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Neutralizing Anti-IL-10 Antibody Blocks the Protective Effect of Tapeworm Infection in a Murine Model of Chemically Induced Colitis¹

Meaghan M. Hunter, Arthur Wang, Christina L. Hirota, and Derek M. McKay²

There is increasing evidence that parasitic helminth infection has the ability to ameliorate other disease conditions. In this study the ability of the rat tapeworm, *Hymenolepis diminuta*, to modulate dinitrobenzene sulfonic acid (DNBS)-induced colitis in mice is assessed. Mice receiving DNBS (3 mg intrarectally) developed colitis by 72 h after treatment. Mice infected 8 days before DNBS with five *H. diminuta* larvae were significantly protected from the colitis, as gauged by reduced clinical disease, histological damage scores, and myeloperoxidase levels. This anticolitic effect was dependent on a viable infection and helminth rejection, because no benefit was observed in mice given killed larvae or in infected STAT6 knockout mice or rats, neither of which eliminate *H. diminuta*. The anticolitic effect of *H. diminuta* was associated with increased colonic IL-10 mRNA and stimulated splenocytes from *H. diminuta*- plus DNBS-treated mice produced more IL-10 than splenocytes from DNBS-only treated mice. Coadministration of an anti-IL-10 Ab blocked the anticolitic effect of prophylactic *H. diminuta* infection. Also, mice infected 48 h after DNBS treatment showed an enhanced recovery response. Finally, using a model of OVA hypersensitivity, we found no evidence of concomitant *H. diminuta* infection enhancing enteric responsiveness to subsequent ex vivo OVA challenge. The data show that a viable infection of *H. diminuta* in a nonpermissive system exerts a profound anticolitic effect (both prophylactically and as a treatment) that is mediated at least in part via IL-10 and does not predispose to enhanced sensitivity to bystander proteins. *The Journal of Immunology*, 2005, 174: 7368–7375.

Although it is clear that the variety of T cell phenotypes is expanding, the observation that the Th cells include two reciprocally inhibitory subtypes, Th1 and Th2 (1), has provided an interesting paradigm for exploring the mechanisms of disease. By promoting a Th2 environment, the expression of diseases that are mediated by a Th1 response could be repressed, because the production of IL-4 and IL-10 will inhibit the differentiation and activity of Th1 cells (2, 3). The mammalian immune response to parasitic helminths involves an increase in Th2 cytokines, aimed at eliminating the parasite (4, 5). It is intuitive to think that this increase in Th2 cytokines will also have the benefit of protecting the host from an unrelated Th1-type disease, such as Crohn's disease, a major form of inflammatory bowel disease (IBD)³ characterized as having a Th1 profile (6).

Previous studies from our laboratory have shown that infection with the tapeworm parasite, *Hymenolepis diminuta*, given both prophylactically and as a treatment, improved the abnormalities in colonic epithelial ion transport that accompany dextran sodium sulfate (DSS)-induced murine colitis (7). Other studies showed that infection with parasitic helminths can reduce the Th1 response

to an unrelated infection (8, 9) and have confirmed an anticolitic effect of infection with parasitic helminths (principally nematodes and *Schistosoma mansoni*) in murine model systems (10–12). Furthermore, some preliminary, but notable, success has been achieved by Weinstock et al. (13) in treating the histopathology and symptoms experienced by cohorts of patients with Crohn's disease with infective eggs of the porcine whipworm, *Trichuris suis*. These studies indicate the promise of helminth therapy in modulating Th1-dominated disease.

However, most of the helminths used to modulate colitis are invasive parasites that evoke varying degrees of tissue damage and pathology, and in the case of *S. mansoni* can be potentially fatal. For this reason, the current study investigated the anticolitic benefits of a more benign helminth, *H. diminuta*, in a chemically induced Th1 model of colitis. *H. diminuta* infection elicits a Th2 response from its host (14), but rather than abrasive hooks or teeth, the worm is equipped with suckers that allow attachment to the villi of the host small intestine. In addition, *H. diminuta* is not auto-infective, and its life cycle requires cyclical passage through an intermediate insect host and a definitive host, the rat. Although *H. diminuta* will chronically parasitize the rat host, it is immunologically expelled from the mouse within 12 days of infection (15–17).

Our results demonstrate that *H. diminuta* infection in the nonpermissive mouse system, both before and after exposure to dinitrobenzene sulfonic acid (DNBS), reduces the severity of the disease, but does not predispose to enhanced allergic-type hypersensitivity. Although the precise mechanism(s) of this anticolitic effect remains to be determined, it is clear that it is dependent on the immunologically mediated rejection of the parasite and, as a consequence of this, the mobilization of an IL-10 response.

Materials and Methods

Helminth infection and induction of colitis

Male BALB/c mice (7–9 wk old; Harlan Animal Suppliers; one experiment used females) were housed in filter-topped cages at McMaster University

Intestinal Disease Research Program, McMaster University, Hamilton, Ontario, Canada
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² Address correspondence and reprint requests to Dr. Derek M. McKay, Intestinal Disease Research Program, HSC-3N5C, McMaster University, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5. E-mail address: mckayd@mcmaster.ca

³ Abbreviations used in this paper: IBD, inflammatory bowel disease; DNBS, dinitrobenzene sulfonic acid; DSS, dextran sodium sulfate; EtOH, ethanol; ir., intrarectal; ΔIsc, maximum change in the net active ion transport response; KO, knockout; MPO, myeloperoxidase; TNBS, trinitrobenzene sulfonic acid.

Central Animal Facility. Mice received five infective *H. diminuta* cysticercoids by oral gavage in 100 μ l of 0.9% NaCl (15). Colitis was induced in lightly anesthetized mice by an intrarectal (ir.) injection of 3 mg of DNBS in 100 μ l of 50% ethanol (EtOH), delivered 3 cm into the colon via a polyethylene catheter (18). In the prophylactic protocol, mice were infected with *H. diminuta*, then 8 days later received DNBS ir., followed by autopsy 72 h or 7 days later. In additional experiments, mice received DNBS 1, 2, or 3 wk after *H. diminuta* infection and were autopsied 72 h after DNBS. In the treatment protocol, mice received DNBS 48 h before *H. diminuta* infection, followed by autopsy 8 days after *H. diminuta* infection. To check for worm infectivity, a segment of the jejunum at the ligament of Trietz was formalin fixed, embedded in paraffin, and stained with periodic acid-Schiff's reagent to identify goblet cells (15). In additional studies, mice were gavaged with five hot EtOH (70% for ~14 h)-killed cysticercoids, 1 mg/100 μ l PBS of adult *H. diminuta* protein Ag, or five excysted larvae (1% pepsin for 10 min, then placed in a 1% trypsin/1% sodium tauroglycocholate solution until the larvae emerged from the cyst). STAT-6 knockout (KO) mice (C57BL/6 background) were infected with *H. diminuta*, and 3 wk later received an ir. instillation of 4.5 mg of DNBS, with autopsy 72 h after DNBS treatment.

Male Sprague-Dawley rats (Harlan Animal Suppliers) were infected by oral gavage with 10 *H. diminuta* cysticercoids (15). In the chronic condition, colitis was induced 3 mo after infection by delivering 22 mg of DNBS in 100 μ l of EtOH into the colon via a 7.0-cm long catheter. In the acute condition, colitis was induced 8 days after infection. In both cases, rats were autopsied 72 h after DNBS treatment. Tissue for histology was taken at the site of DNBS instillation (i.e., 7 cm from anal verge), and a 1-cm portion of colon distal to this was snap-frozen for determination of myeloperoxidase (MPO) activity.

Controls consisted of naive animals, EtOH only-treated, *H. diminuta*-infected only, and DNBS only-treated animals. These experiments conformed to the Canadian guidelines for animal welfare and were in compliance with the regulations specified by the animal care committee at McMaster University.

Macroscopic assessment

Mice and rats were examined daily for signs of disease or ill health: wet feces-stained anal area, anal bleeding, altered behavior, weight loss, and fur ruffling. Upon autopsy, the colon was excised (ileal-cecal junction to the anus) and examined for signs of loose stool, fluid accumulation, bleeding, or macroscopic ulceration. A clinical disease score (maximum, 5) was determined based on the following criteria: >10% loss of body weight (0 or 1); wet anus, soft stool, or empty colon (0–1); anal bleeding/occult blood (0 or 1); macroscopic ulcers present (0 or 1); and death (1). DNBS-induced colitis causes colon shortening, so after excision, the colon was divided based on total length; the distal 30% was discarded, the next 30% was snap-frozen in liquid N₂ for assay of MPO activity, the next 10% was fixed in formalin for histology, and the next 10% was taken for RT-PCR analysis.

MPO assay

The presence of MPO, an enzyme found in granulocytes and often considered a marker of neutrophil infiltration, was assayed according to an established protocol (19). The MPO data are presented as units per milligram of tissue, where 1 U equals the amount of MPO required to degrade 1 μ M H₂O₂/min at room temperature.

Histological assessment

Colonic segments were fixed in 10% neutral buffered formalin, dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin wax. Sections (3 μ m thick) were collected on coded slides, stained with H&E, then independently examined by two investigators (M.M.H. and A.W.). The histology damage score was calculated on a 12-point scale: loss of architecture, 0–3; inflammatory infiltrate, 0–3; goblet cell depletion, 0 or 1; ulceration, 0 or 1; edema, 0 or 1; muscle thickening, 0–2; and presence of crypt abscesses, 0 or 1. Additional sections were stained with periodic acid-Schiff's reagent for goblet cell enumeration per five fields of view.

Colonic cytokine mRNA

Total RNA was extracted from colonic tissue samples using the TRIzol method (Invitrogen Life Technologies), and cDNA was reverse transcribed from 2 μ g of RNA. Each cDNA sample was incubated in 25 μ l of reaction containing 2 mM MgCl₂, Taq polymerase (Invitrogen Life Technologies) and the following nucleotide primers: IL-4: forward, 5'-ATG GGT CTC AAC CCC CAG CTA GT-3'; reverse, 5'-GCT CTT TAG GCT TTC CAG GAA GTC-3'; IFN- γ : forward, 5'-CAT GGC TGT TTC TGG CTG TTA

C-3'; reverse, 5'-TCG GAT GAG CTC ATT GAA TGC-3'; IL-10: forward, 5'-ATG CAG GAC TTT AAG GGT TAC TTG GGT T-3'; reverse, 5'-ATT TCG GAG AGA GGT ACA AAC CAG GTT T-3'; IL-12: forward, 5'-TCC AGG TGT CTT AGC CAG TC-3'; reverse, 5'-CAG ATA GCC CAT CAC CCT GT-3'; and TNF- α : forward, 5'-AGT CCG GGC AGG TCT ACT TT-3'; reverse, 5'-GCA CCT CAG GGA AGA GTC TG-3' (final concentration of cytokine primers, 0.3 μ M). The β -actin housekeeping gene was used as the positive control: forward, 5'-CCA GAG CAA GAG AGG TAT CC-3'; reverse, 5'-CTG TGG TGG TGA AGC TGT AG-3' (final concentration, 0.06 μ M). Primers for IL-4, IL-10, IL-12, and TNF- α were designed using the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), whereas the IFN- γ primer sequence has been published previously (10). For all primers except TNF- α , the thermal cycler was set for 38 cycles at an annealing temperature of 56°C; TNF- α was amplified over 28 cycles at an annealing temperature of 56°C. The final PCR product was then run on a 2% agarose gel containing 0.5 μ g/ml ethidium bromide, and visualized under a UV light. The densities of the bands and the ratio of the cytokine product to the β -actin band were determined.

Cytokine production

Spleens were removed in RPMI-5 medium (containing 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin sulfate, and 50 μ M 2-ME; all from Sigma-Aldrich) and mashed through a 200- μ m mesh nylon screen, the cell suspension was centrifuged, and the pelleted cells were treated with lysis buffer (0.15 M NH₄Cl, 10.0 mM KHCO₃, and 0.1 mM Na₂EDTA) to remove erythrocytes. Splenocytes were rinsed in fresh medium and plated at 5 \times 10⁶ cells/ml plus 0.5 or 2 μ g/ml Con A for 24 or 48 h. Supernatants were collected, and the levels of IL-10 and IL-12 (p40 subunit) were determined in duplicate serial dilutions using ELISA reagents (R&D Systems), with detection limits of 8 and 16 pg/ml, respectively.

Gut hypersensitivity response

Mice received five infective *H. diminuta* larvae by oral gavage at the same time as i.p. injections of *Bordetella pertussis* toxin (50 ng/mouse dissolved in PBS) and OVA (100 μ g/mouse dissolved in 10% (w/v) aluminum potassium sulfate solution) (20). Two weeks later, a single, whole-thickness segment of midjejunum was mounted in an Ussing chamber, and basal ion conduction and short-circuit current recorded under voltage clamp conditions as described previously (20). After a 20-min equilibration period, the maximum change in the net active ion transport response (Δ Isc) to OVA (100 μ g/ml), added to the serosal side of the tissue, was compared among tissues from the OVA-sensitized and *H. diminuta*-infected mice, OVA only-sensitized mice, *H. diminuta*-infected mice, and naive (nonsensitized, noninfected) mice.

Anti-IL-10 Ab administration

Using the prophylactic protocol, mice received five infective larvae of *H. diminuta*, followed by DNBS 8 days later. Three days after *H. diminuta* infection, mice received 50 μ g of an anti-IL-10 Ab (Pierce-Endogen), suitable for in vivo blocking, via i.p. injection (21). This was followed by i.p. injections of 100 μ g of anti-IL-10 Ab on day 7 after infection and an additional 50 μ g of anti-IL-10 Ab on day 9 after infection (i.e., 1 day before and 1 day after DNBS treatment). Controls received an isotype-matched irrelevant Ig (Pierce-Endogen) following the same protocol. Mice were autopsied 72 h after DNBS treatment.

Statistical analysis

Data are presented as the mean \pm SEM, where *n* is the number of mice used. Statistical comparisons were performed via one-way ANOVA, followed by post-hoc pairwise comparisons with Student's *t* test, where *p* < 0.05 was set as the level of acceptable statistical difference.

Results

Prophylactic *H. diminuta* infection blocks DNBS-induced colitis

An increase in jejunal goblet cell numbers in *H. diminuta*-infected mice confirmed the viability of the infection: control, 11 \pm 1; DNBS, 9 \pm 1; *H. diminuta*, 15 \pm 1 (*p* < 0.05 vs control); and *H. diminuta* plus DNBS, 17 \pm 2 (*p* < 0.05 vs control) goblet cells/villus crypt unit (15) (*n* = 4).

By 3 days after DNBS treatment, all mice displayed weight loss, diarrhea, and colonic ulceration; these effects of DNBS were significantly improved in mice previously infected with *H. diminuta*,

as reflected by the clinical disease scores (Fig. 1, A and B). Histological assessment revealed the expected loss of colonic architecture, goblet cell depletion, transmural ulceration, and significant inflammatory cell infiltration in colonic sections of DNBS-treated mice. Remarkably, mice that had been infected with *H. diminuta* had a histological appearance and MPO levels very similar to those of control animals (Fig. 1). Also, only those mice that received DNBS alone had significant reductions in colonic goblet cell numbers: control, 102 ± 3 ; DNBS, 74 ± 6 ($p < 0.05$ vs all other groups); and *H. diminuta* plus DNBS, 105 ± 8 goblet cells/5 fields

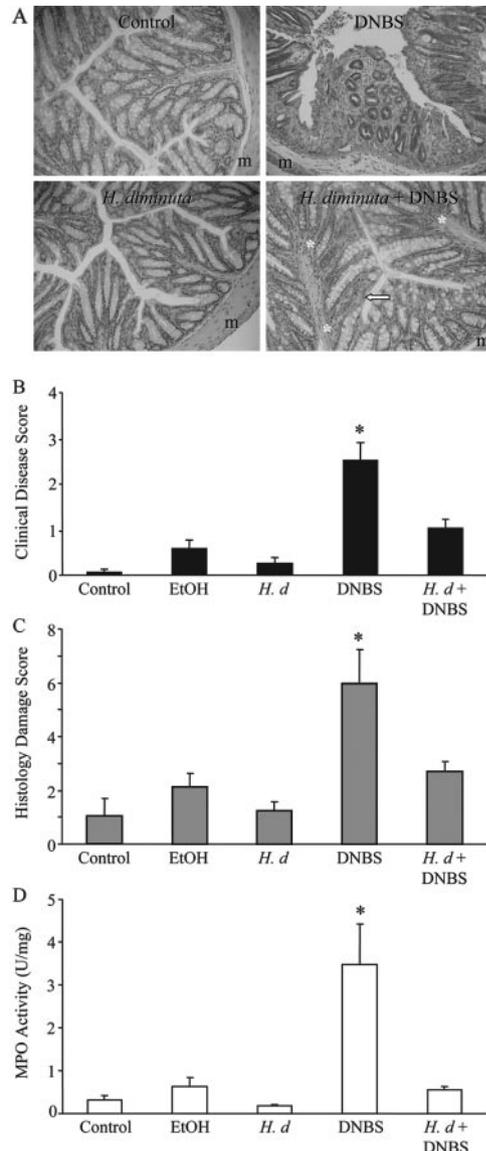


FIGURE 1. Infection with *H. diminuta* (*H. d*) 8 days (i.e., prophylactic protocol) before DNBS administration (3 mg in 100 μ l of 50% EtOH *ir.*) blocks colitis. A, Representative photomicrographs illustrating the loss of colonic architecture and frank ulceration that occur with DNBS, whereas tissues from *H. diminuta*-infected plus DNBS-treated mice have a more regular appearance, with evidence of immune cell infiltration (*) and focal areas of altered epithelium (arrow; m, muscle; original magnification, $\times 200$). Inhibition of colitis by prior *H. diminuta* infection was quantified by clinical disease scores (B), histology damage scores (C), and colonic MPO levels (D). Controls consisted of naive, age-matched male mice, mice that received 50% EtOH *ir.* (vehicle for DNBS), and *H. diminuta* only-infected mice. Mice were killed 72 h after DNBS treatment. Values are the mean \pm SEM ($n = 18$ –20 from four or five experiments). *, $p < 0.05$ compared with all other groups.

of view ($n = 6$). There was no gender bias in the anticolitic effect of *H. diminuta* infection, because female BALB/c mice were equally protected against DNBS-induced colitis ($n = 4$; data not shown).

The anticolitic benefits of *H. diminuta* infection were still evident when mice were assessed 7 days after DNBS treatment (Table I). Subsequent experiments examined the longevity of the anticolitic effect of *H. diminuta* and showed that mice were protected for at least 2 wk after infection, with the response waning by 3 wk after infection (Fig. 2). As predicted by the increased MPO values (Fig. 2B), histological examination of the colon revealed a profound inflammatory cell infiltrate, edema, and significant loss of normal structure and ulceration; tissues from DNBS-treated mice and *H. diminuta*- (3 wk postinfection) plus DNBS-treated mice were virtually identical (damage scores: control, 1.5 ± 0.5 ; DNBS, 9.5 ± 0.5 ($p < 0.05$ vs control); and *H. diminuta* plus DNBS, 8.0 ± 1.8 ($p < 0.05$ vs control) arbitrary units; $n = 4$). However, if mice received a second *H. diminuta* infection (five cysticercoids by oral gavage) 28 days after the primary infection, they were protected from DNBS-induced colitis when they were assessed 72 h after DNBS treatment (data not shown). This protective response of a secondary worm infection was not quantifiably different from that observed when mice were challenged 8 days after a primary *H. diminuta* infection with *ir.* DNBS.

Anticolitic effect of *H. diminuta* is dependent on a viable infection and subsequent rejection response

The anticolitic effect of *H. diminuta* was dependent on the larvae being infective, because killed cysticercoids, excysted larvae, and an adult worm Ag preparation did not prevent the symptoms and histopathology associated with DNBS-induced colitis (Table II). We previously showed that mice lacking the IL-4/IL-13 transcription factor, STAT6, do not expel *H. diminuta* (22). In this study we confirmed those results (i.e., all 11 STAT-6 KO mice infected with *H. diminuta* had mature worms in their small intestine at the time of autopsy); moreover, *H. diminuta*-infected STAT-6 KO mice were not protected from the colitic effects of *ir.* DNBS administration, as shown by clinical disease scores and MPO levels (Fig. 3). Also, colon from STAT-6 KO mice receiving DNBS was histologically indistinguishable from that excised from *H. diminuta*-plus DNBS-treated mice; the colonic lumen was dilated, and there was evidence of edema and a mild inflammatory infiltrate. Small focal epithelial erosions or ulcers were apparent (damage score: DNBS, 4.2 ± 0.4 ; *H. diminuta* plus DNBS, 5.0 ± 0.6 arbitrary units; $n = 3$).

Similarly, the permissive rat host does mount an immune response against *H. diminuta*, but cannot eradicate the helminth. There were no consistent significant differences in DNBS-induced colitis in uninfected rats compared with those infected 8 days previously with *H. diminuta* (i.e., parallel time course to murine studies) or 3 mo previously (i.e., patent chronic infection; Table III).

H. diminuta infection alters the cytokine profile in colitic animals

RT-PCR analysis of colonic tissue excised 72 h after DNBS treatment revealed an increase in mRNA for TNF- α (Fig. 4A). Assessment of IFN- γ and IL-12 mRNA by RT-PCR revealed no significant increase compared with controls (data not shown), nor was there any appreciable change in IL-10 RT-PCR product from tissues excised from the colon 72 h after DNBS treatment (Fig. 4C), and IL-4 mRNA was not detected (Fig. 4B). In contrast, tissues from *H. diminuta* only-infected mice had increased IL-4 and IL-10 mRNA; these findings should be quantified by real-time PCR, but do confirm a Th2 bias in the infected mice. Similarly, *H. diminuta*

Table I. The anticolitic effects of *H. diminuta* infection are still evident at 7 days after DNBS treatment^a

Treatment	Weight Change (g)	MPO (U/mg)	Colon (mm)	Clinical Disease Score	Histology Damage Score
Control	0.8 ± 0.3	0.3 ± 0.2	109 ± 1	0 ± 0	0.5 ± 0.2
<i>H. diminuta</i>	0.6 ± 0.2	0.2 ± 0.1	108 ± 2	0.2 ± 0.1	0.8 ± 0.3
DNBS (3 mg ir.)	-1.1 ± 0.9 ^b	3.4 ± 1.1 ^b	75 ± 4 ^b	2.3 ± 0.7 ^b	5.5 ± 1.9 ^b
<i>H. diminuta</i> + DNBS	-0.6 ± 0.3	0.9 ± 0.2	99 ± 0.3 ^c	0.6 ± 0.1	2.8 ± 0.5 ^c

^a Male BALB/c mice were used. Values are the mean ± SEM ($n = 4$).

^b $p < 0.05$ compared with control.

^c $p < 0.05$ compared with DNBS and control groups.

infection 8 days before DNBS administration resulted in increased colonic expression of IL-4 and IL-10 mRNA compared with DNBS only (72 h after DNBS treatment (Fig. 4)). The pattern of cytokine mRNAs 7 days after DNBS was variable, except that tissues from *H. diminuta*- plus DNBS-treated mice had greater levels of IL-10 than tissue from DNBS only-treated mice (Fig. 4D). In contrast, RT-PCR products from colonic extracts of DNBS-treated STAT-6 KO mice with or without *H. diminuta* or from DNBS-treated BALB/c mice with or without *H. diminuta* infection 3 wk previously were neither significantly elevated nor different between the group comparisons ($n = 3$ and 4, respectively; data not shown). Background levels of RT-PCR IL-10 mRNA product in these latter two studies were similar to those in naive control BALB/c mice (see Fig. 4).

Spleen cells from control, 50% EtOH-treated, or DNBS only-treated mice stimulated in vitro with low dose Con A (i.e., 0.5 μ g/ml) for 24 h showed negligible IL-10 production, whereas splenocytes from *H. diminuta*-infected mice with or without DNBS treatment produced small, but readily detectable, amounts

of IL-10 (Fig. 5A). The magnitude of this effect was increased when spleen cells were cultured for 48 h with 2 μ g/ml Con A (Fig. 5B). Very similar cytokine profiles were observed from Con A-stimulated splenocytes 7 days after DNBS treatment with or without *H. diminuta* infection: DNBS, 350 ± 40; *H. diminuta* plus DNBS, 1070 ± 100 pg/ml IL-10 ($n = 4$; $p < 0.005$). However, there were no statistically significant differences in Con A (2 μ g/ml, 48 h)-induced IL-10 from spleen cells from DNBS-treated STAT6-KO mice with or without *H. diminuta* infection (DNBS, 240 ± 21; *H. diminuta* plus DNBS, 277 ± 113 pg/ml IL-10; $n = 3$) or splenocytes from BALB/c mice given DNBS 3 wk after *H. diminuta* infection (naive, 163 ± 4; DNBS, 84 ± 6; *H. diminuta* plus DNBS, 100 ± 27 pg/ml IL-10; $n = 4$). In all experiments, nonstimulated spleen cells produced minimal amounts of IL-10 (<50 pg/ml).

In contrast to the IL-10 data, IL-12 (p40 subunit) production in response to Con A was only significantly increased in splenocytes from EtOH- or DNBS only-treated mice (Fig. 5C). Similarly, *H. diminuta* infection resulted in reduced stimulated IL-12 when splenocytes were challenged in vitro 7 days after DNBS treatment: DNBS, 290 ± 17; *H. diminuta* plus DNBS, 202 ± 21 pg/ml IL-12 (p40 subunit; $n = 4$; $p < 0.02$).

IL-10 neutralization interferes with the anticolitic benefit of *H. diminuta* infection

Focusing on the potential anti-inflammatory nature of IL-10, mice infected with *H. diminuta* were treated with an anti-IL-10 Ab regimen (a total of 200 μ g given i.p. as three separate injections), in which injections 2 and 3 were performed 1 day before and 1 day after ir. DNBS. In time-matched control mice, DNBS elicited the expected colitis, which was suppressed by prior *H. diminuta* infection. However, mice treated with DNBS, *H. diminuta*, and anti-IL-10 Ab developed colitis that was not appreciably different from that in DNBS only-treated mice (Fig. 6).

H. diminuta infection does not enhance murine gut hypersensitivity reactions

Jejunal segments from mice that were sensitized to OVA and infected with *H. diminuta* displayed no significant enhancement of Ag-induced, short-circuit current responses when challenged in vitro with OVA compared with intestine from OVA only-sensitized mice: OVA-sensitized Δ Isc, 23.7 ± 8.2; OVA-sensitized plus *H. diminuta*-infected Δ Isc, 25.4 ± 4.3 μ A/cm² (mean ± SEM; $n = 8$ mice; $n = 2$ tissues/mouse). Ussing chambered tissues from naive and *H. diminuta* only-infected mice were completely unresponsive to OVA challenge.

Infection with *H. diminuta* after DNBS treatment hastens recovery from colitis

Treatment with viable *H. diminuta* cysticercoids 48 h after administration of DNBS significantly enhanced disease recovery, as

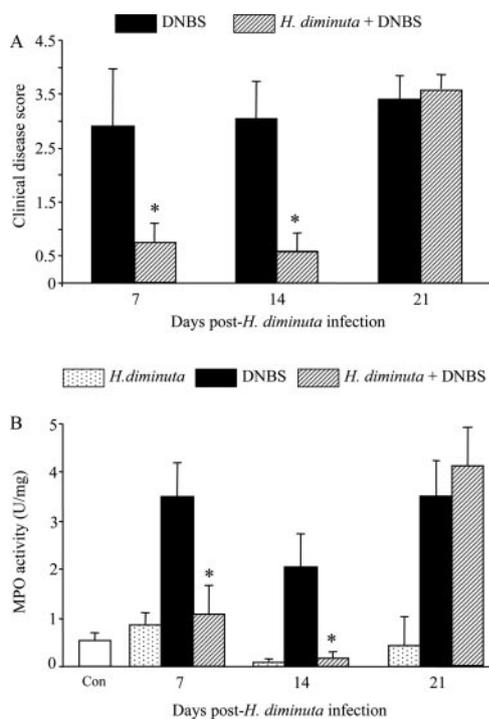


FIGURE 2. The protective effect of *H. diminuta* is not apparent 3 wk after infection, as indicated by the clinical disease scores (A) and colonic MPO levels (B). Values are the mean ± SEM ($n = 4$ –9 from three experiments). *, $p < 0.05$ compared with DNBS only or 72 h after treatment with 3 mg of DNBS ir. *H.d.*, *H. diminuta* (5 cysticercoids). Controls are naive noninfected mice, time-matched to the 21 days postinfected mice. A, Clinical scores for noninfected and *H. diminuta* only-infected mice were <0.5 U ($n = 4$).

Table II. *The anticolic effect of H. diminuta is dependent on a viable infection^a*

Treatment	Weight Change (g)	MPO (U/mg)	Colon (mm)	Clinical Disease Score
Control	0.7 ± 0.2	0.2 ± 0.1	110 ± 1	0.0 ± 0.0
50% EtOH	0.7 ± 0.4	0.2 ± 0.1	106 ± 2	0.2 ± 0.1
Five viable <i>H. diminuta</i> cysts	0.6 ± 0.2	0.1 ± 0.1	100 ± 3	0.0 ± 0.0
Five killed <i>H. diminuta</i> cysts	0.1 ± 0.1	0.9 ± 0.1	108 ± 6	0.2 ± 0.1
Adult worm Ag (100 µg)	0.8 ± 0.3	0.7 ± 0.1	111 ± 4	0.1 ± 0.1
DNBS (3 mg ir.)	-2.5 ± 0.4 ^b	4.1 ± 1.3 ^b	75 ± 1 ^b	2.3 ± 0.3 ^b
Viable <i>H. diminuta</i> cysts + DNBS	-0.4 ± 0.2	0.6 ± 0.1	102 ± 3	0.5 ± 0.2
Excysted ^c <i>H. diminuta</i> + DNBS	-3.4 ± 1.2 ^b	10.3 ± 4.0 ^b	83 ± 5 ^b	2.4 ± 0.5 ^b
Killed <i>H. diminuta</i> cysts + DNBS	-4.6 ± 1.6 ^b	5.1 ± 2.6 ^b	85 ± 10 ^b	1.3 ± 1.2 ^b
Adult worm Ag + DNBS	-1.9 ± 1.2 ^b	9.9 ± 6.3 ^b	85 ± 7 ^b	1.9 ± 0.8 ^b

^a Male BALB/c mice were used. Values are the mean ± SEM ($n = 7$ from two experiments). Mice were infected 8 days before DNBS and killed 72 h after DNBS, cysts, cysticercoids delivered intragastrically.

^b $p < 0.05$ compared with controls.

^c Five excysted worms were gavaged into the stomach.

shown by animal weight gain (Fig. 7A) and reduced clinical disease scores and colonic MPO levels assessed 7 days after infection (i.e., 9 days post-DNBS; Fig. 7B).

Discussion

The concept of a beneficial parasite is not new (23), and from our current understanding of cytokine signaling, it has been repackaged as the concept of immune distraction, essentially promoting an immune response that will dampen or down-regulate the immunopathological events. However, when using biological organisms, the situation is likely to be more complicated than this. We previously showed that infection with *H. diminuta* alleviated the electrophysiological abnormalities that characterize murine DSS-induced colitis (7). Using DNBS to evoke what is considered a Th1-driven colitis (24), the present study shows that *H. diminuta* infection in a prophylactic regimen almost abolished the colitis, as assessed by clinical parameters, histological assessment, and biochemical markers (i.e., MPO).

One interpretation of the data is that *H. diminuta* ameliorates colitis via the release of a particular factor, and indeed, like other parasitic helminths (25), *H. diminuta* Ag can block mitogen-driven T cell responses in vitro (26). However, in the nonpermissive murine system, the helminth never establishes or attains a significant biomass (15). Moreover, our studies indicate that the anticolic effect is not a simple Ag-driven event; rather, it requires a viable infection and a subsequent rejection response, because DNBS-induced colitis in rats and STAT6 KO mice was unaffected by *H.*

diminuta, which establishes in these animals. These data are in accordance with other murine model systems and human studies in which the anticolic effect is correlated with worm expulsion (10, 13). Additionally, it is important to highlight that the protective effect of the helminth wanes and that mice challenged with DNBS 3 wk after infection were not protected. However, from a therapeutic perspective, secondary infection with *H. diminuta* was also associated with an anticolic effect, and these data are reminiscent of those reported by Weinstock et al. (13), who showed that there was benefit of repeated porcine whipworm infection in patients with Crohn's disease.

The fact that *H. diminuta* never enters the colon (except when being flushed from the body) indicates small-to-large intestine communication and therefore likely involvement of a neuroendocrine or immune-derived factor. Enteric neuroendocrine responses to parasitic helminths have been described (27), but based on the starting paradigm for this work, we focused on cytokine responses. Initial RT-PCR analysis of colonic extracts obtained 3 days after DNBS treatment revealed three pertinent findings: 1) there was increased TNF- α mRNA in all groups compared with controls; 2) IL-4 was undetectable in tissue from DNBS-only treated animals, but was detected in DNBS-treated plus *H. diminuta*-infected mice and was significantly increased in *H. diminuta* only-infected mice compared with the other groups; and 3) IL-10 was increased in extracts from *H. diminuta*-infected with or without DNBS-treated mice (IL-10 mRNA was still elevated 7 days after DNBS in tissue from the coinfecting mice). At 3 days after DNBS treatment, we did not detect any consistent changes in colonic levels of IL-12 or IFN- γ by RT-PCR. In comparison with the mRNA analysis, in vitro Con A stimulation produced increased IL-10 output from splenocytes retrieved from mice previously infected with *H. diminuta* (with or without DNBS) and increased IL-12 from cells obtained from DNBS-treated mice, but not those from coinfecting mice.

The data confirm an earlier report that *H. diminuta* infection does provoke a Th2-type response (14). In terms of the anticolic effect of this infection, a number of points should be highlighted. First, the increased IL-4 mRNA is consistent with other investigations that have shown that various anticolic strategies (28), including that of helminth infection or exposure to schistosome eggs, were accompanied by an IL-4 response (10, 12, 29). In the current study we have not attempted to block IL-4 and determine its contribution, if any, to the anticolic effect, but this is an approach that should be pursued. Similarly, the induction of an IL-12 response is characteristic of the DNBS (or trinitrobenzene sulfonic acid (TNBS)) model of colitis and the reduced IL-12 production

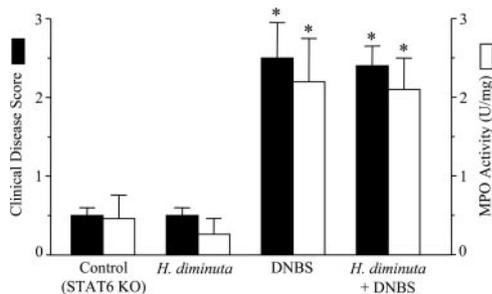


FIGURE 3. Infection of STAT-6 KO with *H. diminuta* 21 days before DNBS administration (3 mg/100 µl 50% EtOH ir.) did not affect colitis, as assessed by clinical disease score or colonic MPO levels. Controls consisted of naive, age-matched male mice and *H. diminuta* only-infected mice. Mice were killed 72 h after DNBS treatment. Values are the mean ± SEM ($n = 4$ for control and *H. diminuta* only-treated mice; $n = 7$ for DNBS-treated and DNBS-treated plus *H. diminuta*-infected mice from two experiments). *, $p < 0.05$ compared with controls.

Table III. *H. diminuta* infection in the permissive rat host does not affect DNBS-induced colitis^a

Treatment	Weight Change (g)	MPO (U/mg)	Clinical Disease Score	Histology Damage Score
Chronic				
Control	1.1 ± 8.8	0.6 ± 0.6	0.3 ± 0.3	2.0 ± 1.0
<i>H. diminuta</i>	2.0 ± 10.0	0.9 ± 0.1	0.0 ± 0.0	2.8 ± 0.8
DNBS (22 mg ir.)	-19.0 ± 7.9 ^b	5.6 ± 6.9 ^b	3.8 ± 0.4 ^b	6.0 ± 2.7 ^b
DNBS + <i>H. diminuta</i>	-10.4 ± 8.2 ^b	7.6 ± 8.0 ^b	2.4 ± 0.9 ^b	4.1 ± 3.3 ^b
Acute				
Control	2.4 ± 1.3	0.4 ± 0.2	0.0 ± 0.0	ND
<i>H. diminuta</i>	2.3 ± 1.1	1.2 ± 0.2	0.0 ± 0.0	ND
DNBS (22 mg ir.)	-9.8 ± 2.0 ^b	2.3 ± 0.2 ^b	2.4 ± 0.2 ^b	ND
DNBS + <i>H. diminuta</i>	-6.2 ± 0.2 ^b	2.2 ± 0.18 ^b	2.0 ± 0.2 ^b	ND

^a Values are the mean ± SEM ($n = 7$ from three experiments in the chronic study and $n = 3$ in the acute study). Rats received 10 *H. diminuta* cysticercoids, DNBS was administered 8 days (acute) or 3 mo (chronic) after infection, and rats were assessed 72 h after DNBS treatment.

^b $p < 0.05$ compared with controls.

from mitogen-stimulated spleen cells from DNBS-treated plus *H. diminuta*-infected mice is consistent with data on the anticolitic effect of abrogating IL-12 activity in this model (30–32). In assessing prototypic Th1 responses, we were surprised by the lack of an increased IFN- γ mRNA response 3 days after DNBS treatment, yet others have shown that elevated IFN- γ mRNA occurs only 1–2 wk after DNBS treatment (33). Indeed IFN- γ signaling is dispens-

able in this model of colitis (30, 31). However, it should also be noted that helminth infection has been associated with reduced IFN- γ mRNA and splenocyte IFN- γ production 7 days after TNBS treatment (10, 12). TNF- α has been implicated in the response to helminth infection (34) and is considered a major player in murine models of colitis (35) and human inflammatory bowel disease (36). Our RT-PCR findings are in accordance with these data. However, given the anticolitic effect of *H. diminuta*, this suggests that the infection, although not reducing the increased TNF- α mRNA (and presumably protein), is blocking or antagonizing the effects of TNF- α .

There is increasing awareness that any benefit of parasitic helminth infection in colitis may extend beyond a simple skewing

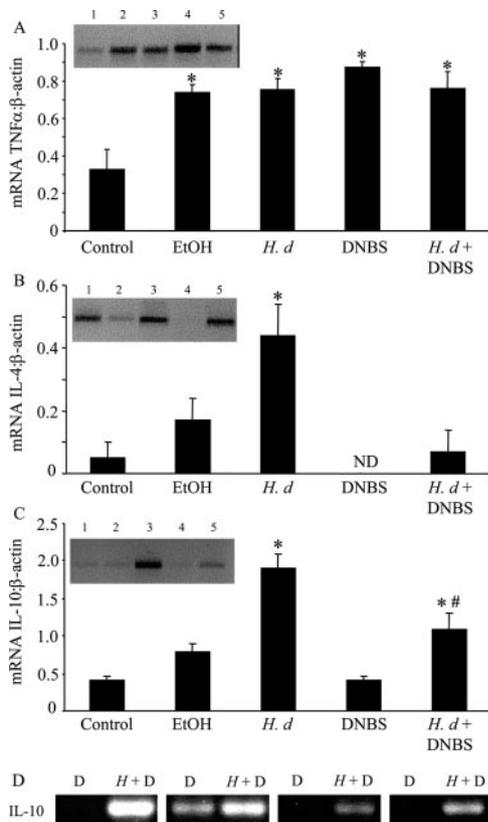


FIGURE 4. Infection with *H. diminuta* (*H. d*) 8 days before DNBS treatment (3 mg/100 μ l 50% EtOH ir.) alters colonic TNF- α (A), IL-4 (B), and IL-10 (C) mRNA levels compared with DNBS only treatment. Values are the mean ± SEM ($n = 5$ from two experiments). *, $p < 0.05$ compared with all other groups; #, $p < 0.05$ compared with *H. d* only. ND, not detected. Cytokine mRNA was compared with β -actin as a housekeeper control gene. *Insets*, Representative RT-PCR gels (1, control; 2, EtOH; 3, *H. d*; 4, DNBS; 5, *H. d* plus DNBS). *D*, RT-PCR mRNA products from colonic tissues from four separate mice given DNBS with or without *H. diminuta* infection (prophylactic protocol) and autopsied 7 days after DNBS treatment.

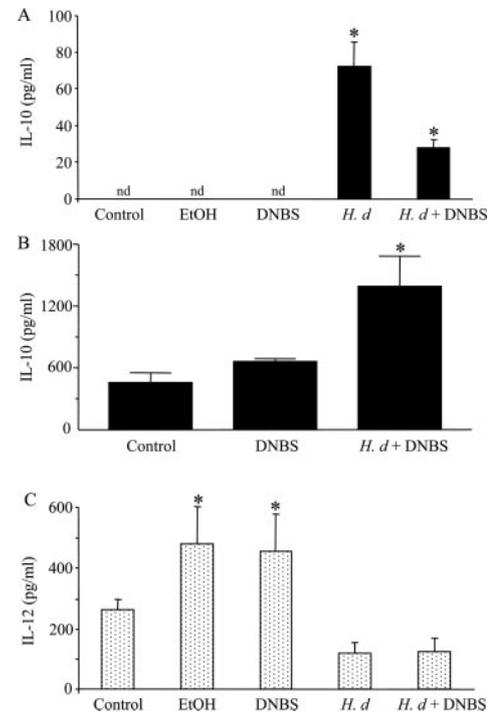


FIGURE 5. Murine splenocytes isolated and challenged in vitro with Con A (0.5 μ g/ml for 24 h (A and C) or 2 μ g/ml (B)) show enhanced production of IL-10 production in *H. diminuta* (*H. d*)-infected mice and an increase in IL-12 (p40 subunit) from spleen cells from DNBS-treated mice that was reduced by prior *H. diminuta* infection. Values are the mean ± SEM ($n = 3$ –5 mice/group). *, $p < 0.05$ compared with control. nd, not detected. Five *H. diminuta* cysticercoids were given 8 days before DNBS (3 mg/100 μ l 50% EtOH ir.), and mice were autopsied 72 h after DNBS treatment. Data are from one representative experiment of two performed.

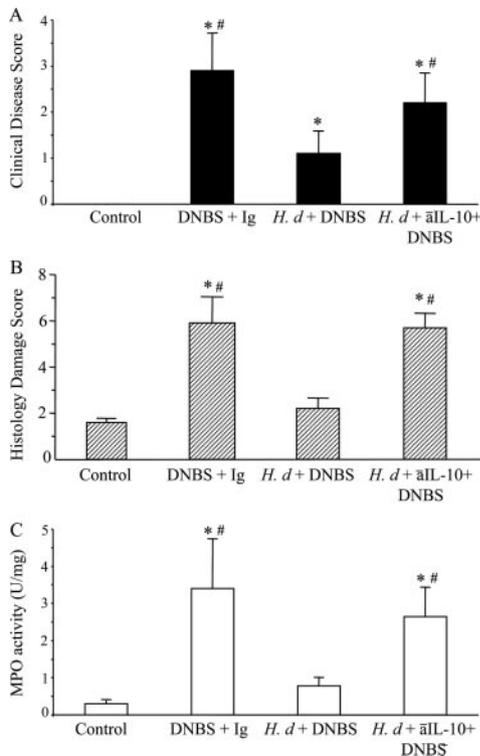


FIGURE 6. Anti-IL-10 Ab (total, 200 $\mu\text{g}/\text{mouse}$ i.p.) reversed the anticolitic effects of *H. diminuta* (*H. d*) as assessed by clinical disease score (A), histology damage score (B), and colonic MPO activity (C). DNBS (3 mg/100 μl 50% EtOH ir.) was administered 8 days after *H. d* infection. Values are the mean \pm SEM ($n = 5$). *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with *H. d*-infected plus DNBS-treated mice. Ig, isotype matched Ig at 200 $\mu\text{g}/\text{mouse}$.

away from Th1-type responses to the generation of a more general immunosuppressed or immunoregulatory environment in which IL-10 might play a key role (37). The induction of a local and a systemic IL-10 response in the current model combined with the recognized anticolitic effect of IL-10 in the TNBS/DNBS models (38, 39) led us to consider a role for this cytokine in the present study. Administering three injections of neutralizing IL-10 Ab at intervals during the course of *H. diminuta* infection and subsequent DNBS challenge virtually abolished the anticolitic effect exerted by the worm. These findings are complemented by the lack of an IL-10 response in *H. diminuta*-infected STAT6 KO mice and in BALB/c mice 3 wk after infection; in both instances, DNBS-induced colitis was not affected by worm infection. Thus, the mobilization of IL-10 in response to *H. diminuta* infection has the added benefit of being capable of reducing the severity of a subsequent procolitic stimulus. In terms of precisely defining the effect of IL-10, it will be necessary to determine the source of the IL-10, which could be T cells, regulatory T cells, B cells (40), or even the epithelium (41). However, recent findings show that the nematode parasite, *Heligmosomoides polygyrus*, inhibits established colitis in the IL-10-deficient mouse (11); clearly, the effector pathway in that model does not involve IL-10. Thus, in addition to IL-10, the anticolitic effect of helminths may include roles for increased IL-4, blockade of IL-12 and TNF- α production or function, induction of other regulatory molecules (e.g., TGF- β (39)), and a plethora of other possibilities. Subsequent studies need to assess these issues in the current and complementary model systems.

As an anticolitic therapy, it would be remiss to overlook studies that suggest that rIL-10 treatment of Crohn's disease has not ful-

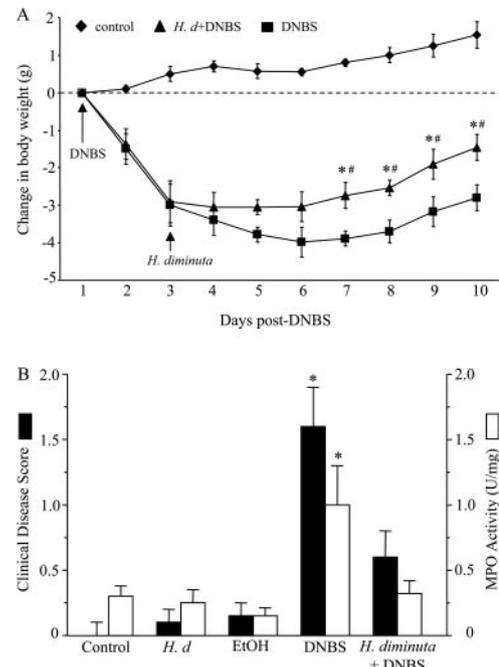


FIGURE 7. Male BALB/c mice infected with five cystercoids of *H. diminuta* (*H. d*) 48 h after DNBS (3 mg/100 μl 50% EtOH ir.) display an accelerated recovery of body weight (A) and evidence of less severe disease as measured by clinical disease score and colonic MPO levels 10 days after DNBS treatment. Values are the mean \pm SEM ($n = 8$ from two experiments). *, $p < 0.05$ vs control; #, $p < 0.05$ vs DNBS-treated mice. Clinical scores and MPO levels are low due to natural recovery from the DNBS insult (compare with Fig. 1).

filled the promise predicted by analyses of animal models of colitis (42). However, rather than abandon the potential value of IL-10 as a treatment, the substantial data from animal models should be viewed as proof-of-concept of its ability to ameliorate colitis; thus, it may be that the targeting or administration regimen of rIL-10 in the human studies is responsible in part for the limited efficacy of these formulations (43).

Finally, this investigation has generated two other pieces of data salient to the consideration of helminth therapy. First, when used as a therapy, rather than a prophylactic, *H. diminuta* enhanced the recovery phase after DNBS challenge, and with the exception of our analysis of DSS-induced colitis and current clinical investigations with *Trichuris suis* (13), this is, to our knowledge, the only other study in which a parasitic helminth infection has been used as a therapy. Second, the concern with promoting helminth-driven Th2 responses is the theoretical possibility of predisposing the individual to ectopic, allergic-type diseases, although epidemiological analyses do not support this (44). Indeed, there are data to suggest that helminth infections might protect against such disorders (45) and, perhaps paradoxically, against ulcerative colitis also (13), although there is dispute as to whether ulcerative colitis is solely a Th2-type disorder (46). We found that *H. diminuta* infection did not enhance enteric hypersensitivity responses to rechallenge with a sensitizing Ag challenge.

There is increasing use of biologicals as anti-inflammatory or immunomodulatory modalities. This is being supplemented by assessment of the usefulness and mechanisms of actions of neutralizing antibodies and probiotics, and one could suggest that helminths represent a special category of the latter group. The data presented in this study show that rejection of a viable *H. diminuta* infection from mice is both a prophylactic and a treatment for DNBS-induced murine colitis and is mediated in large part by IL-10, with

no concomitant predisposition of the animals to increased gut hyper-sensitivity. Thus, although parasitism is by definition a malevolent condition, a more tolerant view may see the strategic use of parasitic helminths (that cause minimal pathology themselves) as beneficial to a cohort of patients with IBD for which traditional therapies are ineffective. Moreover, a comprehensive analysis of parasitic helminths in models of colitis has the potential to elucidate novel anti-inflammatory or restitution mechanisms.

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Disclosures

The authors have no financial conflict of interest.

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