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Heligmosomoides polygyrus Infection Can Inhibit Colitis through Direct Interaction with Innate Immunity

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Inflammatory bowel disease (IBD) and other immune-mediated illnesses are rare in tropical, less-developed countries. Helminth infections common in such countries may prevent IBD (1). Various helminth species used in several animal models of intestinal inflammation can avert or limit disease activity. For instance, rodents receiving nonviable schistosome OVA (2) or intestinal helminth infection, dendritic cells (DCs) from the lamina propria of Rag mice displayed decreased expression of CD80 and CD86, and heightened expression of plasmacytoid dendritic cell Ag-1 and CD40. They were also less responsive to laminae propria, producing less IL-12p40 and IL-10. Also diminished was their capacity to present OVA to OT2 T cells. These experiments infer that H. polygyrus does not require direct interactions with T or B cells to render animals resistant to colitis. DCs have an important role in driving both murine and human IBD. Data suggest that phenotypic alternations in mucosal DC function are part of the regulatory process. The Journal of Immunology, 2010, 185: 3184–3189.

In a Rag-transfer colitis model of IBD, H. polygyrus required CD8+ T cells in vivo to reverse the disease process (7). H. polygyrus infection also elicits a regulatory T cell population able to down-regulate allergen-induced lung pathology (8). Also, after H. polygyrus infection, LP T cells from healthy wild-type mice make large amounts of regulatory cytokines like IL-10 and TGF-β (9).

Interactions with cells of the innate immune system could be part of the protective process. For instance, schistosomes protect BALB/c mice from dextran sulfate sodium enteritis via a macrophage-dependent mechanism not requiring regulatory T cells (10). Protection in animal models of asthma may involve alternatively activated macrophages (11).

We used a Rag IL-10−/− T cell transfer murine model of IBD to further explore the importance of innate immunity in H. polygyrus protection from IBD. This investigation showed that direct interaction alone with the innate immune system is sufficient to allow H. polygyrus to impede intestinal Ag-specific responses and to protect mice from colitis. Data suggest that changes in dendritic cell (DC) function contribute to this regulatory process.

Materials and Methods

Mice

This study used C57BL/6 Rag2, OT2, and IL-10−/− mice (The Jackson Laboratory, Bar Harbor, ME). Breeding colonies were maintained in specific pathogen-free facilities at Tufts University. Animals were housed and handled following national guidelines and as approved by our Animal Review Committee.

Colitis model

Rag mice of similar age were reconstituted with 10⁶ IL-10−/− splenic T cells and 3 × 10⁵ OT2 splenic T cells given i.p. One week later, the animals were administered piroxicam (Sigma-Aldrich, St. Louis, MO) mixed into their feed for 2 wk (piroxicam at 40 mg/250 g chow, week 1; 60 mg/250 g chow, week 2) to induce colitis. The piroxicam was stopped. Two weeks later, the mice were sacrificed; their colons were examined microscopically for colitis; and LP mononuclear cells (LPMCs) were isolated for culture (Fig. 1).
H. polygyrus infection

Infective, encephal H. polygyrus L3 (U.S. National Helminthological Collection 81930) were obtained from fecal cultures of eggs by the modified Baermann method and stored at 4°C. Mice were colonized with 125 H. polygyrus third-stage larvae by oral gavage.

For some experiments, animals were infected for 2 wk with H. polygyrus after induction of colitis (end of piroxicam treatment) and then sacrificed to assess colitis severity (Fig. 1). In other experiments, Rag mice first were infected with H. polygyrus or just received a sham infection for 2 wk, and then both the infected and control groups received a single dose of pyrantel pamoate (0.5 mg/mouse; Sigma-Aldrich) via oral gavage to eliminate H. polygyrus. One week after deworming, the mice were reconstituted with T cells, and some were treated with piroxicam, as described above, to induce colitis, whereas others were kept untreated.

Several control experiments, deworming was confirmed by documenting the absence of adult H. polygyrus in the small bowel 1 wk after receiving the drug.

Dispersion of splenocytes, and splenic T cell enrichment

Single-cell suspensions of splenocytes were prepared by gentle teasing in RPMI 1640 medium (Life Technologies, Grand Island, NY). The cells were washed three times in RPMI 1640. Splenic T cells or CD4+ T cells were isolated by negative selection using the EasySep mouse T cell enrichment kit, as outlined by the manufacturer (19751; StemCell Technologies, Vancouver, Canada). Viability was determined using exclusion of trypan blue dye.

LPMC isolation and LP cell fractionation

Gut LPMCs were isolated from the terminal ileum (TI), as described (6). Cell viability was 90%, as determined by trypan blue exclusion. LP DCs (CD11c+), macrophages (F4/80+), and NK cells (DX5+) were isolated from dispersed LPMCs using appropriate mAbs and FACS. Cells were surface stained with fluorochrome-labeled anti-CD11c, anti-F4/80, and/or anti-DX5 mAb before sorting (see below).

Cell culture

LPMCs from T cell-reconstituted Rag mice were cultured (2 × 10^5 cells/well) for 48 h in 96-well round-bottomed plates. Cells were cultured along with OVA (50 μg/ml; Sigma-Aldrich; or anti-CD3 mAb (2C11; American Type Culture Collection, Manassas, VA) and anti-CD28 mAbs (BD Phar-Mingen, San Diego, CA), each at 0.5 μg/ml. The culture medium was RPMI 1640 containing 10% FCS, 25 mM HEPES buffer, 2 mM l-glutamine, 5 × 10^-3 M 2-ME, 1 mM sodium pyruvate, 100 U/ml penicillin, 5 mg/ml gentamicin, and 100 mg/ml streptomycin (all Life Technologies, Gaithersburg, MD). After culture, the supernatants were assayed for IFN-γ and IL-17A using ELISA (described below).

In the Rag LPMC/OT2 T cell mix experiments, OT2 Thy1.2 splenic T cells were mixed with LPMCs from Rag mice at a ratio of 1:3. Cells (2 × 10^5) were cultured in RPMI 1640 complete medium for 48 h. Some cultures contained OVA at up to 1000 μg/ml to stimulate cytokine release. Supernatants were assayed for IFN-γ and IL-17 after the incubation using ELISA.

In some experiments, OT2 Thy1.2 T cells (1.5 × 10^5) were mixed with isolated CD11c+, F4/80+, and/or DX5+ LPMCs (~4 × 10^5) from each of Rag mice cultured 48 h, as above, with up to 50 μg OVA before the supernatants were assayed for IFN-γ and IL-17.

In other experiments, isolated CD11c+ LP DCs were cultured for 48 h alone with or without 100 ng/ml highly purified LPS derived from Escherichia coli 026B6 (Sigma-Aldrich), or mixed with OT2 T cells and stimulated with OVA (50 μg/ml). Supernatants were assayed for IL-12p40, IL-10, and TGF-β using ELISA. Cells were again cultured in RPMI 1640 complete medium except for TGF-β determinations in which the cultures contained 1%, rather than 10%, FCS with 1 mg/ml albumin (AMRESCO, Solon, OH).

Sandwich ELISAs

ELISAs were performed using paired Abs (R&D Systems, Minneapolis, MN), according to manufacturer’s instructions. IL-17 ELISA was done using primary capture Ab from (R&D Systems) and biotinylated anti-IL-17A Ab (R&D Systems). IL-12 (p40) ELISA was performed using primary capture Ab (BD Pharmingen, Franklin Lakes, NJ) and biotinylated secondary Ab (BD Pharmingen). IL-10 was captured with anti–IL-10 mAb (R&D Systems) and detected with biotinylated mAb (R&D Systems). To measure IFN-γ, plates were coated with a mAb to IFN-γ (HB170; American Type Culture Collection) and incubated with supernatant. IFN-γ was detected with polyclonal rabbit anti–IFN-γ (gift from Dr. M. Wilson, University of Iowa, Iowa City, IA), followed by biotinylated goat anti-rabbit IgG (Axell, Westbury, NY). Total TGF-β was measured using acid-treated supernatant and mAb240 for capture and biotinylated chicken IgY BAF240 for detection (both R&D Systems).

Flow cytometry analysis

LPMCs were washed twice, adjusted to 10^5 cells/ml in FACS buffer (lymphocyte growth medium), and stained with saturating amounts of conjugated mAb for 30 min at 4°C. Following staining, cells were washed twice and resuspended in lymphocyte growth medium for analysis on a FACSCalibur using CellQuest software (BD Biosciences, Mountain View, CA). Before adding labeled mAb, each tube received 1 μg anti-Fc mAb (eBioscience, San Diego, CA) to block nonspecific binding of conjugated Abs to FcRs. The mAbs used for staining or cell sorting were anti–Thy1.2-FITC, or –PECy5, or–allophycocyanin; anti–CD11c-PE-Cy5; anti–F4/80-PE-Cy5; anti–CD4-PE or–PE-Cy5; anti–DX5-PE (all from eBioscience).

Statistical analysis

Data are means ± SE of multiple determinations. Differences between two groups was compared using Student t test. Multiple group comparisons used analysis of variation and Dunnett t test. The p values <0.05 were considered significant.

Results

Development of an IBD model in which gut LPMCs respond to OVA Ag

Rag mice reconstituted with IL-10−/− T cells develop severe colitis after piroxicam exposure (12). Intestinal luminal Ags that drive LP T cells are poorly defined. To permit the study of Ag-specific responses in the intestine LP, we reconstitute Rag mice with IL-10−/− T cells mixed with OT2 T cells bearing MHC class II-dependent TCR that recognize OVA. These mice were highly susceptible to colitis upon piroxicam administration. LPMCs isolated from such mice cultured with OVA in vitro released large amounts of IFN-γ and IL-17, which were secreted minimally in the absence of OVA (Fig. 2). LPMCs from mice reconstituted only with IL-10−/− T cells did not respond to OVA, suggesting that the cytokine response was OT2 T cell dependent.

H. polygyrus infection stops colitis and reduces the LPMC cytokine response to OVA

H. polygyrus can reverse colitis in Rag mice reconstituted with IL-10−/− T cells (6, 13). Using the Rag IL-10 knockout (KO)/OT2 T cell reconstitution model of IBD, we determined whether infection with H. polygyrus after induction of colitis could suppress intestinal inflammation and decrease cytokine release from LPMCs isolated from these mice. Fig. 3 shows that H. polygyrus markedly suppressed the intestinal inflammation and that isolate LPMCs from H. polygyrus–infected animals produced much less IFN-γ and IL-17 after OVA stimulation compared with the sham-infected controls. No IL-4 was detected.

Additional experiments reconstituted Rag mice with IL-10 KO and OT2 T cells, as above, but 1 wk later, the animals were infected with H. polygyrus and never given piroxicam to induce colitis. Two weeks postinfection, LPMCs isolated from the TI and cultured as in Fig. 3 also made much less IFN-γ and IL-17 upon OVA stimulation (10 μg/ml) compared with the LPMCs isolated from uninfected control animals (IFN-γ, 470 ± 40 versus 51 ± 10 pg/ml; IL-17, 220 ± 21 versus 105 ± 15 pg/ml; uninfected versus infected, n = 3, mean ± SE).

Rag mice exposed to H. polygyrus only before T cell reconstitution are protected from colitis

Immune effector and regulatory pathways of both innate and adaptive immunity help drive colitis. Experiments explored whether H. polygyrus interacting exclusively with cells of the innate immune system would suffice to control disease. Rag mice devoid of func-
tional T and B cells were exposed to *H. polygyrus* for 2 wk and then treated with a single dose of pyrantel pamoate to eliminate the worms. Control mice received a sham infection and drug treatment. One week after drug treatment, mice were reconstituted with IL-10−/− and OT2 T cells, exposed to piroxicam for 2 wk, and then sacrificed 2 wk after stopping the piroxicam (Fig. 1). Fig. 4 shows that *H. polygyrus* exposure before introduction of T cells was sufficient to block inflammation and to suppress both anti-CD3/CD28 mAb- and OVA-induced cytokine responses from the LPMCs. Once more, there was no detectable IL-4.

Additional experiments determined whether the reduced LPMC cytokine response to OVA was only secondary to improvement in inflammation. Following a brief infection with *H. polygyrus* and reconstitution of adaptive immunity, as described above, mice were maintained for 3 wk, but they were not given piroxicam to induce colitis. LPMCs isolated from these animals made substantially less IFN-γ and IL-17 and no IL-4 after either OVA or anti-CD3/CD28 mAb stimulation in vitro compared with the uninfected controls (Fig. 5).

**In Rag mice without T cell reconstitution, *H. polygyrus* exposure alters intestinal DC function, cell surface protein expression, and cytokine secretion**

It was assumed that *H. polygyrus* interactions with cellular components of innate immunity caused the above described protection from colitis and alterations in cytokine production. To test this hypothesis, Rag mice were infected with *H. polygyrus* for 2 wk. After 2 wk of infection, LPMCs isolated from Rag mice were mixed with splenic OT2 T cells and then cultured in vitro with or without various concentrations of OVA to stimulate cytokine release. After incubation for 48 h, the supernatants were assayed for IFN-γ and IL-17 content. LPMCs isolated from age-matched Rag mice that never had *H. polygyrus* infection served as controls. Fig. 6 shows that LPMCs from Rag mice with previous *H. polygyrus* infection supported OVA-induced IFN-γ production poorly. IL-17 secretion was minimally affected. LPMCs cultured without OT2 T cells and OT2 T cells cultured without LPMCs released no detectable IFN-γ or IL-17 upon OVA or LPS stimulation. IL-4 was not detected in any cultures.

The alteration noted in the supportive role of isolated LPMCs in the OT2 T cell IFN-γ response could have reflected *H. polygyrus*-induced changes in the cellular composition of the isolated, dispersed LPMCs. Flow analysis of the isolated LPMCs from *H. polygyrus*-infected and control mice revealed no significant alterations in the composition of DCs (CD11c+), macrophages (F4/80+), or NK cells (DX5+) (Fig. 7).

Additional experiments explored the potential role of these three cell types in modulating the OT2 IFN-γ response. Using FACS, CD11c+ DCs were isolated from the dispersed LPMCs from Rag mice previously exposed to *H. polygyrus* or from mice that never received this infection. CD11c+ DCs were mixed with OT2 T cells and then cultured with OVA for 48 h. CD11c+ intestinal DCs from mice previously infected with *H. polygyrus* were less proficient at supporting the IFN-γ response compared with DCs from the controls, whereas there was no change in IL-17 secretion (Fig. 8). F4/80+ and DX5+ LP cells from either *H. polygyrus*-infected or control Rag mice mixed with OT2 T cells individually or in combination did not support OVA-induced IFN-γ or IL-17 secretion. Adding F4/80+ macrophages or DX5+ NK cells to the CD11c+ LP DC/OT2 T cell mix did not affect OVA-induced IFN-γ or IL-17 secretion either.

DCs support or modulate T cell responses by cell-to-cell contact and through secretion of cytokines. Flow cytometric analysis was used to study cell surface proteins associated with DC function. We examined CD11b, MHCII, CD8a, CD103, CD80, CD86, plasmacytoid dendritic cell Ag (PDCA)-1, and CD40. DCs from the TI of Rag mice exposed to *H. polygyrus*, compared with DCs from control Rag animals, displayed a relative decrease in surface expression of CD80 and CD86 with enhanced expression of PDCA-1 and CD40 (Fig. 9). We also examined CD11c+ DCs in LPMCs isolated from the colon. These cells showed a similar pattern of changes in cell surface protein expression.

DCs can produce various cytokines, such as IL-12, IL-10, and TGF-β, that can stimulate, modulate, and/or inhibit T cell development and secretion. To see whether *H. polygyrus* exposure altered the capacity of intestinal CD11c+ DCs to produce cytokines, DCs from Rag mice previously infected with *H. polygyrus* or DCs from control animals that never experienced this infection were cultured alone or with LPS for 48 h to stimulate cytokine release. DCs isolated

![FIGURE 1. Experimental design.](http://www.jimmunol.org)
from mice previously exposed to H. polygyrus released less IL-12p40 and IL-10 in response to LPS than did comparable DCs isolated from the control animals (Table I). In other cultures, the DCs from either H. polygyrus-exposed or unexposed Rag mice were mixed with OT2 cells and then cultured with OVA (100 µg/ml). After OVA stimulation, supernatants from cultures containing H. polygyrus-exposed DCs also contained less IL-10 and IL-12p40. In all experiments, cellular supernatants did not contain measurable amounts of TGF-β.

Discussion

The most important observation of this study is that Rag mice briefly exposed to H. polygyrus before reconstitution with IL-10−/− colitogenic T cells are protected from colitis. Moreover, the protection is as profound as that seen in Rag mice exposed to H. polygyrus after T cell reconstitution. T cells drive the pathology in this animal model of IBD (12). The data presented in this study suggest that H. polygyrus regulates the pathogenic T cell response that causes IBD via direct interaction with cellular components of the innate immune system requiring no direct interface with T cells.

The intestinal mucosal immune system responds to many molecules within the gut. It is difficult to study the control of T cell responses to intraluminal Ags in the LP because a multitude of poorly defined Ags shapes mucosal immunity. To overcome this limitation, we injected OT2 transgenic T cells, which recognize OVA in a class II-dependent fashion, into the IL-10−/− murine model of IBD. When introduced at the time of IL-10−/− T cell transfer, OT2 T cells appear in the LP, and isolated LPMCs cultured in vitro with OVA produce large amounts of IFN-γ and IL-17. IFN-γ and IL-17 are proinflammatory cytokines incriminated in driving colitis in both human and many murine models of IBD (13).

Exposure to H. polygyrus before introduction of IL-10−/− and OT2 T cells reduced the capacity of the intestinal mucosa to make IFN-γ and IL-17 after either anti-CD3 mAb or OVA stimulation. This depressed cytokine response was evident even in the absence of colitis, suggesting that the downmodulation in proinflammatory cytokine secretion was not just secondary to improvement in intestinal inflammation.

It was assuming that innate immunity, influenced by H. polygyrus exposure, rendered mice resistant to colitis and the LP less prone to secrete colitogenic cytokines like IFN-γ and IL-17. The dispersed LPMCs from these mice were comprised mostly of DCs, macrophages, and NK cells, which were the focus of this study. H. polygyrus infection did not alter the relative proportion of these three cell subsets in the dispersed LPMC preparations. Our in vitro studies using various combinations of these three cell subsets revealed that DCs poorly supported OVA-specific IFN-γ production after H. polygyrus exposure. Neither the macrophages nor the NK cells had a substantial effect on this Ag-specific response.

DCs within the intestines function to limit local immune responses to the mostly harmless luminal Ags (14). Disruption in DC function may be one of the factors promoting IBD. The aim of this study was not to categorize all the various DC subsets expressed in the gut after H. polygyrus infection, but to determine whether H. polygyrus exposure substantially affected the phenotypic and functional expression of intestinal DCs in Rag mice expressing no functional T or B cells. We examined the state of DC activation after 2 wk of infection because the Rag mice were dewormed at that time point.
H. polygyrus infection had a prominent effect on the capacity of DCs to display various surface proteins and to secrete cytokines. There was decreased expression of the costimulatory molecules CD80 and CD86 associated with a decrease in IL-12p40 secretion. These alterations in DC function could explain why the intestinal DCs isolated from mice with H. polygyrus infection were less efficient at presenting OVA to OT2 T cells, stimulating IFN-γ production. IL-10 suppresses IL-12 secretion, depresses Th1 cell function, and has other immunoregulatory properties important for maintaining mucosal immune homeostasis (15). H. polygyrus exposure actually decreased, rather than increased, the capacity of the DC population to produce IL-10, suggesting that IL-10 did not participate in the downregulation of DC CD80/86 expression, IL-12 secretion, or OT2 stimulation. Soluble TGF-β, another important mucosal immunoregulatory cytokine (15), and IL-4 were not detected within culture supernatants, suggesting that these cytokines were not critical for this regulation. Our study did not rule out the possible participation of cell surface-bound TGF-β in the regulatory process.

PDCA-1 is a marker of plasmacytoid DCs and was more widely expressed on the intestinal DCs after H. polygyrus infection. Activation of T cells by plasmacytoid versus conventional DCs appears to favor generation of regulatory-type T cells (16). Thus, the appearance of more intestinal DCs expressing PDCA-1 after H. polygyrus colonization could be of regulatory significance.

It is notable that H. polygyrus exposure did not affect the overall level of MHC class II expression, which is centrally important for Ag-induced OT2 cell activation. Also notable was the failure of H. polygyrus infection to alter the number of DCs displaying CD8, CD11b, and CD103, which are markers of intestinal DC subtypes reported to influence Th and regulatory T polarization (14).

Expression of CD40 on DCs increased after H. polygyrus colonization. DCs express CD40 upon activation following interactions with some pathogens and microbial products (17). Some data suggest that the expression of CD40 on DC subsets is more dependent on the nature of the stimulus rather than on the phenotype of the DCs. Ligation of CD40 can result in enhanced expression of costimulatory molecules and proinflammatory molecules like IL-12 and has been implicated in DC-mediated induction of effector T cell responses (18). The physiological significance of H. polygyrus induction of CD40 on the intestinal DCs remains obscure.

It is assumed, although not yet proven, that changes in DC function lead to the protection. It still remains possible, although less likely, that the intestinal DCs assimilated H. polygyrus Ags after the infection and that this retained Ag drove the protection.

H. polygyrus mostly inhabits the proximal small intestine in our Rag mice. We evaluated the function of DCs from the distal TI. It remains unknown how H. polygyrus communicated with the DCs in distal regions of the intestine. DCs can extend dendrites across
the epithelial barrier to sample luminal molecules. Perhaps *H. polygyrus* Ags were sampled in the fecal stream. At least one secretory product of *H. polygyrus*, calreticulin, has been characterized (19). It binds to the scavenger receptor type A on DCs promoting Th2 responses. Crude supernatants containing excrete-ory-secretory products from *H. polygyrus* blunts CpG-stimulated bone marrow-derived DC activation (20). These supernatants also impair DC-induced Ab responses, but enhance their ability to promote regulatory T development. These observations support the hypothesis that Hpr-modulated intestinal DCs function via release of immune modulatory molecules.

**H. polygyrus** infection induces a significant shift in the abundance and relative distribution of intestinal bacteria (21). Among the various changes, there is a prominent increase in the Lacto-bacillaceae family of organisms, which contains bacterial species reported to decrease intestinal inflammation in murine models of IBD (22). We speculate that *H. polygyrus* also affects intestinal DC function through altering the complex intestinal bacterial flora.

As a group, a broad array of helminths and their products condition DCs through triggering some distinctly different intracellular signaling pathways to support Th2 rather than Th1 responses (23). The response of DCs to helminths is substantially different and much more blunted compared with their response to microbial pathogens. Helminths also impede DC maturation, which favors a tolerogenic response.

Helminth infection can antagonize the effects of bacterial products recognized via TLRs. This was demonstrated in our study showing that the LPS response of intestinal DCs was blunted after *H. polygyrus* infection. Helminths can signal via TLRs on DCs to promote a Th2/regulatory T cell response rather than the usual microbial-induced Th1 response (23). It is tempting to speculate that this could be one of their mechanisms of action.

The experiments reported in this work did not directly prove that *H. polygyrus*-induced changes in intestinal DC function protected the mice from colitis. However, there are ample data suggesting that DCs have an important role in driving both murine and human the mice from colitis. However, there are ample data suggesting that DCs have an important role in driving both murine and human IBD (24). We speculate that *H. polygyrus* also affects intestinal DC function through altering the complex intestinal bacterial flora.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


