Infection with a Helminth Parasite Prevents Experimental Colitis via a Macrophage-Mediated Mechanism[1]

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The propensity of a range of parasitic helminths to stimulate a Th2 or regulatory cell-biased response has been proposed to reduce the severity of experimental inflammatory bowel disease. We examined whether infection with *Schistosoma mansoni*, a trematode parasite, altered the susceptibility of mice to colitis induced by dextran sodium sulfate (DSS). Mice infected with schistosome worms were refractory to DSS-induced colitis. Egg-laying schistosome infections or injection of eggs did not render mice resistant to colitis induced by DSS. Schistosome worm infections prevent colitis by a novel mechanism dependent on macrophages, and not by simple modulation of Th2 responses, or via induction of regulatory $CD4^+$ or $CD25^+$ cells, IL-10, or TGF-β. Infected mice had marked infiltration of macrophages ($F4/80^+CD11b^+CD11c^-$) into the colon lamina propria and protection from DSS-induced colitis was shown to be macrophage dependent. Resistance from colitis was not due to alternatively activated macrophages. Transfer of colon lamina propria $F4/80^+$ macrophages isolated from worm-infected mice induced significant protection from colitis in recipient mice treated with DSS. Therefore, we propose a new mechanism whereby a parasitic worm suppresses DSS-induced colitis via a novel colon-infiltrating macrophage population. *The Journal of Immunology,* **2007, 178: 4557–4566.**

The incidence of inflammatory bowel disease (IBD)[3] is increasing in developed countries. IBD is the collective name for a family of chronic, relapsing inflammatory conditions clinically subdivided into Crohn's disease (CD) and ulcerative colitis (UC). The reasons for the increased incidence of IBD in developed societies are complex and multifactorial, but are associated with a breakdown in homeostasis between gut Ags and immunity (1, 2). One hypothesis to explain this disruption of the gut-immunity balance that may lead to IBD is the reduced presence of parasitic helminth worm infections in modern societies (3).

In experimental colitis models in rodents, it has been shown that infection with a number of parasitic worms can reduce the severity of colitis (4–8). Although not fully elucidated, studies to date propose that the propensity of parasitic worm infections to induce Th2-like responses, with IL-4, IL-5, and IL-13 release and/or regulatory cytokines (IL-10, TGF-β) and regulatory ($CD4^+CD25^+$ cells) cellular responses may mediate the reduced colon inflammation. These experimental animal studies have now been extended to worm infections of humans. UC or CD patients that were infected with *Trichuris suis*, a worm that infects pigs, were shown to have reduced disease activity indices (9, 10). Additionally, infection of CD patients with the human hookworm *Necator americanus* has also been shown to have therapeutic potential (11). Although there are no empirical data on the mechanism whereby worm infections of human IBD patients ameliorate disease, it is predicted that protection is mediated by the same induction of Th2 cell or regulatory cell responses as seen in worm-infected experimental animals (12).

The parasitic helminth *Schistosoma mansoni* infects people in tropical countries in Africa and South America. *S. mansoni* has been shown in a number of studies to be a potent modulator of unrelated diseases (13). The adult worms of *S. mansoni* reside in the mesenteric vasculature, where they produce eggs that have to pass through the intestinal wall to be excreted in the feces. The excretion of *S. mansoni* eggs is an immune-mediated process and while regulated inflammation is necessary for the migration of eggs through the intestine, excessive tissue inflammation is suppressed by the parasite to prevent eggs perforating the intestine, resulting in sepsis that would kill the host (14, 15). Following experimental *S. mansoni* infection in mice there is an acute phase, 7–8 wk postinfection, coinciding with egg laying and the stimulation of marked type 2 immunity. Infection then progresses to a chronic phase, after 14–16 wk, which is characterized by down-modulation of the immune response. Consistent with schistosomes regulating inflammation in the intestines, infection of rats with *S. mansoni* has been shown to ameliorate hapten-induced colitis (6). It has been demonstrated that the schistosome egg stage is also able to suppress intestinal inflammation induced in an experimental model of colitis, with injection of mice with eggs ameliorating hapten-induced colitis via suppression of pathogenic Th1 cells (16). Indeed, schistosome egg-derived glycans have been shown to have potent suppressive activity against $CD4^+$ cells (17, 18). Previously, we have shown that the worm stage of *S. mansoni* infection also modulates the immune response (19–21). These earlier studies involved an experimental model where mice were infected with only the male *S. mansoni* cercariae resulting in an infection

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Received for publication October 16, 2006. Accepted for publication January 18, 2007.

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[1] This work was supported by The Wellcome Trust, Science Foundation Ireland, and the Higher Education Authority Programme for Research in Third Level Institutions.

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[3] Abbreviations used in this paper: IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; DSS, dextran sodium sulfate; DAI, disease activity index; eGFP, enhanced GFP; LP, lamina propria; iNOS, inducible NO synthase; nor-NOHA, N^{ω}-hydroxy-nor-L-of arginine.

with no eggs produced due to the absence of female worms. Mice infected with male schistosome worms are rendered refractory to anaphylaxis and lung inflammation, via the regulatory cytokine IL-10 (20, 21). In the current study, we investigated whether infection with *S. mansoni* worms can also protect mice from experimental colitis.

Materials and Methods

Mice

BALB/c and C57BL/6 strain mice were from the Bioresources Unit (Trinity College, Dublin, Ireland). Double IL-4- and IL-13-deficient (IL-4/13$^{-/-}$) mice on a BALB/c background were bred in-house. RAG-1$^{-/-}$ on a BALB/c background were purchased from The Jackson Laboratory and bred in-house. Mice expressing enhanced GFP (eGFP) on a β-actin promoter were provided by Prof. P. Humphries (Genetics, Trinity College, Dublin, Ireland) and bred in-house. Mice were housed in individually ventilated and filtered cages under positive pressure (Tecniplast). Food and water were supplied ad libitum. Sentinel mice were screened to ensure specific pathogen-free status. All animal experiments were performed in compliance with Irish Department of Health and Children regulations and approved by the Trinity College Bioresources Ethical Review Board.

Parasitology

A Puerto Rican strain of *S. mansoni* was maintained by passage in albino *Biomphalaria glabrata* snails and outbred CD-1 strain mice. Female 6- to 8-wk-old mice were infected percutaneously with 60 male cercariae for worm-only infections and 30 male and female cercariae for egg-laying infections. Infected mice were treated with dextran sodium sulfate (DSS) to induce colitis 7–8 wk postinfection. This time point was chosen to coincide with the acute phase of infection which is associated with peak immune responses. Infected mice were also treated with DSS during the chronic, 14–16 wk postinfection, when immune responses are down-modulated. The sex of cercariae shed from individual snails was determined by PCR as described (19). To induce a schistosome Th2 response, mice were injected i.p. with 5000 *S. mansoni* eggs weekly for 5 wk. Induction of colitis commenced 1 day after the final injection of eggs.

Induction of DSS-induced colitis

DSS (35–50,000 kDa; MP Biomedicals) was dissolved in the drinking water of mice. Fresh DSS solution was provided every second day. BALB/c mice were exposed to 5% DSS for 7 days. C57BL/6 mice received 3% DSS for 5 days, followed by normal drinking water for 3 days. The mice were checked each day for morbidity and weight was recorded. Induction of colitis was determined by weight loss, fecal blood, and, upon autopsy, length of colon. Blood in feces was detected using a Hemdetect occult blood detection kit (Dipro).

To quantify induction of colitis, a disease activity index (DAI) was determined based on previous studies of DSS-induced colitis (22). DAI was calculated for each mouse daily based on body weight loss, occult blood, and stool consistency/diarrhea. A score of 1–4 was given for each parameter, with a maximum DAI score of 12. Score 0: no weight loss, normal stool, no blood; score 1: 1–3% weight loss; score 2: 3–6% weight loss, loose stool, blood visible in stool; score 3: 6–9% weight loss; score 4: <9% weight loss, diarrhea, gross breeding. Loose stool was defined as the formation of a stool that readily becomes paste upon handling. Diarrhea was defined as no stool formation. Gross bleeding was defined as fresh blood on fur around the anus with extensive blood in the stool.

Colon histology

At autopsy, the length of the colon was measured and a 1-cm section of colon was fixed in 10% formaldehyde-saline. H&E-stained sections were graded based on a scoring system modified from a previous study (23). Histology scoring was performed in a blinded fashion. A combined score of inflammatory cell infiltration and tissue damage was determined as follows: cell infiltration: score 0, occasional inflammatory cells in the lamina propria (LP); 1, increased infiltrate in the LP predominantly at the base of crypts; 2, confluence of inflammatory infiltrate extending into the mucosa; 3, transmural extension of infiltrate. Tissue damage: score 0, no mucosal damage; 1, partial (up to 50%) loss of crypts in large areas; 2, partial to total 50–100% loss of crypts in large areas, epithelium intact; 3, total loss of crypts in large areas and epithelium lost.

mAbs and cell depletions

Anti-IL-10R (1B1.3a), anti-TGF-β (1D11.16.8), anti-CD25 (PC61 5.3), and a control mAb (1B7.11; anti-trinitrophenyl) were all purchased from American Type Culture Collection. The anti-IL-10R mAb was obtained subject to an Material Transfer Agreement from DNAX Research Institute. Anti-CD4 (YTS191) was provided by Prof. A. Cooke (University of Cambridge, Cambridge, U.K.) and Prof. H. Waldman (University of Oxford, Oxford, U.K.). Hybridomas were grown and mAb isolated as described (20). All Abs were tested for endotoxin contamination and confirmed to have <0.5 endotoxin units/mg (chromogenic LAL; BioWhittaker). All mAbs were administered on each of days 0, 3, and 5 of DSS treatment. On these days, each mouse received an i.p. injection of 250 μg of mAb per mouse. In all individual cell depletions experiments, the mAb-treated mice were checked by flow cytometry to ensure efficient (>95% of CD4$^+$ or CD25$^+$ spleen cells) depletion of the target cells.

Macrophages were depleted by the treatment of mice with liposomes containing dichloromethylene bisphosphonate (clodronate-liposomes), prepared as described (24). Clodronate was a gift from Roche Diagnostics. Mice were injected with 0.2 ml of a suspension of clodronate-liposomes or PBS-liposomes, injected i.v. on the day before exposure to DSS, and on days 1 and 3 during DSS treatment. This regimen was first confirmed to deplete >90% of F4/80$^+$ macrophages, detected by flow cytometry, in the mesenteric lymph nodes and colon LP. Arginase activity was inhibited by daily administration (i.p.) of 100 μg of N^{ω}-hydroxy-nor-L-of arginine (nor-NOHA; Calbiochem) per mouse (25).

Flow cytometry

LP cell surface phenotyping was analyzed using anti-F4/80 (clone F4/80), anti-CD11c (clone HL3), and anti-CD11b (Mac-1; clone M1/70). Cells (1×10^6) were washed three times with FACS buffer (PBS with 2% FCS, 0.05% sodium azide). LP macrophages were identified as F4/80$^+$ cells that were CD11b$^+$ and CD11c$^-$ (26). Fluorochrome-conjugated Abs were diluted in FACS buffer to 1 μg/ml. The washed cells were resuspended in 100 μl of the diluted Ab and incubated for 45 min in the dark on ice. The cells were then washed three times. In eGFP$^+$ LP macrophage transfer experiments, the number of eGFP$^+$F4/80$^+$ cells recovered in the LP of recipient mice were determined by flow cytometry analysis. Phagocytosis of dextran by LP macrophages was assessed using FITC-conjugated dextran (FITC-dextran). Briefly, 0.5 mg/ml FITC-dextran (m.w. = 40,000; Sigma-Aldrich) was incubated with LP cells for 30 min at 37°C; uptake was stopped by washing the cells in ice-cold FACS buffer. Control for dextran internalization was established by incubating cells with FITC-dextran on ice. Data were acquired using a FACSCalibur flow cytometry machine and analyzed using CellQuest software (BD Biosciences).

Isolation of colon LP cells

The colons were excised and cut longitudinally and washed three to five times with 30 ml of cold Ca^{2+}/Mg^{2+}-free HBSS (Sigma-Aldrich). The tissue was incubated in 20 ml of HBSS/EDTA for 30 min at 37°C with regular manual shaking to ensure that the epithelial cells are disrupted from the mucosa. The colon pieces were then washed once in HBSS and cut into very small pieces with a scalpel. The finely chopped tissue was then transferred to a fresh 50-ml tube and digested for 1 h at 37°C with 1.6 mg/ml collagenase D (Roche) (equivalent to 4000 Mandl units/ml) and 40 μg/ml DNase (Roche) in IMDM (Sigma-Aldrich) supplemented with 10% FCS (Biosera), 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen Life Technologies). The supernatant was then filtered through 100-μm cell strainers and then 40-μm cell strainers. The cells were resuspended in 20 ml of 30% Percoll (Sigma-Aldrich) and the cell suspension was layered over 25 ml of 70% Percoll. The monocytes were removed from the 70:30% interface and washed twice in DPBS.

Macrophages were isolated from colon LP cells from uninfected and infected mice by magnetic separation using anti-F4/80 mAb as described (19). Isolated cells were confirmed by flow cytometry to be LP macrophages (F4/80$^+$CD11b$^+$CD11c$^-$), see above. A total of 3×10^5 isolated LP macrophages was administered to mice i.v. on days 0 and 4 of the DSS experimental model, with each mouse receiving a total of 6×10^5 cells. Colon LP macrophages were isolated from infected and uninfected eGFP-expressing mice and transferred i.v. to mice, as above.

Cell preparation and cytokine analysis

Spleens or mesenteric lymph nodes were removed and cells isolated for cell culture. Cells were cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% (v/v) heat-inactivated FCS (Labtech), 100 mM L-glutamine (Invitrogen Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies). Cells (5×10^6/

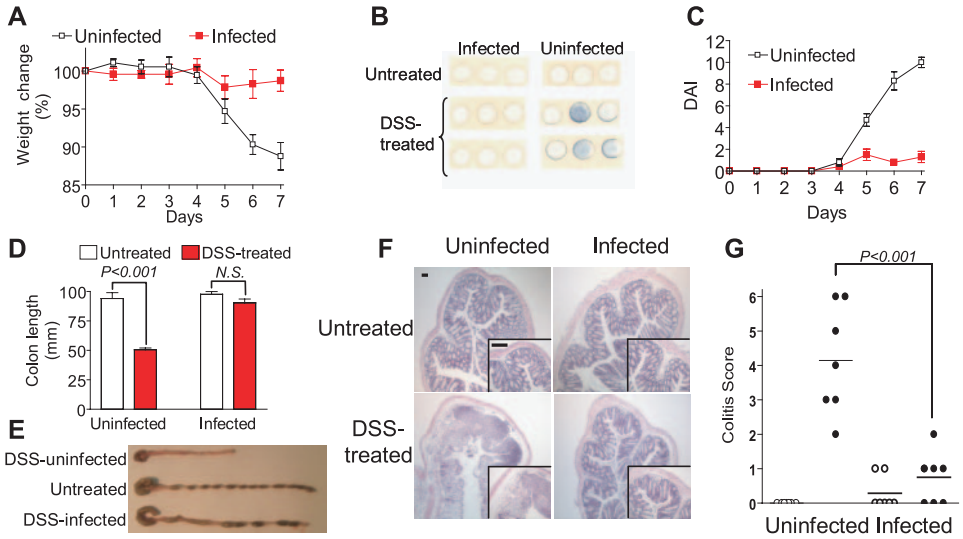

FIGURE 1. Schistosome worm-infected BALB/c strain mice are protected from DSS-induced colitis. Uninfected and 8 wk-infected BALB/c strain mice were treated with 5% DSS for 7 days. *A*, Weight change during treatment, expressed as percentage change from day 0. *B*, Presence of blood in the feces of individual mice on day 7, as shown by blue coloration on Hemdetect indicator paper. *C*, Weight loss, stool consistency, and fecal blood were scored to provide DAI for each group. *D* and *E*, Upon autopsy on day 7, the colons were removed and the length was measured; representative colons are shown. Student's *t* test was used to test for statistical differences between groups. *F*, H&E staining of colon tissue sections. Scale bars, 0.4 mm. *G*, Histology scores for colon cellular infiltration and tissue disruption. Untreated mice, ○; DSS-treated mice, ●. Scores from individual mice are plotted and the group mean is shown as a bar. Statistical differences between groups were analyzed by Mann-Whitney *U* test. Data are from six to eight mice per group and are representative of at least three separate experiments.

ml) were unstimulated (medium) or stimulated with plate-bound anti-CD3 (clone 145-2C11; 10 μg/ml adhered for 2 h at 37°C) for 72 h at 37°C. Supernatants were harvested after 72 h and IL-10 and TGF-β measured by ELISA. IL-10 was measured using a DuoSet ELISA Development System from R&D Systems Total TGF-β (acidified samples) was measured by ELISA according to the manufacturer's instructions (Promega).

Real-time PCR

RNA was extracted from colons from individual mice using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNA was also obtained from F4/80$^+$ cells isolated from a pool of LP cells prepared from colons from four to eight uninfected or infected mice. cDNA was reverse-transcribed from 1 μg of RNA using the QuantiTect Reverse Transcription kit (Qiagen). Real-time PCR was performed on an Applied Biosystems 7300 System. Relative quantities of mRNA for genes were determined using TaqMan Universal PCR Master Mix. Primers for arginase-1, inducible NO synthase (iNOS), and β-actin were from TaqMan Gene Expression Assays (Applied Biosystems). mRNA levels for each sample were normalized to β-actin mRNA levels then quantified relative to uninfected controls.

Statistical analysis

All in vivo experiments were performed at least two separate times, with 4–10 mice per group. For statistical analysis of differences in cell frequency detected in flow cytometry of tissue, cells were prepared from 5 individual mice per group. Difference between groups was analyzed by Student's *t* test. Colitis scores were analyzed by Mann-Whitney *U* test. Values of $p < 0.05$ were considered significant.

Results

Schistosome worm-infected BALB/c strain mice are resistant to DSS-induced colitis

Uninfected BALB/c strain mice treated with 5% DSS develop progressive weight loss (Fig. 1*A*), with mice developing diarrhea with blood in the feces (Fig. 1*B*), and increased DAI from the fourth to seventh day (Fig. 1*C*). In striking contrast, mice infected with schistosome worms did not develop these symptoms (Fig. 1, *A–C*). Additionally, uninfected mice treated with DSS had significant shortening of the colon ($p < 0.001$), whereas there was a nonsignificant reduction in the colon length of DSS-treated infected mice (Fig. 1, *D* and *E*). Schistosome-infected mice were also protected from DSS-induced damage to the colon (Fig. 1*F*). When the colon pathology was quantified, worm-infected mice treated with DSS had significantly lower histological scores ($p < 0.001$) than uninfected DSS-treated mice (Fig. 1*G*). However, infected mice not treated with DSS did have elevated scores for colitis as compared with age-matched uninfected mice (Fig. 1*G*), which was due to the presence of sporadic infiltration of cells within the colon LP. The data in Fig. 1 are from mice infected for 7–8 wk, with similar protection from DSS-induced colitis obtained in mice with chronic 12–16 wk infections (data not shown).

Parasite eggs do not induce protection from DSS-induced colitis

To assess the role of the egg stages of infection in DSS-induced colitis, mice were infected with male and female cercariae, which leads to the production of eggs during infection. When mice infected with male and female cercariae were treated with DSS at 8–9 wk postinfection, ~4 wk after egg laying had commenced, the infected mice developed more severe colitis than seen in uninfected mice, as demonstrated by an early increase in DAI between days 2 and 3 of DSS treatment and a consistently higher DAI throughout the course of treatment (Fig. 2*A*). Infected mice that did not undergo DSS treatment had elevated initial DAI values and their colons were significantly shorter ($p < 0.05$) than age- and sex-matched uninfected mice (Fig. 2, *A* and *B*). Pathology in the absence of DSS in infected mice may be due to parasite eggs migrating through the intestinal tissue: indeed, a mean of 648 eggs per colon (SEM 60, $n = 6$) was detected in tissue digests. Therefore, the exacerbation of disease during DSS treatment of mice infected with male and female cercariae may be due to the inflammation induced by passage of parasite eggs through the colon. We have also exposed male and female cercariae-infected mice to DSS during the chronic stages of infection. DSS treatment from the 15th week postinfection also resulted in more severe colitis in infected mice compared with age-matched uninfected mice (data not shown).

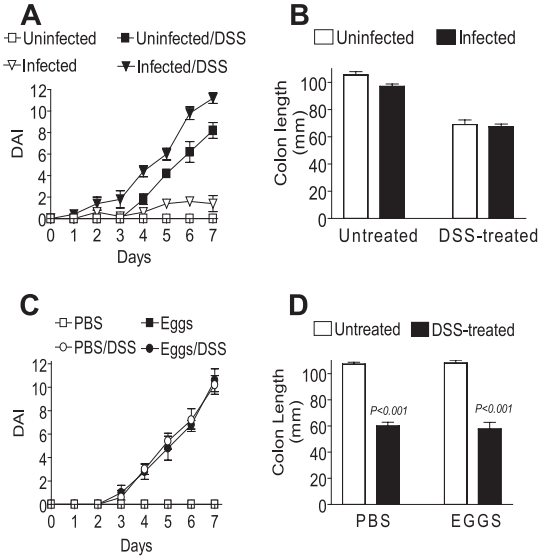

FIGURE 2. BALB/c strain mice infected with an egg-laying *S. mansoni* male and female worm infection or injected with schistosome eggs are susceptible to DSS-induced colitis. BALB/c strain mice infected with 30 (male and female) cercariae were treated with 5% DSS 8 wk postinfection. *A*, DAI of uninfected and infected mice that were untreated or given DSS for 7 days. *B*, Colon lengths after 7 days of DSS treatment of both uninfected and infected mice caused a comparable significant ($p < 0.001$) reduction compared with untreated mice. (*C*) DAI and (*D*) colon length of mice injected with PBS or with schistosome eggs (5000 eggs i.p. per week for 5 wk) treated with 5% DSS for 7 days commencing after the last egg injection. Values of p represent significance of difference between colon length of untreated vs DSS-treated mice. Data are the mean ± SEM from eight mice per group.

To investigate the effects of schistosome eggs on DSS-induced colitis without the confounding factor of eggs migrating through the colon, schistosome eggs were injected into the peritoneum of uninfected mice before DSS treatment. This protocol has previously been shown to prevent colitis induced by 2,4,6-trinitrobenzene sulfonic acid (16). However, mice injected with eggs weekly for 5 wk were fully susceptible to DSS-induced colitis (Fig. 2, *C* and *D*). Taken together, these data demonstrate that protection from DSS-induced colitis is mediated by the worm stage of infection. Thus, from here on, experiments refer to those conducted on mice infected with male worms only.

Resistance from colitis is independent of regulatory cytokines

Worm infection of mice increases the production of the regulatory cytokines, TGF-β and IL-10 (19–21), with such regulatory responses ameliorating intestinal inflammation (27, 28). Although infected mice have elevated IL-10 and TGF-β production from mesenteric lymph node cells (Fig. 3, *A* and *D*), treatment with anti-IL-10R and anti-TGF-β mAbs did not alter the resistance from colitis in infected mice (Fig. 3, *B*, *C*, *E*, and *F*). However, in the same experiments, blocking the activity of IL-10 or TGF-β with mAbs exacerbated disease severity in uninfected mice, as shown by the increase in DAI and significant ($p < 0.05$) reduction in colon length obtained relative to uninfected mice treated with control mAb (Figs. 3, *B*, *C*, *E*, and *F*). The effects shown here in uninfected mice treated with mAbs to block IL-10 or TGF-β support previous studies demonstrating that both cytokines have a protective role during DSS-induced colitis (29, 30). Therefore, schistosome worm infection prevents colitis induced by DSS via a mechanism independent of two regulatory cytokines that are implicated in suppressing intestinal inflammation.

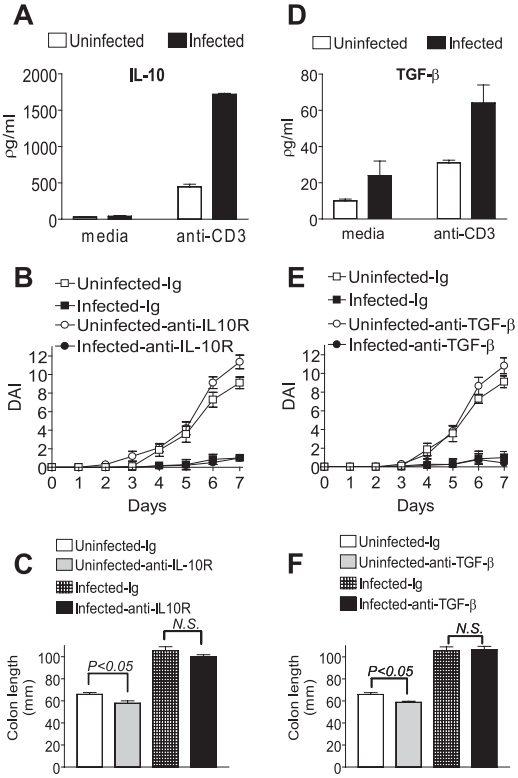

FIGURE 3. No role for regulatory cytokines in resistance of infected BALB/c strain mice to DSS-induced colitis. (*A*) Production of IL-10 and (*D*) TGF-β by mesenteric lymph node cells from uninfected and infected mice. Mesenteric lymph node cells from three to four BALB/c strain mice were pooled. Data are representative of four separate experiments. (*B*, *C*, *E*, and *F*) DAI and colon lengths of DSS-treated, uninfected, and infected BALB/c strain mice administered with 250 μg of mAb i.p. against IL-10R, TGF-β, or a control mAb (Ig) on each of days 0, 3, and 5 of DSS treatment. Student's t test was used to test for statistical differences between groups. Data are the mean ± SEM from 5 to 10 mice per group and are representative of three separate experiments.

Protection from colitis is mediated by a mechanism independent of T regulatory cells and other lymphocytes

To exclude a role for $CD4^+CD25^+$ regulatory cells in resistance of infected mice from colitis, mAbs were administered before and during DSS treatment with >95% of $CD4^+$ or $CD25^+$ cells depleted (data not shown). Depletion of $CD25^+$ cells had no affect on the resistance of infected mice to colitis (Fig. 4*A*). Recently, the use of anti-CD25 mAb treatment as a protocol for in vivo depletion of T regulatory cells has been called into question (31). Therefore, to ensure all $CD4^+$ T cells, including regulatory and Th1 or Th2 cells, were removed, mice were also treated with anti-CD4-depleting mAbs. In the absence of $CD4^+$ cells, infected mice remained protected from colitis (Fig. 4*A*).

To specifically address whether the mechanism underlying the resistance of infected mice to DSS-induced colitis was independent of lymphocytes, RAG-$1^{-/-}$ mice, which are deficient in T and B cells, were infected with worms and treated with DSS. Although uninfected RAG-$1^{-/-}$ mice developed colitis, concomitant worm infection of RAG-$1^{-/-}$ mice rendered the animals refractory to colitis (Fig. 4*B*).

Resistance of infected mice to colitis is macrophage dependent

Histological studies showed that infected mice have increased infiltration of cells within the colon LP before DSS treatment (Fig. 1*F*). We used flow cytometry on colon LP cells to characterize the

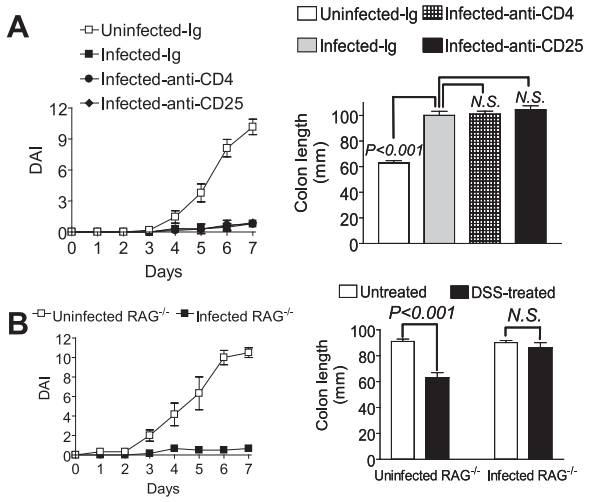

FIGURE 4. Infection protects BALB/c strain mice by a mechanism independent of $CD4^+$ or $CD25^+$ cells and infected RAG-$1^{-/-}$ are protected from DSS-induced colitis. *A*, DAI and colon lengths of DSS-treated, uninfected, and 8 wk-infected BALB/c strain mice administered i.p. with 250 μg of anti-CD4 or anti-CD25 and a control mAb (Ig) on each of days 0, 3, and 5 of DSS treatment. *B*, DAI and colon lengths of uninfected and 8-wk infected RAG-$1^{-/-}$ mice treated with 5% DSS for 7 days. DAI and colon length of five to seven mice per group are shown. Student's *t* test was used to test for statistical differences between groups. Data are representative of two separate experiments.

cell infiltrate. A previously described method for mouse colon LP cell phenotyping was used (26), with LP macrophages shown to be $CD11b^+$ and $F4/80^+$, whereas the $CD11c^+$ LP dendritic cells were $F4/80^-$ (Fig. 5*A*). Thus, in subsequent studies on individual mice, F4/80 and CD11b were used as dual markers for flow cytometry detection of LP macrophages. The infiltrating cells were predominantly $F4/80^+$ macrophages, with 2- to 3-fold more macrophages within the colon LP of infected mice compared with uninfected mice ($p < 0.01$; Fig. 5, *B* and *C*). In uninfected mice, exposure to DSS causes a marked infiltration of macrophages into the colon LP, an observation reported previously (32), with a significant increase ($p < 0.01$) in macrophages in colons of DSS-treated mice compared with levels in colons of untreated mice (Fig. 5*C*). In contrast to uninfected mice, DSS treatment of infected mice caused a nonsignificant increase in the percentage of macrophages detected by flow cytometry within the colon LP (Fig. 5*C*). Thus, although there are more macrophages in the colon LP of infected mice, treatment with DSS did not elicit further local recruitment of macrophages.

To determine the biological significance of the increased frequencies of macrophages in the colon LP during worm infection, we used clodronate-liposomes to deplete macrophages (24) in mice during DSS treatment. Cells were isolated from the colon LP and mesenteric lymph node from uninfected and infected mice treated with clodronate-liposomes or PBS-liposomes and the depletion of $CD11b^+F4/80^+$ cells in clodronate-treated groups confirmed by flow cytometry (data not shown). Worm-infected mice that were treated with clodronate-liposomes were fully susceptible to DSS-induced colitis, with comparable elevations in DAI and reduction in colon length as DSS-treated uninfected mice (Fig. 6, *A* and *B*). The mechanism whereby worm infection modulates immunity to render mice refractory to DSS-induced colitis is therefore dependent on macrophages. This mechanism is not due to macrophages within the colons of infected mice being unable to phagocytose DSS, as macrophages isolated from the colon LP from infected mice were able to internalize FITC-dextran in vitro to the same degree as cells from uninfected mice (Fig. 6, *C* and *D*).

Protection from colitis is mouse strain dependent and not mediated by alternatively activated macrophages

To further investigate the mechanism of protection from colitis, C57BL/6 strain mice, which are more susceptible to DSS-induced colitis than BALB/c strain mice (33), were infected and colitis was induced by administration of DSS. In contrast to infected BALB/c

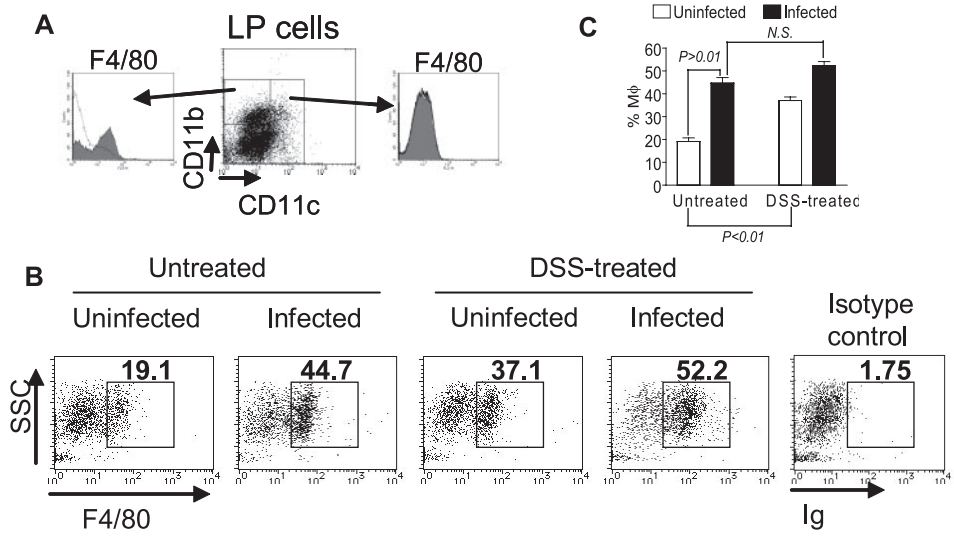

FIGURE 5. Worm infection of BALB/c strain mice causes an infiltration of macrophages into colon LP. *A*, Flow cytometry on colon LP cells from BALB/c strain mice stained with anti-CD11b or anti-CD11c mAb (*middle panel*). *Left panel*, $CD11b^+$ gated cells express F4/80, consistent with being colon LP macrophage, with absence of F4/80 on $CD11c^+$ gated dendritic cells (*right panel*). Isotype control is shown as clear shading and anti-F4/80 mAb-stained cells are shaded. Flow cytometry was done on LP cells obtained from pools from four to six colons. *B*, Flow cytometry detection of cells expressing the macrophage-specific marker F4/80 in $CD11b^+$ gated LP cells isolated from the colon. Representative dot plots of LP cells from BALB/c strain mice following different treatments, $F4/80^+$ cells vs side scatter (SSC), and an isotype control Ab vs SSC are shown. *C*, Bar graph shows the mean percentage of $F4/80^+CD11b^+$ macrophages in the LP of colons from five to seven individual mice per group. Student's *t* test was used to test for statistical differences between groups. Data are the mean ± SE from five to seven mice per group. Data shown are representative of two to three separate experiments.

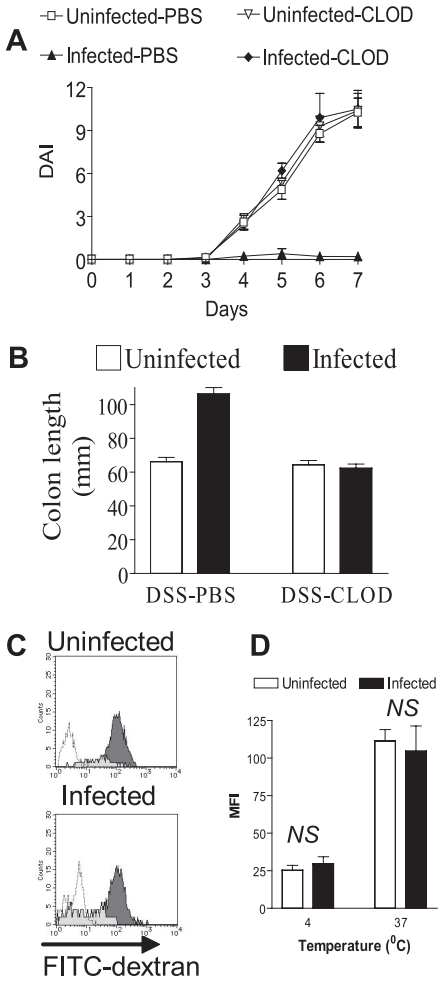

FIGURE 6. Worm infection-mediated protection from colitis in BALB/c strain mice is macrophage dependent. (*A*) DAI and (*B*) colon length of uninfected and infected BALB/c strain mice depleted of macrophages by treatment with clodronate-liposomes (CLOD) or control PBS-liposomes (PBS). *C*, Representative histograms of uptake of FITC-dextran by colon LP F4/80$^+$CD11b$^+$ macrophages from uninfected and infected BALB/c strain mice when cultured at 37°C (dark-shaded histogram) compared with cells incubated on ice (0°C; light-shaded histogram). The dotted line represents unstained LP macrophages incubated in medium alone. *D*, Bar graph showing mean plus SE mean fluorescence intensity for FITC staining of LP macrophages, at 4°C and 37°C, from five to seven individual uninfected or infected mice. Data are the mean ± SE from five to seven mice per group. Data shown are representative of two to three separate experiments.

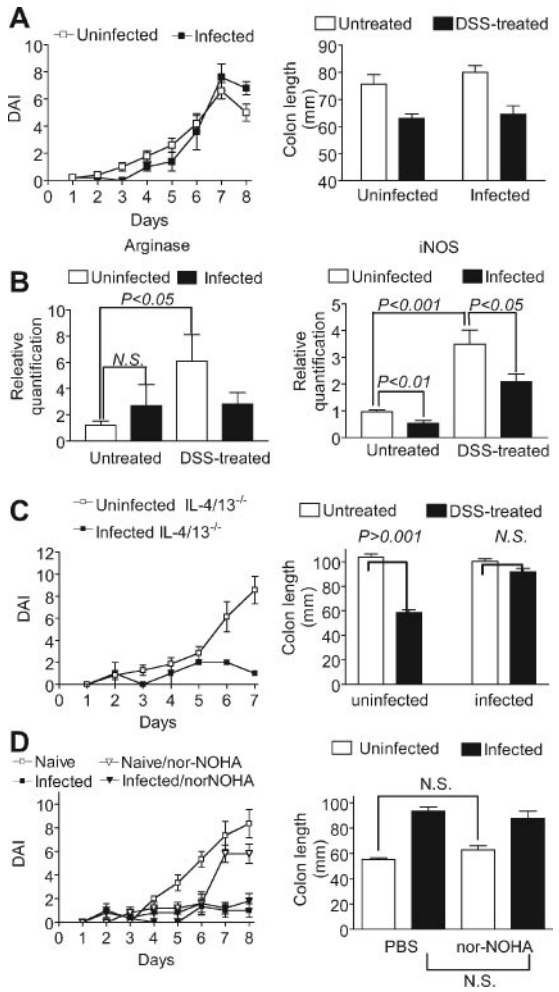

FIGURE 7. Resistance to DSS-induced colitis is mouse strain dependent and is not mediated by alternatively activated macrophages. *A*, C57BL/6 strain mice were infected with *S. mansoni* worms. Eight weeks postinfection, infected mice and age-matched uninfected control mice were treated with 3% DSS for 5 days and then normal water for 3 further days. DAI and colon length of six mice per group are shown. *B*, Real-time PCR quantification of arginase and iNOS mRNA from the colons of untreated uninfected and infected BALB/c strain mice and mice treated for 6 days with DSS. Data shown are from six mice per group and are representative of two separate experiments. *C*, IL-4/13$^{-/-}$ BALB/c strain mice were infected with *S. mansoni* worms. Eight weeks postinfection, infected mice and age- and sex-matched IL-4/13$^{-/-}$ mice were treated with 5% DSS for 7 days. DAI and colon length of six to eight mice per group are shown. Data are representative of two separate experiments. *D*, DAI and colon lengths of DSS-treated, uninfected, and infected BALB/c strain mice given i.p. injections of 100 μg of arginase inhibitor (nor-NOHA) and PBS daily during DSS treatment. There were six to eight mice per group and data are representative of two separate experiments. All data are the mean ± SE from six to eight mice per group. Student's *t* test was used to test for statistical differences between groups.

mice, infected C57BL/6 were fully susceptible to DSS-induced colitis, with comparable DAI values and reduction in colon length as uninfected C57BL/6 strain mice (Fig. 7*A*). Infected C57BL/6 mice did not develop the significant increased infiltration of macrophages into the colon LP that was seen in infected BALB/c strain mice (Fig. 5*B*), with uninfected C57BL/6 having 10.4 ± 2.8% F4/80$^+$ colon LP cells compared with a nonsignificant increase to 12.2 ± 3.7% F4/80$^+$ cells in colon LP of infected C57BL/6 mice (mean ± SD from five individual mice).

In the murine model of leishmaniasis, disease severity is mediated by strain-dependent differences in macrophage function, with BALB/c mice being more susceptible than C57BL/6 strains via alternative activation of macrophages and induction of arginase (34). The stimulation of alternatively activated macrophages has been shown to be a characteristic of helminth modulation of immune responses (35), with alternatively activated macrophages implicated in schistosome egg-induced intestinal inflammation (15). We therefore quantified expression of arginase and the classically activated macrophage gene iNOS in colons from individual mice. In the colons of infected BALB/c mice, the mRNA expression levels of arginase was not significantly increased compared with uninfected mice, with colons from infected mice having significantly reduced ($p < 0.01$) expression of iNOS (Fig. 7*B*). DSS treatment of uninfected mice induced significant elevation in both arginase and iNOS in the colons ($p < 0.05$ and $p < 0.001$, respectively; Fig. 7*B*), whereas infected mice treated with DSS had

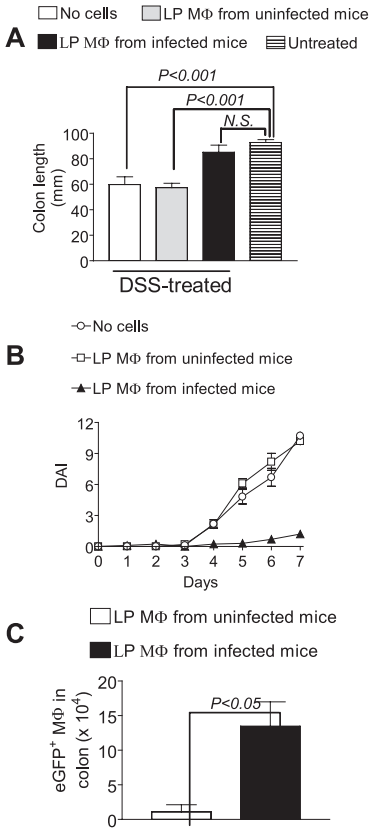

FIGURE 8. Colon LP macrophages (Mϕ) from infected BALB/c strain mice transfer protection from colitis in recipient mice. LP F4/80$^+$CD11b$^+$ CD11c$^-$ Mϕ were isolated from the colons of uninfected and infected BALB/c strain mice and injected i.v. into naive BALB/c strain mice on days 0 and 4 of a 7-day DSS treatment regime. (*A*) Colon length and (*B*) DAI of recipient mice treated with DSS. *C*, LP F4/80$^+$ from uninfected and infected eGFP$^+$ mice were injected into a recipient mouse as described above. eGFP$^+$ F4/80$^+$CD11b$^+$ Mϕ in the LP of recipient mice were detected by flow cytometry on day 7 of a DSS treatment regime. Mice received a total of 6×10^5 LP macrophages each, and the mean (plus SEM) numbers of eGFP$^+$ Mϕ recovered per colon from four to five individual mice are shown. All Mϕ transfer experiments were done on two (eGFP mice) or three (infected mice) separate occasions. Student's *t* test was used to test for statistical differences between groups.

no change in colon arginase expression with iNOS levels elevated, but significantly lower ($p < 0.05$), than that detected in uninfected mice treated with DSS (Fig. 7*B*). In additional experiments, arginase and iNOS expression levels in F4/80$^+$ LP cells, isolated from pools of cells from the LP of colons from four to eight mice, showed F4/80$^+$ cells from the colons LP of infected mice had iNOS and arginase expression that was comparable to that detected in F4/80$^+$ LP cells from uninfected mice (data not shown). Taken together, these data suggest that the increase in F4/80$^+$ cells in the LP of infected mice shown in Fig. 5 is not alternatively activated macrophages.

Detection of the expression of arginase or iNOS can only infer associations between gene expression and the functional significance of alternative or classic macrophage activation in the colons. Therefore, to determine whether resistance to colitis in worm infection was mediated by alternatively activated macrophages, mice deficient in both IL-4 and IL-13 (IL-4/13$^{-/-}$), which have defects in alternatively activated macrophages (36), were infected with schistosome worms and exposed to DSS. Infected IL-4/13$^{-/-}$ mice remained refractory to colitis (Fig. 7*C*), indicating that protection was unlikely to be mediated by alternatively activated macrophages. Additionally, infected BALB/c mice were treated with the arginase inhibitor nor-NOHA (25) during DSS administration. Inhibition of arginase activity in infected mice had no effect on the progression of colitis compared with untreated mice (Fig. 7*D*). Taken together, these data suggest that the macrophage-dependent resistance of worm-infected mice from DSS-induced colitis is not due to alternative activation.

Colon LP macrophages from infected mice transfer protection from colitis

To specifically address whether the macrophages infiltrating the colon during worm infection were directly mediating protection from colitis, we isolated F4/80$^+$ macrophages from the LP of colons from uninfected and infected mice and transferred them to uninfected mice and treated them with DSS. Mice injected with colon macrophages from uninfected mice developed colitis when exposed to DSS, comparable to disease observed in mice not injected with cells (Fig. 8, *A* and *B*). In contrast, transfer of colon macrophages from infected mice rendered recipient mice refractory to DSS-induced colitis (Fig. 8, *A* and *B*).

To track the in vivo migration of transferred cells, LP macrophages were isolated from the colons of uninfected and infected mice with ubiquitous expression of eGFP. When mice were culled 3 days later, flow cytometry analysis of colons from recipient mice showed that significantly more eGFP$^+$ F4/80$^+$ macrophages isolated from colons of infected mice migrated to the colon compared with infiltration of eGFP$^+$ macrophages from the LP of uninfected mice ($p < 0.05$; Fig. 8*C*). Therefore, LP macrophages isolated from the colons of worm-infected mice preferentially migrate to the colon when injected into naive mice and they prevent DSS-induced colitis in recipients.

Discussion

There is accumulating experimental evidence that infection with parasitic worms may reduce the severity of disease in models of colitis (4, 5, 7, 8). Furthermore, the potential of parasitic worms as a therapeutic for IBD has been tested in patients with UC or CD (9–11). The data presented here demonstrate that mice infected with schistosome worms are refractory to DSS-induced colitis. The resistance to DSS-induced diseases was by a process not simply associated with modulation through Th2 or T regulatory cells or cytokines. We have identified that protection is mediated by a novel mechanism involving schistosome worm infections inducing modulated colon LP macrophages that can prevent inflammation in the colon.

DSS-induced colitis is a well-established model which exhibits many of the symptoms observed in human IBD such as diarrhea, bloody feces, mucosal ulceration, shortening of the colon, and weight loss (37, 38). It is thought that DSS (a sulfated polymer) induces mucosal injury and inflammation initially through a direct toxic effect on epithelial cells, allowing intestinal bacteria to penetrate the injured mucosa and perpetuate mucosal inflammation (39). Disease in the DSS model is not dependent on T or B cells, although both Th1 and Th2 cells have both been shown to influence the later phases of disease (40, 41). Although a specific cell type has not been identified as central to the induction of DSS-induced colitis, inflammation is associated with elevated levels of TNF-α, IL-1β, and IL-6 in the colon; as disease occurs in RAG-1$^{-/-}$ and SCID mice macrophages are implicated as a possible source for these cytokines (1). In support of a role for macrophages in DSS colitis, it has recently been shown that LP macrophages have a suppressive influence on DSS-induced colitis (42), suggesting that macrophages are central to both induction and regulation of DSS-induced colonic inflammation.

As schistosome worm infection induces a Th2 response bias, the resistance of the infected mice to DSS could be mediated by elevated Th2 cytokines which can inhibit Th1 cytokine production. However, induction of a Th2 response using schistosome eggs in the absence of infection failed to alter the course of colitis. The 5-wk egg injection protocol was used to mimic the prolonged priming of eggs during infection, with mesenteric lymph nodes cells from mice injected with eggs in this manner producing comparable secretion of IL-4 as worm-infected mice (data not shown). We have also used a shorter egg-priming regime, $2 \times 10{,}000$ eggs i.p. at 2-wk intervals, and with this protocol egg-injected mice were also not protected from DSS-induced colitis. In support for no role for Th2 cytokines in protection, worm infection of mice deficient in both IL-4 and IL-13 were refractory to DSS-induced colitis. These findings that a schistosome-induced Th2 response was not protective in this model are consistent with work investigating the effect of another helminth, *Hymenolepis diminuta* on colitis, which although inducing a robust Th2 response, also did not reduce DSS-induced tissue damage (43). However, other gastrointestinal parasitic worms may induce a regulatory type 2 response that can suppress colon inflammation in different models of colitis. This is illustrated by infection of IL-10-deficient mice with the mouse gastrointestinal helminth *Heligmosomoides polygyrus*, ameliorating colitis via a mechanism involving the inhibition of Th1 cytokine responses (7).

Experimental infections in this study consisted primarily of only male worms. In contrast, male and female worm-infected mice were more susceptible to DSS-induced colitis. However, *S. mansoni* infection of rats, as a nonpermissive host no eggs are excreted, resulted in reduced severity of hapten-induced colitis (6). In male and female cercariae-infected mice, eggs pass through the intestinal wall causing tissue inflammation, this damage to the colon by tissue-migrating eggs coupled with the "double hit" of disruption of the epithelial barrier by DSS may be lead to the exacerbated disease. In view of the role of innate immune responses in the DSS model (44), this egg-induced damage and exposure to luminal contents may predispose to proinflammatory responses in the colon. In contrast, worm-infected mice do not have the egg-induced damage to the intestines.

Regulatory cells and cytokines can suppress experimental colitis (45). However, protection from DSS-induced colitis in schistosome-infected mice was shown not to be dependent on regulatory T cells or regulatory cytokines, because infected mice depleted of $CD4^+$ or $CD25^+$ cells or with IL-10 or TGF-β neutralized remained refractory to the effects of DSS administration. Indeed, the potency of worm infection-mediated resistance to DSS-induced disease was sufficient to prevent the increase in colon inflammation that was seen in uninfected mice treated with mAb against IL-10 or TGF-β. A recent study has demonstrated that infection with *H. polygyrus* induces a $CD8^+$ regulatory cell population that may protect mice from colitis (46). Although we did not directly investigate the role of $CD8^+$ cells in this study, as infected RAG-$1^{-/-}$ mice were protected from DSS-induced colitis, it is indicative that schistosome infection induces protection from disease independently of T or B cells.

We have identified that there is selective infiltration of macrophages into the colon LP of infected mice and demonstrated using clodronate depletion that schistosome-induced protection from disease was mediated by macrophages. Recently, Qualls et al. (42) have shown that intestinal mononuclear phagocytes have a protective role limiting the extent of intestinal inflammation in the DSS model of colitis. In our study, schistosome worm infection elicited the expansion of a macrophage population in the colon LP, when these cells were isolated and transferred to mice they induced protection from DSS-induced colitis. In contrast, macrophages isolated from the colon LP of uninfected mice did not transfer protection to recipient mice that were exposed to DSS, which highlights the need for schistosome infection to modulate the colon LP macrophages to become protective. There were mouse strain differences in the extent of macrophage infiltration of the colon LP after schistosome worm infection. There was significantly increased infiltration of macrophages within the colons of infected BALB/c strain mice, whereas infection of C57BL/6 strain mice did not induce marked macrophage infiltration. Our observations of mouse strain differences in macrophage infiltration of the colon has also been shown following infection of mice with a colon-dwelling helminth, *Trichuris muris*, with greater infiltration of $F4/80^+$ cells into the colon LP of BALB/c (Th2) strain mice than in a Th1-biased (AKR) mouse strain (47). Additionally, while schistosome-infected BALB/c strain mice were refractory to DSS-induced colitis, infected C57BL/6 mice were fully susceptible to DSS-induced colitis. Preliminary studies have also shown that LP macrophages isolated from infected C57BL/6 strain do not transfer protection from DSS-induced inflammation (data not shown), which contrast with the same cells from infected BALB/c mice (Fig. 8, *A* and *B*). However, it is worth noting that susceptibility to DSS-induced colitis and the diminished macrophage infiltration of the colon LP in C57BL/6 mice maybe two unconnected phenomena.

The C57BL/6 (Th1) and BALB/c (Th2) strain difference in susceptibility to *Leishmania major* infection involves alternative activation of macrophages (34). As helminth infections, including *S. mansoni*, are associated with the induction of alternatively activated macrophages (15, 35, 36, 48, 49), we investigated whether these cells mediated resistance to colitis. Recently, the induction of functional alternatively activated macrophages by the gastrointestinal helminth *H. polygyrus* was abrogated in vivo in wild-type mice, by depletion with clodronate-liposomes or blocking arginase enzymatic activity (50). In our study, using similar strategies to block the same cells, we failed to show a role for alternatively activated macrophages in schistosome-induced resistance from DSS-induced colitis. It is noteworthy that when arginase activity was inhibited in uninfected mice, there was some reduction in DAI (Fig. 7*D*), suggesting that alternatively activated macrophages may have a role in disease in the DSS colitis model.

Macrophages with suppressive activity that are not alternatively activated cells have been induced in mice by injection with schistosome egg glycans, with these $F4/80^+Gr1^+$ cells suppressing T cell activation (17, 18). We have shown previously suppressive $F4/80^+$ macrophages from the spleens of worm-infected mice down-regulate Gr1 expression (19). Furthermore, depletion of $Gr1^+$ cells with anti-Gr1 mAb (RB6-8C5) treatment did not render infected mice susceptible to DSS-induced colitis (data not shown). Thus, schistosome worm infection stimulates a novel macrophage population, that is not an alternatively activated or $Gr1^+$ macrophage, that preferentially migrates to the colon LP where they can suppress colonic inflammation.

We have also shown previously that schistosome worms can modulate splenic macrophages to induce T cell anergy via a mechanism involving the costimulatory surface marker PD-L1 (19). Interestingly, PD-L1 is involved in colitis as its expression is upregulated in inflamed colons from both IBD patients and in SCID mice after transfer of $CD4^+CD45RB^{high}$ T cells (51). However, rather than playing an inhibitory role, PD-L1 was shown to contribute to the proinflammatory response because administration of blocking Ab ameliorated colitis in SCID mice (51). The recent generation of PD-L1-deficient mice (52, 53) will facilitate future

in-depth investigations on the possible role of PD-L1 in the observations reported here.

Schistosome infections are the cause of morbidity and death in humans and even if humans were infected with a worm-only infection, as used here, despite the absence of eggs, which are the major cause of pathology, there are concerns with side effects from aberrant migration of worms to, for example, the CNS. Therefore, although humans have already been deliberately infected with parasitic worms as a potential therapy for IBD, we do not advocate infection of patients with schistosome worms. Hence, to exploit schistosomes as a therapy for IBD, and other diseases, a rational strategy is to identify the desirable protective mechanism, as done here, and isolate the parasite molecule(s) that elicits the protection, as shown recently with a schistosome-derived anti-inflammatory molecule (54). Future work is required to characterize the protective intestinal macrophages induced by schistosome worms and to determine the mechanism whereby they suppress inflammation in the colon.

Acknowledgments

We thank Prof. Peter Humphries for providing the eGFP mice and Dr. Tom Connor for access to real-time PCR equipment.

Disclosures

The authors have no financial conflict of interest.

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