IL-4, IL-10, and transforming growth factor β) and to develop significantly lower gastric levels of proinflammatory Th1 cytokines (IFN-γ, TNF-α, and IL-1β) (13). However, unlike the results obtained from mice coinfect ed with *H. polygyrus* and *H. felis* showing a reduced *H. felis*-mediated pathology in the stomach (13), our results demonstrate that concurrent infection with *H. polygyrus* and *C. rodentium* results in the development of a more severe intestinal pathology. This difference may be attributable to the different anatomical location of infection, the different helmint infection protocols (challenge versus primary infection), and/or to the distinct biological characteristics of *H. felis* versus *C. rodentium* with the latter presumably having a greater propensity to multiply and spread when host defenses are compromised. Thus, our results indicate that helmint-induced damping of host protective Th1 responses may allow microorganisms that are usually confined to the superficial layer of the gut to spread deeper and systemically, leading to more severe tissue damage and disease.

We have also observed that *H. polygyrus* coinfection altered colonic cytokine responses with an upregulation of IL-4, TNF-α and a downregulation IFN-γ expression. An effect of IL-4 on intestinal epithelial cell function has been reported in *H. polygyrus* challenged mice by anti-IL-4 MAb treatment, which prevented changes in epithelial function such as increased mucosal permeability and an increased Cl− secretory response to prostaglandin E2 (35). Furthermore, overexpression of IL-4 in the colonic mucosa results in the development of severe colitis in mice (46). In line with our observation, it has been shown that IFN-γ-deficient mice expressed an increased level of colonic TNF-α and developed an enhanced susceptibility and inflammation (41). As a central mediator of intestinal inflammation, increased levels of TNF-α may be another factor contributing to tissue injury (30, 32, 33). Taken together, we conclude that helmint-induced alterations in intestinal cytokine response may contribute to the functional alterations of the colonic epithelial barrier and to the enhanced colonic pathology in coinfect ed host.

In conclusion, our model of coinfection provides a suitable tool to investigate the complex interactions between Th1, Th2, and T regulatory cell responses during infections with multiple enteric pathogens. This model can also be used to explore the feasibility of using parasites, parasite components, or products as therapeutic agents in the treatment of intestinal inflammato- 

dious diseases, as has recently been suggested (11). The poten-
tial for intestinal helmint infection to alter immune responses to luminal bacteria and orally inoculated antigens, such as oral vaccines, has been an unappreciated area of study but one which has broad and important public health implications.

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