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Heligmosomoides polygyrus bakeri Induces Tolerogenic Dendritic Cells that Block Colitis and Prevent Antigen-Specific Gut T Cell Responses

Arthur M. Blum,* Long Hang,* Tommy Setiawan,* Joseph P. Urban, Jr.,† Korynn M. Stoyanoff,* John Leung,* and Joel V. Weinstock*

Immunological diseases such as inflammatory bowel disease (IBD) are frequent in less developed countries, possibly because helminths provide protection by modulating host immunity. In IBD murine models, the helminth *Heligmosomoides polygyrus bakeri* prevents colitis. It was determined whether *H. polygyrus bakeri* mediated IBD protection by altering dendritic cell (DC) function. We used a Rag IBD model where animals were reconstituted with IL10+/− T cells, making them susceptible to IBD and with OVA Ag-responsive OT2 T cells, allowing study of a gut antigenic response. Intestinal DC from *H. polygyrus bakeri*-infected Rag mice added to lamina propria mononuclear cells (LPMC) isolated from colitic animals blocked OVA IFN-γ/IL-17 responses in vitro through direct contact with the inflammatory LPMC. DC from uninfected Rag mice displayed no regulatory activity. Transfer of DC from *H. polygyrus bakeri*-infected mice into Rag mice reconstituted with IL10+/− T cells protected animals from IBD, and LPMC from these mice lost OVA responsiveness. After DC transfer, OT2 T cells populated the intestines normally. However, the OT2 T cells were rendered Ag nonresponsive through regulatory action of LPMC non-T cells. The process of regulation appeared to be regulatory T cell independent. Thus, *H. polygyrus bakeri* modulates intestinal DC function, rendering them tolerogenic. This appears to be an important mechanism through which *H. polygyrus bakeri* suppresses colitis. IFN-γ and IL-17 are colitogenic. The capacity of these DC to block a gut Ag-specific IFN-γ/IL-17 T cell response also is significant. *The Journal of Immunology*, 2012, 189: 2512–2520.

Many immune-mediated diseases such as inflammatory bowel disease (IBD) are rare in less developed countries. Helminths are worm-like organisms common in the geographic regions with a low prevalence of these conditions. Helminths strongly interact with the immune system of their hosts to modulate immune reactivity. A growing body of data suggests that helminthic infections can protect the host from developing these immunological diseases (1). Helminths also are being tested that helminthic infections can protect the host from developing immune-mediated diseases such as *T. muris* (2), *Trichinella spiralis* (4), *H. polygyrus bakeri*, or *Hymenolepis diminuta* (5) or that receives nonviable schistosome ova (6) are protected from IBD.

The mechanisms through which helminths function to alleviate disease remain incompletely understood. The protection probably involves induction of several independent immune regulatory processes. At least part of the protection depends on parasite induction of regulatory-type cytokines in the host. After *H. polygyrus bakeri* infection, lamina propria (LP) T cells from healthy wild-type (WT) mice make much more regulatory cytokines such as IL-10 and TGF-β (7). Regulatory T cells also are important. T cells from the mesenteric lymph nodes (MLN) of *H. polygyrus bakeri*-infected IL10−/− mice abrogate established colitis when transferred into IL10−/− recipients (3). Helminth colonization induces Foxp3 expression in MLN and LP T cells. In a Rag-IL10−/− T cell transfer colitis model of IBD, *H. polygyrus bakeri* acquire CD8+ T cells to reverse the disease process (8). *H. polygyrus bakeri* infection also elicits a regulatory T cell population that improves allergen-induced lung pathology (9).

Interactions with cells of the innate immune system may be part of the regulatory process. Schistosomes protect BALB/c mice from dextran sodium sulfate enteritis via a macrophage-dependent mechanism not requiring regulatory T cells (10). Alternatively activated macrophages may protect animal models from asthma (11).

We previously showed that Rag mice briefly exposed to *H. polygyrus bakeri* only before T cell reconstitution are rendered resistant to colitis (12). This suggested that interactions of *H. polygyrus bakeri* just with cellular components of innate immunity are sufficient to protect these animals from IBD. LP dendritic cells (DC) from *H. polygyrus bakeri*-infected Rag mice present Ag poorly compared with the DC from the unaffected animals. Following *H. polygyrus bakeri* infection, intestinal DC of a Rag mouse show
changes in expression of cell surface molecules. This raised the possibility that alterations in DC function were part of the protective process.

The current study found that intestinal and MLN DC from Rag mice infected with *H. polygyrus bakeri* function as immune regulatory cells that can limit antigenic responses in the gut. Moreover, transfer of these DC into colitis-prone mice is sufficient to protect the animals from IBD even in the absence of *H. polygyrus bakeri* infection. The tolerogenic DC require direct contact with the proinflammatory LP mononuclear cells (LPMC) to block antigenic responses and do not appear to function through altering the frequency of Foxp3+ T cells in the gut or MLN, or by stimulating IL-10 or TGF-β production.

### Materials and Methods

**Mouse and *H. polygyrus bakeri* infection**

This study used C57BL/6 Rag2, OT2, and IL10−/− mice (The Jackson Laboratory, Bar Harbor, ME). In some experiments, we used C57BL/6 OT2 CD45.1 mice (a gift from Dr. A.L. Fuhlbrigge, Brigham and Women’s Hospital, Boston, MA) or IL10 knockout (KO) Foxp3 gEFP reporter mice (a gift from Dr. C. Nagler, University of Chicago, Chicago, IL). 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.

For these experiments, 5- to 6-wk-old mice were colonized with 125 *H. polygyrus bakeri* third-stage larvae by oral gavage. Infective enshathed *H. polygyrus bakeri* L3 (U.S. National Helminthological Collection number 81930) were obtained from fecal cultures of eggs by the modified Baermann method and stored at 4°C. To deworm mice, animals were given a single dose of pyrantel pamoate (0.5 mg/mouse; Sigma-Aldrich, St. Louis, MO) via oral gavage. Deworming was confirmed by the absence of adult worms in the duodenum at time of animal sacrifice and by preliminary experiments that showed the absence of worms in the gastrointestinal tract of mice 1 wk after taking pyrantel pamoate.

**Dispersion of splenocytes or MLN cells and splenic T cell enrichment**

Single-cell suspensions of splenocytes or MLN cells were prepared by gentle teasing in RPMI 1640 medium (Life Technologies, Grand Island, NY). The cells were washed three times in RPMI 1640 medium. 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 (number 19751; StemCell Technologies, Vancouver, BC, Canada). Viability was determined using exclusion of trypan blue dye.

**LPMC isolation and LP cell fractionation**

Gut LPMC were isolated from the terminal ileum (TI) or colon as described previously (3). Cell viability was 90% as determined by trypan blue exclusion. DC (CD11c+) were isolated from dispersed LPMC or MLN cells with mouse CD11c+ selection kit number 18758 from StemCell Technologies that was used according to kit directions. The beads used to isolate CD11c+ cells from the MLN and gut recovered ~85% of these cells at ~95% purity as determined by FACS. The beads displayed equal efficiency at isolating both the CD11c+ and CD11c− subsets.

**Colitis model**

Rag mice of similar age (usually 6–7 wk old) were reconstituted i.p. with 4 × 10^6 IL10−/− splenic T cells. In some experiments, mice also received 2 × 10^6 OT2 splenic T cells given i.p. One week later, the animals were administered piroxicam mixed into their feed for 2 wk (piroxicam at 40 mg/250 g chow week 1 and 60 mg/250 g chow week 2). Two weeks after induction of colitis, the piroxicam (Sigma-Aldrich) was stopped, and the colitis was studied 1 wk later (week 4 after cell transfer). The colons after induction of colitis, the piroxicam (nonsteroidal anti-inflammatory drug [NSAID]), as outlined above, to induce colitis. One week after stopping the piroxicam, the colon was examined and scored blinded for severity of colitis on a four-point scale (13). LPMC were isolated from the gut for cell culture with or without OVA to measure IFN-γ and IL-17 release, or they were subject to flow analysis (see Fig. 4).

**Sandwich ELISAs**

ELISAs were performed using paired Abs (R&D Systems, Minneapolis, MN), according to the 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-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 supernatants. IFN-γ was detected with polyclonal rabbit anti–IFN-γ (a gift from Dr. M. Wilson, University of Iowa), followed by biotinylated goat anti-rabbit IgG (AlexCell, Westbury, NY). Total TGF-β was measured using acid-treated supernatant and mAbs240 for capture and biotinylated chicken IgY BAF240 for detection (both R&D Systems).

**Flow cytometry analysis**

LPMC were washed twice and adjusted to 10^7 cells/ml in FACS buffer (LGM) and stained with saturating amounts of conjugated mAb for 30 min at 4°C. Following staining, cells were washed and resuspended in LGM 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 -PE-Cy5, anti–CD4-PE or -PE-Cy5, anti–CD45.2-allophycocyanin, or cell sorting were anti–Thy1.2-FITC or -PE 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 -PE-Cy5, anti–CD4-PE or -PE-Cy5, anti–CD45.2-allophycocyanin, anti–CD8-PE-Cy5, anti–CD95-PE-Cy5, and anti–CD122-PE-Cy5 (all from eBioscience). Statistical analysis. Data are means ± SE of multiple determinations. Difference between two groups was compared using Student t test. Multiple group comparisons used analysis of variation and Dunnett’s t test. A p value < 0.05 was considered significant.

**Results**

To study colitis, experiments used a well established Rag IL10−/− T cell transfer model of IBD, which renders these animals sus-
FACS was used to determine the percentage of CD4+ T cells expressing Foxp3. This model is relevant to human IBD because defects in the IL10 signaling pathway predispose people to IBD (15). Many such models develop colitis inconsistently. To enhance expression of disease, 1 wk after cell transfer, mice were fed an NSAID (piroxicam) for 2 wk. This resulted in all animals developing severe colitis that was evident 1 wk thereafter stopping the NSAID. (Four weeks after cell transfer.) The NSAID disrupts the production of protective arachidonic acid metabolites in the mucosa (13), making the animals more prone to IBD. This is relevant to human IBD, because administration of many types of NSAIDs worsen the disease (16, 17). We also adoptively transferred transgenic OT2 T cells bearing MHC class II-dependent TCR that recognize OVA into the Rag mice concomitantly with the other cells so that we could study an Ag-specific T cell response in the gut LP. Isolated LPMC from these T cell-reconstituted Rag animals respond to OVA with IFN-γ and IL-17 release. These cytokines were of particular interest, because these cytokines help drive the disease in human IBD and in many animal models of this condition (18–20).

Rag mice exposed to *H. polygyrus bakeri* only before T cell reconstitution are protected from colitis (12). Because Rag mice lack functional T and B cells, this suggested that interactions of *H. polygyrus bakeri* just with cellular components of innate immunity are sufficient to provide this protection. To extend this observation, these experiments tested the hypothesis that infection with *H. polygyrus bakeri* induces tolerogenic DC in the gut, which are an important component of the protective process.

**DC isolated from Rag mice infected with H. polygyrus bakeri cultured in vitro with LPMC block OVA-induced cytokine secretion**

In the initial experiments, it was determined if DC from *H. polygyrus bakeri*-infected Rag mice could impair the natural interaction of intestinal proinflammatory DC with their effector T cells such as to impede antigenic responses. Rag mice were reconstituted with IL10−/− splenic T cells, and treated with piroxicam to induce colitis. LPMC were isolated from these animals, mixed with splenic OT2 T cells and cultured in vitro with or without OVA Ag to stimulate cytokine production. Some wells also received supplemental DC isolated from the intestines of a separate group of Rag mice that had been infected with *H. polygyrus bakeri* for 2 wk before sacrifice (Hpb DC). The control for *H. polygyrus bakeri* infection was wells given DC isolated from the gut of age-matched Rag mice that never had *H. polygyrus bakeri* infection (DC no Hpb) (Fig. 1A).

Isolated LPMC from the mice with colitis produced IFN-γ and IL-17 constitutively and secreted substantially more when cultured with OVA (Fig. 1B). Addition of Hpb DC, given at a ratio of 1:5 (DC/LPMC), did not affect constitutive cytokine secretion, but addition of splenic OT2 T cells and cultured in vitro with or without OVA Ag to stimulate cytokine production. Some wells also received supplemental DC isolated from the intestines of a separate group of Rag mice that had been infected with *H. polygyrus bakeri* for 2 wk before sacrifice (Hpb DC). The control for *H. polygyrus bakeri* infection was wells given DC isolated from the gut of age-matched Rag mice that never had *H. polygyrus bakeri* infection (DC no Hpb) (Fig. 1A).

Isolated LPMC from the mice with colitis produced IFN-γ and IL-17 constitutively and secreted substantially more when cultured with OVA (Fig. 1B). Addition of Hpb DC, given at a ratio of 1:5 (DC/LPMC), did not affect constitutive cytokine secretion, but totally blocked OVA-induced cytokine stimulation. Addition of the DC no Hpb control cells did not block cytokine release (Fig. 1B).

**FIGURE 1.** Intestinal DC from *H. polygyrus bakeri*-infected Rag mice inhibit IFN-γ and IL-17 production. (A) The experiment design. Rag mice were reconstituted with 10⁶ IL10 KO T cells or 10⁶ IL10 KO/Foxp3eGFP T cells, given i.p., and exposed to piroxicam (NSAID) to induce colitis as outlined above in (A) and in Materials and Methods. One week after stopping the piroxicam, Rag mice were infected with *H. polygyrus bakeri* for 2 wk, and then, the *H. polygyrus bakeri* were eliminated by treating the animals with a single oral dose of pyrantel pamoate. Age-matched control animals with a single oral dose of pyrantel pamoate. Age-matched control

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### Materials and Methods

Colitis LP cells cultured at 2 × 10⁹ cells/well in RPMI 1640 complete medium for 48 h in 96-well round-bottom plates with or without OVA (10 µg/ml) to stimulate IFN-γ and IL-17 release. However, adding DC from mice with no prior infection (DC no Hpb) had no such effect. Data are mean ± SE of three to four independent experiments. *p < 0.01, for IFN-γ and IL-17, LPMC ± OVA or LPMC + DC (no Hpb) ± OVA. (C) FACS analysis. The table in (C) shows that LPMC maintained in culture loose most of their Foxp3+ T cells and that adding Rag intestinal DC to the LPMC cultures does not maintain Foxp3+ T cell content. Data are means of three experiments ± SE.
Supernatants from these cell cultures also were examined for IL-10, TGF-β, and IL-4 content, which are cytokines that can regulate or alter antigenic responses. IL-10 or TGF-β was secreted in similar amounts under all culture situations (Fig. 1B). OVA added to the cultures did not increase either IL-10 or TGF-β release. No IL-4 was detected in any cultures.

Other experiments used DC isolated from the MLN of Rag mice with or without *H. polygyrus bakeri* infection. DC from the MLN of *H. polygyrus bakeri*-infected mice when added to the isolated LPMC from the colitic animals (1:5 cell ratio) also blocked OVA-induced cytokine responses equally as well as gut DC. The OVA response was not affected by the addition of MLN DC from Rag mice who never received *H. polygyrus bakeri* infection (data not shown).

**DC regulation of the OVA response requires cell contact**

Using Transwell plates, it next was determined whether DC regulation of OVA-induced cytokine production required cell contact. Once more, LPMC isolated from colitic animals were mixed with splenic OT2 T cells and cultured in the outer wells of a Transwell plate alone or with gut *Hpb* DC. Some wells also receive OVA to stimulate cytokine release. As expected, OVA induced strong cytokine responses only in the absence of the supplemental DC (Fig. 2). However, *Hpb* DC did not alter OVA-induced cytokine secretion when the *Hpb* DC were placed in the inner well separated by a semipermeable membrane from the OT2 T cells and colitic LPMC present in the outer well.

The intestines of Rag mice protected from colitis by *H. polygyrus bakeri* infection contain tolerogenic DC

The above experiments suggested that infection of Rag mice with *H. polygyrus bakeri* induces DC within the gut that can block the intestinal OVA response in vitro. To further test the significance of this observation, it was determined whether animals protected from colitis by *H. polygyrus bakeri* infection display tolerogenic-type DC within their intestines.

Rag mice were reconstituted with IL10−/− T cells and OT2 T cells and then treated with piroxicam to induce colitis. One week after stopping the piroxicam, the mice were sacrificed, and their colitis also was scored for intensity of inflammation by examining histological sections. In these mice, the intestine was dissociated to isolate DC, which were added to LPMC from the severely colitic mice and then cultured in vitro to see whether these DC would affect the LPMC OVA response (Fig. 3A).

**FIGURE 2.** DC regulation requires cell contact. The experiment was designed as outlined in Fig. 1, and 96-well Transwell plates were used in this experiment. Each well contained complete RPMI 1640 medium (200 μl/well). LPMC were mixed with splenic OT2 T cells (ratio 2:1) and placed in the outer chamber (2 × 10⁵ cells/well). In some cases, the outer chamber also contained DC (4 × 10⁵ cells/well) derived from TI of Rag mice infected with *H. polygyrus bakeri* for 2 wk (*Hpb* DC). Still, other wells contained *Hpb* DC placed in the inner chamber with the LPMC + OT2 T cells in the outer chamber separated by a 0.4-μm semipermeable membrane. Data are mean ± SE of three independent experiments. *p < 0.01, LPMC ± OVA or LPMC ± OVA cultured without contact with *Hpb* DC.

**FIGURE 3.** *H. polygyrus bakeri* infection induces tolerogenic DC in the Gut upon suppression of the colitis. (A) The experiment design. To obtain LPMC from colitic mice that would respond to OVA, Rag mice first were reconstituted i.p. with 10⁵ splenic T cells from IL10 KO mice and 3 × 10⁵ OT2 T cells from WT OT2 mice. Then, the Rag mice were given piroxicam (NSAID) to induce colitis. One week after stopping the piroxicam, LPMC were isolated from the TI. To produce DC that could block the LPMC antigenic response, a second group of Rag mice were exposed to *H. polygyrus bakeri* for 2 wk before reconstitution with IL10 KO T cell or IL10 KO/Foxp3eGFP reporter T cells and OT2 T cells. Then, they were give piroxicam. DC or Foxp3+ T cells were isolated from the TI 1 wk after stopping the piroxicam. (B) Cytokines analysis. Colitis LP cells cultured at 2 × 10⁵ cells/well in RPMI 1640 complete medium for 48 h in 96-well round-bottom plates with or without OVA (10 μg/ml) to stimulate IFN-γ and IL-17 release. To determine whether these intestinal DC or Foxp3+ T cells could modulate the LPMC cytokine response to Ag (OVA), the DC (*Hpb* DC) or Foxp3+ T cells (*Hpb Foxp3) were added to some of the LPMC cultures at the ratio of 1:5. DC isolated from the TI of mice devoid of the infection, but otherwise manipulated similarly to the mice that received the infection, served as a source for control DC (DC no *Hpb*). Data are mean ± SE of three independent experiments. *p < 0.01, LPMC ± OVA, LPMC+DC no *Hp b* ± OVA, or LPMC+Foxp3 ± OVA.
IL10 KO T cell-reconstituted Rag mice developed severe colitis after piroxicam treatment, unless they were infected with *H. polygyrus bakeri* before the T cell reconstitution (no *H. polygyrus bakeri* infection versus *H. polygyrus bakeri* infection: inflammatory score 3.7 ± 0.4 versus 1.2 ± 0.3, mean ± SE, *n* = 3 separate experiments; *p* < 0.01). Isolated LPMC from the colitic mice (no *H. polygyrus bakeri* infection) cultured in vitro-produced IFN-γ and IL-17 whose production was enhanced by OVA stimulation (Fig. 3B). When the LPMC were mixed with DC from the Rag mice protected from colitis by *H. polygyrus bakeri* infection and cultured, OVA stimulation failed to excite more cytokine release. However, mixing LPMC with intestinal DC from mice never infected with *H. polygyrus bakeri* did not affect either constitutive or OVA-induced IFN-γ or IL-17 production (Fig. 3B).

**Hpb DC are sufficient to prevent colitis**

The next series of experiments ascertained whether DC from *H. polygyrus bakeri*-infected Rag mice could adoptively transfer protection from colitis. Rag mice received IL10−/− and OT2 T cells administered i.p. Some mice also received DC isolated from the MLN of Rag mice that had been infected with *H. polygyrus bakeri* for 2 wk. An additional group of animals received DC from the MLN of age-matched Rag mice that never experienced *H. polygyrus bakeri* infection. After NSAID administration, the animals were sacrificed at the appropriate time to assess severity of colitis and the responsiveness of isolated LPMC to OVA stimulation (Fig. 4A).

Fig. 4B shows, as expected, that severe colitis developed in mice receiving no DC. Mice receiving DC from *H. polygyrus bakeri*-infected animals displayed much less colonic inflammation. Adoptive transfer of DC from animals never infected with *H. polygyrus bakeri* did not affect the intensity of the inflammatory response.

LPMC isolated from mice that received no DC or DC from uninfected mice produced both IFN-γ and IL-17 constitutively when cultured in vitro and even more after OVA stimulation. However, LPMC from Rag mice receiving DC from the infected animals lost their responsiveness to OVA stimulation (Fig. 4C). Culture supernatants also contained IL-10. OVA stimulation did not increase IL-10 secretion. LPMC isolated from the colons of mice that received no DC or DC from uninfected mice produced comparable amounts of this cytokine. However, LPMC from mice receiving DC from the infected animals actually made much less IL-10 (Fig. 4C).

Also measured were TGF-β and IL-4. All cultures produced comparable amounts of TGF-β, and OVA did not stimulate more TGF-β release (Fig. 4C). IL-4 was not detected in any cell culture.

Additional experiments were conducted as described above, except that mice received DC isolated from the TI of Rag mice with or without *H. polygyrus bakeri* infection. Comparable to DC from MLN, gut DC from *H. polygyrus bakeri*-infected animals transferred into *H. polygyrus bakeri*-naive recipients protected the mice from colitis and blocked mucosal responsiveness to OVA. Once again, intestinal DC from uninfected mice had no effect (data not shown).
Adoptive transfer of DC from H. polygyrus bakeri-infected mice into Rag recipients does not impair OT2 T cell accumulation in the gut and MLN

The loss of LPMC OVA responsiveness after adoptive transfer of DC from H. polygyrus bakeri-infected mice could have signified that DC transfer simply interfered with normal OT2 T cell accumulation in the LP. T cells from C57BL/6 mice express the molecule CD45.2. We thus reconstituted Rag mice with OT2 T cells from transgenic C57BL/6 mice expressing CD45.1 so that OT2 cells within the isolated LPMC could be distinguished from the IL10−/− T cells through differential CD45 display. In this mouse, transgenic TCR expressed on the CD4+ OT2 T cell subset recognize the OVA.

One group of Rag mice received CD45.1+ OT2 T cells and CD45.2+IL10−/− T cells and then were exposed to piroxicam to induce colitis. Another group of Rag animals were reconstituted with T cells and treated as above, except they also received DC from the MLN of Rag mice infected with H. polygyrus bakeri to abolish the intestinal response to OVA. In the colitic mice, <0.5% of the LP or MLN CD4+ T cells displayed CD45.1 (Fig. 5). The relative number of LP or MLN CD4+ T cells expressing CD45.1 did not diminish in Rag mice receiving the DC.

After DC transfer, isolated intestinal OT2 T cells display retained capacity to produce cytokines after OVA stimulation when cultured in a permissive environment

Because OT2 T cells readily accumulated in the gut even after tolerogenic DC transfer, it next was determined whether the unresponsive intestinal OT2 T cells could regain OVA responsiveness in a more permissive environment. Once again, one group of Rag mice received CD45.1+ OT2 T cells and CD45.2+IL10−/− T cells and then were exposed to piroxicam to induce colitis. Another group of Rag animals were reconstituted with T cells and treated as above, except they also received DC from the MLN of Rag mice infected with H. polygyrus bakeri to abolish the intestinal response to OVA. One week after stopping the piroxicam, LPMC were isolated from the TI of both groups, and CD45.1+ OT2 T cells were separated from the dispersed LPMC using FACS (Hpb OT2 and OT2). Also, the residual LPMC were depleted of T cells (Hpb non-T and non-T). OT2 T cells were mixed with either one or the other non-T cell preparation and cultured with or without OVA. All cultures produced comparable amounts of IFN-γ and IL-17 without OVA stimulation (Table I). OT2 cells from either source did not respond to OVA if the cells were mixed with T cell-depleted LPMC isolated from the intestines of Rag mice that received DC (Table I). However, OT2 cells responded well to OVA if they were cocultured with T cell-depleted LPMC from mice that did not receive DC. These experiments suggested that OT2 T cell Ag unresponsiveness was at least partly secondary to the presence of the non-T cell element of the LP and that they do not lose the capacity to respond to OVA.

H. polygyrus bakeri infection does not increase the number of T cells expressing Foxp3 in LPMC cultures, the intestines, or MLN

Using our murine model of IBD, the above experiments showed that H. polygyrus bakeri infection induced a regulatory-type intestinal DC that could inhibit colitis and antigen-specific T cell responses in the gut. DC can drive expansion of Foxp3+ T cells in tissues. Thus, it was deemed important to the investigator whether DC regulation likely was working through induction of Foxp3+ T cells.

It first was determined whether mixing tolerogenic DC with LPMC expanded the number of Foxp3+ T cells in LPMC cultures. For these experiments, Rag mice were reconstituted with IL10 KO/Foxp3eGFP reporter T cells before induction of colitis so that Foxp3+ T cells could be monitored, by their endogenous expression of eGFP, in the subsequent LPMC cell cultures. Fig. 1C shows that LPMC from the mice with colitis when cultured in vitro will lose most of their Foxp3+ T cells. Culturing LPMC with intestinal DC from H. polygyrus bakeri-infected mice did not expand the number Foxp3+ T cells within these mixed cell cultures.

Using one of the experimental protocols used to generate tolerogenic intestinal DC, it also was ascertained whether the frequency of Foxp3+ T cells in the TI, colon, or MLN would increase if Rag mice were infected with H. polygyrus bakeri before T cell reconstitution. Rag mice were infected with H. polygyrus bakeri for 2 wk and then dewormed by treatment with pyrantel pamoate. One week later, the mice received Foxp3eGFP IL10−/− reporter T cells and OT2 T cells. In some experiments, mice received piroxicam to induce colitis, whereas others received no such treatment and remained free of colitis (Fig. 6A). At the time of sacrifice, dispersed MLN cells and LPMC isolated from the TI and colon were analyzed for Foxp3 T cell expression using flow analysis to detect eGFP+ T cells. Approximately 98% of the Foxp3+ T cells in the MLN, TI, and colon were CD4+. In mice that did not receive piroxicam (no colitis), H. polygyrus bakeri infection induced a substantial decrease in the relative number of CD4− T cells expressing Foxp3 in the three tissues studied (Fig. 6B, 6C, no colitis). In the mice fed piroxicam to induce colitis, colonic inflammation was associated with a drop in the proportion of CD4+ T cells expressing Foxp3. Moreover, H. polygyrus bakeri exposure protected the animals from colitis (inflammatory score: no H. polygyrus bakeri versus H. polygyrus bakeri 3.5 ± 0.5 versus 1.0 ± 0.2, ± SE, n = 3) without significantly altering the proportion of CD4− T cells expressing Foxp3 (Fig. 6B, 6C, colitis).

Unlike intestinal DC, intestinal Foxp3+ T cells from H. polygyrus bakeri-infected mice display no regulatory activity

Studies also examined whether coculturing Foxp3+ T cells, isolated from the gut of mice protected from colitis by H. polygyrus bakeri infection, with LPMC from the colitic animals would suppress IFN-γ or IL-17 secretion. These experiments once again used IL10 KO/Foxp3eGFP reporter T cells to allow identification and isolation of gut Foxp3+ T cells using FACS. Unlike the iso-

FIGURE 5. DC transfer does not diminish the number of OT2 T cells that accumulate in the gut or MLN. Rag mice received 4 × 10^6 splenic IL10 KO T cells displaying CD45.2 and 2 × 10^6 OT2 T cells expressing CD45.1 given by i.p injection. Some mice also received DC (CD11c+) (4 × 10^7/mouse) from the MLN of Rag mice previously infected with H. polygyrus bakeri, whereas other animals did not receive DC (no CD11c). At the end of the experiment, LPMC were isolated from the TI of either the control or CD11c transfer group. Also studied were dispersed MLN cells. Cells in the lymphoid gate were subject to flow analysis to determine the relative number of LP T cells expressing CD45.1. For each group, flow analysis was performed on pooled LPMC or MLN cells isolated from four individual mice. Data are representative of three independent experiments.
purity. The beads displayed equal efficiency at isolating both the CD11c+ population were not changed by separate experiments (CD11chi or CD11clo, uninfected versus infected after H. polygyrus bakeri infection (1.1 6 SE, three experiments). The relative number of CD11c cells in MLN did not change after H. polygyrus bakeri infection (1.1 6 SE), and the relatively number of CD11chi 14.6 6 0.9% (mean 6 0.01). CD103 expression was noted almost exclusively in the CD11c+ population (CD11c+ 14.6 6 2.1%, CD11c0 1.7 6 0.6%, n = 3, 6 SE), and the relatively number of CD11chi and CD11clo cells expressing CD103 did not significantly change after H. polygyrus bakeri infection (data not shown).

In the gut, only one CD11c+ cell subset could be readily distinguished by the expression of CD11c staining intensity. The percentage of LPMC expressing CD11c was not affected by H. polygyrus bakeri infection (uninfected 2.6 6 0.7% versus infected 2.3 6 0.8%, mean 6 SE, n = 3; p > 0.05). CD103 expression on the CD11c+ population were not changed by H. polygyrus bakeri infection (uninfected 25.0 6 1.4% versus infected 24.0 6 1.8%, mean 6 SE, n = 3; p > 0.05).

The beads (StemCell Technologies) used to isolate CD11c+ cells from the MLN and gut recovered ~85% of these cells at ~95% purity. The beads displayed equal efficiency at isolating both the CD11c+ and CD11c0 subset.

Discussion

A critical point made by this investigation is that exposure of Rag mice to H. polygyrus bakeri induces tolerogenic type DC in the intestines. This implies that H. polygyrus bakeri does not require direct interaction with T or B cells to render intestinal DC regulatory. These tolerogenic DC appear to be an important mechanism in H. polygyrus bakeri induction protect from colitis, because DC transfer is sufficient to render animals resistant to IBD even if the recipient mice never experienced H. polygyrus bakeri infection.

The experiments presented in this paper showed that adoptive transfer of DC isolated from the MLN or intestines of Rag mice infected with H. polygyrus bakeri could block colitis and intestinal OVA-induced cytokine responses in vivo. Transfer of DC from uninfected Rag mice had no effect. Thus, the process of infection altered the function of the DC in the gut and MLN of Rag mice, without the aid of T or B cell interactions, rendering these DC capable of modulating mucosal immunity and protecting animals from IBD.

The loss of LPMC antigenic responsiveness after transfer of DC from H. polygyrus bakeri-infected mice could have signified that DC transfer interfered with normal OT2 T cell population of the LP. Our studies using OT2 T cells that expressed CD45.1, which allowed visualization of the OT2 T cells in the LP, showed that this was not the case. Because OT2 T cells populated the LP appropriately, it also was determined whether the LP CD45.1+ OT2 T cells, which were not responsive to OVA stimulation in their natural environment after DC transfer, would respond to Ag if placed in a more permissive environment. Our data suggest that the OT2 T cells retained the capacity to respond to Ag but were being held in check by the non-T cell elements of the LP.

Various additional in vitro experiments revealed that OT2 T cells mixed with LPMC from colitic mice produced large amounts of IFN-γ and IL-17 upon OVA stimulation. However, adding intestinal or MLN DC only from the H. polygyrus bakeri-infected mice blocked this response. Although helminths interacting with DC can drive a Th2 response (21–23), the cultures contained no measurable IL-4, suggesting that the loss of IFN-γ and IL-17 secretion in response to OVA was not simply a shift from a Th1/Th17 response to that of Th2.

The composite of all these data support the contention that H. polygyrus bakeri infection modulates DC function, rendering these cells highly regulatory. Because inappropriate T cell activation to luminal Ags is believed to underlie the etiology of human IBD, the capacity of these DC to block an Ag-specific T cell response in the gut could be an important mechanism through which these cells work to suppress colitis.

The in vitro DC/LPMC mix experiments suggested that these tolerogenic DC interfered with effector T cell/proinflammatory DC interactions. The Transwell experiments showed that the tolerogenic DC required direct physical interaction with other LPMC to block the OVA-induced cytokine response.

We previously showed that H. polygyrus bakeri infection had a prominent effect on the capacity of intestinal DC in Rag mice to

<table>
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<th>IFN-γ</th>
<th>Hpb OT2 + Non-T</th>
<th>Hpb OT2 + Hpb Non-T</th>
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<tr>
<td>Cells</td>
<td>BD*</td>
<td>BD</td>
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<td>BD</td>
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<td>+OVA</td>
<td>93 ± 5</td>
<td>72 ± 10</td>
<td>142 ± 22</td>
<td>80 ± 6</td>
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Intestinal OT2 T cells isolated from Rag mice who received DC from H. polygyrus bakeri-infected animals (Hpb OT2) respond to OVA when cultured with T cell-depleted LPMC (non-T) from Rag mice who did not receive DC. Conversely, the OT2 cells fail to respond to Ag when they are cultured with their own LPMC (Hpb OT2 + Hpb non-T). Moreover, intestinal OT2 T cells that normally respond to OVA (OT2 + non-T) will fail to do so if they are cultured with Hpb non-T. The experimental design is outlined in Fig. 4A, except that the recipient mice received CD45.1+ OT2 T cells to allow OT2 T cell isolation via FACS. Some mice also received DC from the MLN of Rag mice infected with H. polygyrus bakeri for 2 wk (Hpb DC), whereas other mice received no DC. At the time of sacrifice, OT2 T cells were isolated from dispersed LPMC using FACS and anti-CD45.1 mAb. LPMC-deleted T cells (non-T) were obtained using FACS and anti-Thy 1.2 mAb. OT2 T cells (106) from the TI group (Hpb non-T or non-T, no DC) and cultured without or with OVA (10 µg/ml) for 48 h. Data are mean picograms per milliliter ± SE of three independent determinations.

*p < 0.01, cytokine versus cytokine + OVA.
altered the proportion of CD4+ T cells expressing Foxp3 within these tissues. Data are representative of three separate experiments. For each group, flow analysis was performed on pooled LPMC isolated from three to four individual mice. (C) The mean flow data from three individual experiments \( \pm \) SE.

display various surface proteins (12). This included a decreased expression of the costimulatory molecules CD80 and CD86. More widely expressed were PDCA-1, a marker of plasmacytoid DC (24), and CD40. Studying MLN, we observed an increase in DC that were CD11c\(^{lo}\). This shift was previously reported using WT mice with \( H. polygyrus bakeri \) infection (25). In our study, a CD11c\(^{hi}\) subset was not observed in the intestine of our Rag mice before or after \( H. polygyrus bakeri \) infection. DC in MLN, Peyer’s patches, and the LP are continuously exposed to a multitude of luminal Ags. In different regions of the intestines, various DC subsets have been characterized based on the expression of cell surface molecules such as CD103, CX3Crl, and CD11b (26). It is yet to be determined whether these \( H. polygyrus bakeri \)-induced tolerogenic DC can be identified through a unique display of cell surface molecules.

Intestinal DC also can produce regulatory cytokines such as IL-10 and TGF-\( \beta \) or can induce regulatory-type T cells to make these cytokines (26), which in turn can modulate T cell responses. Adding tolerogenic DC to the LPMC/OT2 T cell cultures did not enhance either IL-10 or TGF-\( \beta \) production. Isolated LPMC from Rag mice that received tolerogenic DC in vivo to prevent colitis actually displayed a decreased capacity to produce IL-10. Under all culture conditions, the release of IL-10 and TGF-\( \beta \) was independent of OVA stimulation and thus assumed to be independent of the T cell response. There even was no response to anti-CD3/CD28 mAb stimulation. Except for the OT2 T cells, the T cells used to reconstitute Rag mice were derived from IL-10\(^{-/-}\) mice further supporting this contention. The failure of \( H. polygyrus bakeri \)-induced DC to increase IL-10 or TGF-\( \beta \) production in the cultures suggests that rising levels of these soluble regulatory cytokines were not a critical part of the regulatory mechanism. We previously showed that DC isolated from the gut of \( H. polygyrus bakeri \)-infected Rag mice, compared with DC from uninfected control animals, do not produce more IL-10 or TGF-\( \beta \) when cultured in vitro with or without LPS or CpG oligo stimulation (12).

A diverse array of DC phenotypes has been reported to help induce and maintain immune tolerance. CD11c\(^{hi}\) DC that lack the usual markers of plasmacytoid DC (e.g., CD8, CD103, PDCA, and Siglec-H) expand in the MLN of immunologically intact mice after \( H. polygyrus bakeri \) infection (25). This subset can drive Ag-specific CD4\(^{+}\)Foxp3\(^{+}\) T cell expression in cell cultures (25). \( H. polygyrus bakeri \) infection expands the number of CD4\(^{+}\)Foxp3\(^{+}\) T cells in the MLN of infected mice, suggesting that this CD11c\(^{lo}\) DC has relevance to the natural infection. It also is reported that \( H. polygyrus bakeri \) and other helminths secrete proteins that can induce de novo Foxp3 expression in T cells without the aid of DC (27). There are reports that CD11c\(^{hi}\) DC expressing CD103 also can induce regulatory T cell expression in vitro (28, 29), although there is some dispute whether they can function in this capacity in the colon (30). We did not detect a relative increase in the number of DC expressing CD103. Although our DC isolation technique was able to recover both the CD11c\(^{hi}\) and CD11c\(^{lo}\) subsets from the MLN, we did not determine which specific DC subset was responsible for control of colitis.

As presented in this paper, \( H. polygyrus bakeri \)-induced intestinal DC that block colitis in our IL10\(^{-/-}\) Rag model of IBD, and which inhibit Ag-induced cytokine secretion in the gut, do not appear to mediate their action through expansion of the proportion of T cells expressing Foxp3. This was shown in several different experiments. Among these experiments included a demonstration that intestinal regulatory T cell could not substitute for tolerogenic DC in our in vitro regulation experiments. The previous reports suggesting that \( H. polygyrus bakeri \) promotes Foxp3 expression in the MLN are not necessarily at odds with our findings. The IBD model used in this study is unique in that the Rag mice, which lack functional T and B cells, are only exposed to \( H. polygyrus bakeri \) before T cell reconstitution. \( H. polygyrus bakeri \) infection after T cell reconstitution can enhance Foxp3 expression in Rag mice (3). It is likely that helminths function through several indepen-
ient regulatory pathways to protect mice from IBD. Tolerogenic DC can limit T cell responses via regulatory T cell-independent mechanisms including induction of effector T cell anergy (31, 32) or apoptosis through upregulation of Fas ligand expression (33). Thus, there is precedence for our observation. DC can promote immune tolerance and protect mice from colitis. Tolerance induction can occur through DC induction of regulatory-type T cells that can express Foxp3 and/or produce IL-10 or TGFB (34, 35). The DC also can induce effector T cell deletion or anergy as discussed above. The H. polygyrus bakeri-induced tolerogenic DC appear to function in our IBD model via the latter mechanism. Although the effector T cells are not lost following exposure to the tolerogenic DC, it will be interesting to explore if the proinflammatory DC that drive the immune response are subject to apoptosis, which is a newly appreciated mechanism that can lead to immune tolerance (36). As shown in this paper and elsewhere, tolerogenic-type DC induced by H. polygyrus bakeri exposure can be adoptively transferred to murine recipients to prevent IBD (37), and tolerogenic DC can do likewise in other immunologic diseases (38). It is tempting to speculate that induction of potent tolerogenic DC in vitro through exposure to helminthic products will afford a powerful new approach for the treatment of IBD.

Disclosures

The authors have no financial conflicts of interest.

References