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**Heligmosomoides polygyrus bakeri** Infection Activates Colonic Foxp3+ T Cells Enhancing Their Capacity To Prevent Colitis

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

Helminthic infections protect mice from colitis in murine models of inflammatory bowel disease and also may protect people. Helminths like *Heligmosomoides polygyrus bakeri* can induce regulatory T cells (Treg). Experiments explored whether *H. polygyrus bakeri* infection could protect mice from colitis through activation of colonic Treg and examined mechanisms of action. We showed that *H. polygyrus bakeri* infection increased the number of T cells expressing Foxp3 in the colon. More importantly, Foxp3+/IL-10− and Foxp3+/IL-10− T cell subsets isolated from the colon of *H. polygyrus bakeri*–infected mice prevented colitis when adoptively transferred into a murine model of inflammatory bowel disease, whereas Treg from uninfected mice could not provide protection. Only the transferred colonic Foxp3+/IL-10− T cells from *H. polygyrus bakeri*–infected mice readily accumulated in the colon and mesenteric lymph nodes of recipient mice, and they reconstituted the Foxp3+/IL-10− and Foxp3+/IL-10+ T cell subsets. However, transferred Foxp3+/IL-10+ T cells disappeared. IL-10 expression by Foxp3+ T cells was necessary for colitis prevention. Thus, *H. polygyrus bakeri* infection activates colonic Foxp3+ T cells, making them highly regulatory. The Foxp3+ T cells that fail to express IL-10 may be critical for populating the colon with the Foxp3+/IL-10− T cells, which are required to control colitis. The *Journal of Immunology*, 2013, 191: 1927–1934.

Inflammatory bowel disease (IBD) emerged as a growing health problem in highly developed countries in the latter half of the 20th century, and it presently is advancing in developing countries. Hygiene associated with modern day living, causing alterations in intestinal flora and fauna, is postulated to be a major risk factor (1, 2). Helminthic infections are exceedingly strong inducers of regulatory circuits and cytokines. For example, *Heligmosomoides polygyrus bakeri* infection in mice promotes the production of regulatory molecules in the gut such as IL-10, TGF-β, and PGE2 (3). Loss of helminthic infections could be one of the factors underlying the rise in IBD. Several clinical and epidemiologic studies support this concept (4–6).

Various animal models of IBD suggest that regulatory-type T cells are important for maintaining mucosal immune homeostasis and for controlling colitis (7). *H. polygyrus bakeri* infection stimulates Foxp3 mRNA expression in T cells (8) and expands the number of Foxp3+ T cells in the mesenteric lymph nodes (MLN) (9). Secretions from *H. polygyrus bakeri* can induce T cells to express Foxp3 (9), T cells from the MLN of *H. polygyrus bakeri*–infected, IL-10−deficient mice transferred into helminth-naive animals will arrest colitis, attesting to the importance of T cells in the control of IBD (8). All of this suggests that modulation of regulatory T cell (Treg) function could be an important mechanism through which helminths work to prevent IBD.

IL-10 is a regulatory cytokine important for immune homeostasis in the gut. Mice deficient in IL-10 (10) or IL-10R (11) develop spontaneous colitis. Humans with a mutation in the IL-10R are prone to IBD, further highlighting the importance of IL-10 in the protection from this disease process (12). IL-10 comes from several sources. However, recent evidence suggests that IL-10 from Treg has particular importance in protecting the mucosa from inflammation (13).

Because *H. polygyrus bakeri* can inhibit colitis and is reported to promote Treg development, we used this organism to study the effect of helminth infection on intestinal Foxp3+ T cells. Moreover, we used a Foxp3/IL-10 double-reporter mouse to assess the effect of *H. polygyrus bakeri* infection on Treg subsets distinguished by their differential capacity to make IL-10. Both subsets are naturally expressed in the colon. Experiments revealed that *H. polygyrus bakeri* infection modestly increased the number of colonic T cells expressing Foxp3. Colonic Foxp3+/IL-10− and Foxp3+/IL-10+ T cells from *H. polygyrus bakeri*–infected mice could prevent colitis when adoptively transferred into a murine model of IBD. Colonic Treg from uninfected mice could not mediate such protection. Thus, it appears that *H. polygyrus bakeri* infection activates colonic Foxp3+ T cells, making them highly regulatory. The transferred colonic Foxp3+/IL-10− T cells, from *H. polygyrus bakeri*–infected mice, readily accumulated in the colon and MLN of recipient animals reconstituting both the Foxp3+/IL-10− and Foxp3+/IL-10+ T cell subsets, whereas transferred Foxp3+/IL-10− T cells disappeared. However, only Foxp3+ T cells that make IL-10 could prevent colitis. These additional observations suggest that Foxp3+ T cells that fail to express IL-10 may be critical for populating the colon with Foxp3+/IL-10− T cells, which, in turn, are the most important Treg for control of colitis.
Materials and Methods

Mice
This study used C57BL/6 Rag2 and C57BL/6 wild-type (WT) mice obtained from The Jackson Laboratory (Bar Harbor, ME). Also used were C57BL/6 OT2 CD45.1 mice (a gift of Dr. A. Fuehrbrigg, Brigham and Women’s Hospital, Boston, MA) and IL-10 knockout (KO) Foxp3 mice enhanced GFP (eGFP) reporter mice (gift of Dr. C. Nagler, University of Chicago). Foxp3 mRFP/-IL-10 eGFP double-reporter mice were produced by cross-breeding Foxp3 mRFP, and IL-10 eGFP single-reporter mice were obtained from R. Flavell (Yale University). 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.

H. polygyrus bakeri infection
For the experiments, 5- to 6-week-old mice were colonized with 125 H. polygyrus bakeri third-stage larvae by oral gavage. Infected mice were used after 2 wk. Infective, encephalitogenous H. polygyrus bakeri third-stage larvae (U.S. National Helminthological Collection 81930) were obtained from fecal cultures of eggs by the modified Baermann method and stored at 4°C.

Dispersion of splenic T cells and MLN and splenic T cell enrichment
Single-cell suspensions of splenocytes and 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. Splenic CD4+CD25+ T cells were labeled with FITC-CD4 and PE-CD25 mAbs (eBioscience, San Diego, CA). Then cells were sorted using FACS-MultiLaser system (Cytomation, Fort Collins, CO) with Summit V4.3 software. Viability was determined using exclusion of trypan blue dye.

Lamina propria mononuclear cell isolation, and lamina propria mononuclear cell and MLN cell fractionation
Gut lamina propria (LP) mononuclear cells (LPMC) were isolated from the colon, as described (8). Foxp3 mRFP+/-IL-10 eGFP+ T cells and Foxp3 mRFP+/-IL-10 eGFP+ T cells from dispersed LPMC or MLN cells were isolated by FACS. The viability of the isolated cells always was >95%, as determined using exclusion of trypan blue dye.

Adoptive cell transfer
Rag mice of similar age (usually 5–6 wk old) were reconstituted i.p. with 1 x 107 C57BL/6 WT CD4+CD25+ splenic T cells and 3 x 104 OT2 CD45.1 splenic T cells. In some experiments, mice also received 1 x 105 Foxp3+3, Foxp3+/-IL-103, Foxp3+/-IL-104 or IL-10 KO Foxp3+ colon LPMC T cells given by i.p. injection. In other experiments, they received Foxp3+ MLN T cells.

Colitis model
Mice received CD4+CD25+ splenic T cells from WT and OT2 mice. In some experiments, the animals also received unfractionated colonic Foxp3+3 T cells, or colonic Foxp3+/-IL-103 or Foxp3+/-IL-104 T cells from Foxp3+/-IL-10 double-reporter mice. Some of these reporter mice were infected with H. polygyrus bakeri for 2 wk before sacrifice. One week after T cell transfer, Rag mice were administered piroxicam, a nonsteroidal anti-inflammatory drug (NSAID), mixed into their feed for 2 wk (42 mg/kg). The piroxicam (Sigma-Aldrich) was stopped, and colitis was studied 1 wk after sacrifice. Immuonological data were collected at 4 h, 24 h, and 7 d after�the start of colitis.

Flow cytometry analysis
Isolated LPMC were washed twice, adjusted to 107 cells/ml in LGM, and stained with saturating amounts of conjugated mAb for 30 min at 4°C. Following staining, cells were washed twice, resuspended in LGM for analysis by a BD LSRII Flow Cytometer (BD Biosciences, Mountain View, CA), and analyzed by Summit V4.3 software. Before adding labeled mAb, each tube received 1 μg anti-Fc mAb (eBioscience) to block non-specific binding of conjugated Abs to FcRs. The mAbs used for staining or cell sorting were anti-CD3-FITC, anti-CD4-PE, and anti-CD8-allophycocyanin, anti-CD4-PE-Cy5, anti-CD25-PE, and anti-CD45.1-allophycocyanin, anti-CD8-allophycocyanin, and anti-γ/δ-allophycocyanin (all from eBioscience). Usually, 106 cells were stained, and 105 cells were analyzed.

Immunohistochemical staining for Foxp3 and IL-10 in colon tissue sections
Foxp3 RFP/-IL-10 eGFP reporter mice were left uninfected or infected with H. polygyrus bakeri for 2 wk. Colonies then were fixed in 4% parafomaldehyde (Electron Microscopy Science, Hatfield, PA; catalog 15710) in PBS for 2 h on ice, washed six times in PBS, and stored in 30% sucrose in PBS overnight at 4°C. The tissue then was placed in OCT fluid (Tissue Tek, Torrance, CA; 4583), frozen in liquid nitrogen, and stored at −80°C.

Staining was performed on 4-μm-thick frozen tissue sections. Primary Abs were sheep anti-eGFP (Thermo Fisher, Rockford, IL; O900005W) and rabbit anti-RFP (Life Technologies, Carlsbad, CA; R0367) polyclonal Abs. Secondary Abs were donkey anti-sheep Alexa fluor 488 (713-545-147) (green) and donkey anti-rabbit Alexa fluor 594 (711-585-152) (red) and antibody-purified Abs both from Jackson Immunoresearch Laboratories (West Grove, PA). Frozen sections were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature, rinsed with PBS, and then treated with PBS containing 5% normal donkey serum (Jackson Immunoresearch Laboratories) for 1 h. Slides were washed three times in PBS with Triton X-100 0.1%. Slides were incubated with primary Abs at 1:500 on the same tissue sections for 4 h at room temperature and again washed three times with PBS with Triton X-100. Then slides were incubated with secondary Abs at 1:200 for 1 h in the dark at room temperature and again washed, as described above. Control slides were treated similarly, except no primary Ab was used or they were treated with nonspecific sheep and rabbit Abs. After the incubations and washings, slides were coverslipped using Fluoromount G (Southern Biotech, Birmingham, AL) and viewed using a fluorescent microscope with appropriate filters for the two Alexa dyes in the Tufts Center for Neuroscience Research P30 NS047243.

Quantitative real-time RT-PCR
Total RNA was isolated from individual samples using Quick-RNA Mini Prep (ZymoResearch), as per manufacturer’s instructions. RNA was reverse transcribed using RT2 Easy First Strand Kit (Qiagen). Real-time quantitative RT-PCR was performed by TaqMan analysis using an ABI 7300 instrument (Applied Biosystems). GAPDH levels were used to normalize the data. TaqMan real-time probes for Foxp3, IL-10, IL-4, TGF-β1, IFN-γ, and IL-17 were obtained from Applied Biosystems. Using the average mean cycle threshold (Ct) value for GAPDH and the gene of interest for each sample, the equation 1.8(CtGAPDH−Ctgeneofinterest)10−Ct was used to obtain normalized values.
**Histological evaluation**

A pathologist blinded to the experimental condition graded the severity of the colonic inflammation using a well-described 4-point scale (14).

**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. The p values <0.05 were considered significant.

**Results**

**H. polygyrus bakeri infection induced an increase in the proportion of colonic LPMC CD4+ T cells expressing Foxp3**

T cells that express Foxp3 are plentiful in the gut and help to limit mucosal immune responses. Because *H. polygyrus bakeri* can protect mice from colitis (8, 15), it was determined whether *H. polygyrus bakeri* infection increased the relative number of Foxp3+ T cells in the colonic mucosa of healthy C57BL/6 Foxp3 reporter transgenic mice. Foxp3 mRFP/IL-10 eGFP double-reporter mice were used to allow visualization and then isolation of Treg subsets distinguished by their differential capacity to express IL-10.

In the colon, TI, and MLN of healthy transgenic mice, Foxp3 mRFP was seen only in T cells, and nearly all the Foxp3+ T cells were CD4+ (~97%). In the colonic LP, ~25% of the CD4+ T cells were Foxp3+, and ~63% of them also expressed IL-10 (Fig. 1). In the TI, Foxp3+ T cells comprised only 10% of the total CD4+ T cell population, and, compared with the colon, a smaller proportion of these cells expressed IL-10 (~40%). IL-10 eGFP also was seen in some CD4+ T cells that were negative for Foxp3 expression, appearing in similar proportions in the colon and TI, relative to the total CD4+ T cell subset. The MLN contained very few Foxp3+/IL-10+ or Foxp3+/IL-10+ CD4+ T cells (<1% of total CD4+ T cells). A small number of CD8+ T cells and γδ T cells in the colon, TI, and MLN also expressed Foxp3 (~1% of each subset) (data not shown). There were no Foxp3+/IL-10+ T cells in these two T cell subsets.

**FIGURE 1.** *H. polygyrus bakeri* infection increased the number of Foxp3+ Treg in the colon of Foxp3/IL-10 reporter mice. Some Foxp3/IL-10 double-reporter mice were infected with *H. polygyrus bakeri* for 2 wk before isolation of colonic LPMC for flow analysis (H. polygyrus bakeri), whereas age-matched control mice remained uninfected (no *H. polygyrus bakeri*). Isolated colonic LPMC were analyzed for Foxp3 and IL-10 expression using FACS. The FACS plot shows the results from a single representative experiment gated on the CD4+ T cell subset. Data in Supplemental Table 1 are mean percentages ± SE of CD4+ colonic LP T cells expressing Foxp3 and/or IL-10 from three separate experiments. Each group in each individual experiment used pooled cells from at least three individual mice.

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**RT-PCR analysis of cytokine expression in colonic Foxp3+/IL-10- and Foxp3+/IL-10+ T cells**

RNA was extracted from Foxp3+/IL-10-, Foxp3+/IL-10+, and Foxp3+ T cells isolated from dispersed colonic LP cells using FACS. Reverse-transcribed RNA was analyzed for Foxp3, IL-10, TGF-β1, IFN-γ, and IL-17 transcripts using RT-PCR. As expected, Foxp3+ T cells, identified by mRFP+ fluorescence, contained Foxp3 transcripts, which were nearly undetectable in the Foxp3− T cells. The Foxp3+/IL-10− (eGFP+) T cells had many more IL-10 transcripts compared with the other two cell subsets. *H. polygyrus bakeri* infection increased IL-10 expression in the Foxp3+/IL-10+ T cell subset. Foxp3+/IL-10− and Foxp3+/IL-10+ T cells expressed TGF-β transcripts. *H. polygyrus bakeri* infection did not alter TGF-β expression in any of the cell types.

**Colonic Foxp3+ T cells from *H. polygyrus bakeri*-infected mice prevented colitis and reduced the release of IFN-γ and IL-17 from the colonic LPMC**

To study colitis, experiments employed a well-established Rag CD4+/CD25+ T cell transfer model of IBD (16). Many such models develop colitis inconsistently. To enhance expression of disease, 1 wk after cell transfer, mice were fed NSAID (piroxicam) for 2 wk. This resulted in all animals developing severe colitis that was evident 1 wk thereafter stopping the NSAID (4 wk after cell transfer). The NSAID disrupts the production of protective arachidonic acid metabolites in the mucosa (14), making the animals...
The Rag mice were given piroxicam and sacrificed 4 wk after cell infection. Foxp3+ T cells from the colon of infection affected the functionality of the colonic Foxp3+ T cells in this condition (19, 20). Drives the disease in human IBD and in many animal models of IBD. Study an Ag-specific T cell response in the colonic LPMC. OT2 T cells were isolated from the colon using FACS. RNA was extracted from each subset and converted to cDNA. RT-PCR was used to determine the relative expression of Foxp3, IL-10, TGF-β1, IL-4, IFN-γ, and IL-17 transcripts in the three cell subsets before infection and postinfection. Each experiment used three to four mice, and data are means ± SE of three independent experiments. *IL-4, no H. polygyrus bakeri versus H. polygyrus bakeri, p < 0.02. **IL-10, no H. polygyrus bakeri versus H. polygyrus bakeri, p < 0.01. ‡IL-4 or IL-17, no H. polygyrus bakeri versus H. polygyrus bakeri, p < 0.05.

More prone to IBD. This is relevant to human IBD, because administration of many types of NSAIDs worsens the disease (17, 18). We also adoptively transferred CD4+CD25 T cells from C57BL/6 mice expressing CD45.1 so that OT2 cells within the splenic T cells, but no colonic Foxp3+ T cells. The Rag mice were given piroxicam and sacrificed 4 wk after cell transfer to assess the severity of the colitis. Fig. 3 shows that only colonic Foxp3+ T cells from H. polygyrus bakeri–infected mice protected the mice from IBD.

Colonized LPMC were isolated from the colitis-induced Rag mice 4 wk after cell transfer and cultured with or without OVA. Colonized LPMC cultured without Ag produced substantial amounts of IFN-γ and IL-17, but much more in the presence of OVA. Only adoptive transfer of colonic Foxp3+ T cells from H. polygyrus bakeri–infected reporter animals into Rag recipients decreased constitutive and OVA–stimulated cytokine release from the colon LPMC (Fig. 3). Cultures also were assayed for IL-4 and TGF-β, which were only detected at <100 pg/ml. Neither Treg transfer nor OVA or anti-CD3/CD28 stimulation changed the rate of IL-4 or TGF-β secretion (data not shown).

The loss of LPMC OVA responsiveness after adoptive transfer of colonic Treg from H. polygyrus bakeri–infected mice could have signified that Treg transfer interfered with normal OT2 T cell accumulation in the LP rather than inhibited their function. T cells from C57BL/6 mice express the molecule CD45.2. We reconstituted Rag mice with CD4+CD25− OT2 T cells from transgenic C57BL/6 mice expressing CD45.1 so that OT2 cells within the isolated LPMC could be distinguished from the other T cells through differential CD45 display.

Compared with Rag mice receiving no supplemental Treg, the relative number of colonic LP cells in the lymphoid gate expressing CD45.1 did not diminish in Rag mice reconstituted with colonic Foxp3+ T cells. In colitic Rag mice that did not receive colonic

![FIGURE 2. Cytokine expression in colonic Foxp3+/IL-10−. Foxp3+/IL-10−, and Foxp3+ CD4+ T cells before and after H. polygyrus bakeri infection. Some Foxp3+/IL-10 double-reporter mice were infected with H. polygyrus bakeri for 2 wk before isolation of colonic LPMC (H. polygyrus bakeri), whereas age-matched control mice remained uninfected (no H. polygyrus bakeri). Foxp3+/IL-10−, Foxp3+/IL-10−, and Foxp3+ CD4+ T cells were isolated from the colon using FACS. RNA was extracted from each subcell and converted to cDNA. RT-PCR was used to determine the relative expression of Foxp3, IL-10, TGF-β1, IL-4, IFN-γ, and IL-17 transcripts in the three cell subsets before infection and postinfection. Each experiment used three to four mice, and data are means ± SE of three independent experiments. *IL-4, no H. polygyrus bakeri versus H. polygyrus bakeri, p < 0.02. **IL-10, no H. polygyrus bakeri versus H. polygyrus bakeri, p < 0.01. ‡IL-4 or IL-17, no H. polygyrus bakeri versus H. polygyrus bakeri, p < 0.05.

![FIGURE 3. Colonized Foxp3+ T cells from H. polygyrus bakeri–infected mice blocked the development of colitis in a CD4+CD25− T cell transfer model of IBD. (A) Some Foxp3+/IL-10 reporter mice were infected with H. polygyrus bakeri for 2 wk, whereas age-matched control mice were not infected. Then Foxp3+ T cells were isolated from dispersed colonic LPMC using FACS. These cells from either H. polygyrus bakeri–infected (H. polygyrus bakeri) or uninfected animals (no H. polygyrus bakeri) were adoptively transferred via i.p. injection into Rag mice along with CD4+CD25− WT and OT2 splenic T cells. After 1 wk, the mice received piroxicam in their food for 2 wk to stimulate colitis. The animals were sacrificed 1 wk after stopping the piroxicam to evaluate colitis severity. (B) Colitis was scored for severity on a 4-point scale in stained histological sections. (C) The pictures represent the severity of inflammation in colons of mice receiving no Foxp3+ T cells (no transfer), or adoptively transferred colonized Foxp3+ T cells from either uninfected (no H. polygyrus bakeri) or H. polygyrus bakeri–infected (H. polygyrus bakeri) mice. The sections, stained with H&E, were photographed at ×40. (D) Dispersed colonized LPMC were cultured 48 h in vitro without or with OVA (10 μg/ml) to stimulate cytokine release. Culture supernatants were assayed for IFN-γ and IL-17 using ELISA. Data are means ± SE from three separate experiments. Each experiment used five mice/group. (B) No transfer or no H. polygyrus bakeri transfer Foxp3 versus H. polygyrus bakeri transfer Foxp3, p < 0.01. No transfer versus no H. polygyrus bakeri /transfer Foxp3, p = NS. (D) No transfer or no H. polygyrus bakeri /transfer Foxp3 versus H. polygyrus bakeri/transfer Foxp3, unstimulated or OVA stimulated, p < 0.01. No transfer versus no H. polygyrus bakeri /transfer Foxp3, p = NS. Unstimulated versus OVA stimulated, p < 0.01.](http://www.jimmunol.org/)
Foxp3+ T cell transfer, the percentage of dispersed colonic LPMC in the lymphoid gate that expressed CD45.1 was 4.4 ± 0.7%. The relative number remained the same after either transfer of colonic Foxp3+ T cells from mice without or with H. polygyrus bakeri infection (not infected versus infected, 4.4 ± 0.3% versus 4.5 ± 0.8%). (Data are means ± SE from each of nine independent experiments.) This suggests that the low cytokine response to OVA stimulation in vitro is a result of loss of OT2 T cell responsiveness, not due to lack of OT2 T cell number. Mice with colitis develop a mononuclear infiltrate in the LP. These data also suggest that the mice with colitis have more T cells and OT2 T cells in the colonic lining than mice with blunted colitis as a result of Treg transfer.

**Fluorescent colonic Foxp3+ T cells from H. polygyrus bakeri–infected reporter mice readily accumulated in the colon and MLN of Rag recipients**

It was also determined whether colonic reporter Foxp3+ T cells from H. polygyrus bakeri–infected mice transferred into Rag recipients led to accumulation of fluorescent Foxp3+ T cells in colons of the cell recipients. Following such transfers, the colons of Rag mice contained large numbers of CD4+Foxp3+/IL-10− and Foxp3+/IL-10+ T cells (Fig. 4). All tissues were examined at the usual time of sacrifice for this colitis model (4 wk after Foxp3 T cell transfer). Examination of the MLN yielded similar results, although, compared with the colon, the relative number of CD4+ T cells expressing Foxp3 was lower in this tissue (Fig. 4).

Transfer of colonic Foxp3+ T cells from uninfected reporter mice into Rag recipients resulted in proportionately fewer T cells in the colon that were Foxp3+/IL-10− compared with mice receiving cells from infected animals (∼70% less) (Fig. 4). Moreover, there were nearly any Foxp3+/IL-10− T cells present. Results were similar for MLN. Also noted was a small, but definite CD4+ T cell subset that was Foxp3−/IL-10−.

Rag that received colonic Treg from H. polygyrus bakeri–infected animals developed much less colitis than mice receiving Treg from uninfected mice. The colons of the colitic mice yielded ~30% more LPMC upon dissolution (Treg, no H. polygyrus bakeri infection versus Treg, H. polygyrus bakeri infection; 8.2 ± 1.2 versus 5.7 ± 0.5 × 106 cells/colon, ±SD, n = 6). CD4+ T cells were present in similar proportions in LPMC isolates from mice receiving Treg from either infected or uninfected mice (no Treg versus Treg, H. polygyrus bakeri, no H. polygyrus bakeri; 9.7 ± 2 versus 8.7 ± 2.6%, ±SD, n = 6). This suggests that there was an absolute as well as a relative increase in the number of Treg in the colons of mice transferred Treg from H. polygyrus bakeri–infected reporter mice.

**Foxp3+ T cells from MLN also inhibit colitis after H. polygyrus bakeri infection**

H. polygyrus bakeri infection also affected the functionality of the MLN Foxp3+ T cells. Adoptive transfer of Foxp3+ T cells from MLN of H. polygyrus bakeri–infected reporter mice into Rag recipients blocks colitis. (Colitis score: no cell transfer, 3.7 ± 0.2, versus Foxp3+ Foxp3− T cell transfer from H. polygyrus bakeri–infected mice, 0.36 ± 0.1, p < 0.01; ±SE, three separate experiments.) Colitis was only minimally affected with transfer of MLN Foxp3+ T cells from uninfected animals. (Colitis score: Foxp3+ T cell transfer from uninfected mice, 2.9 ± 0.3, versus no cell transfer, p < 0.05.)

**Colonic Foxp3+/IL-10− and Foxp3+/IL-10+ T cell subsets protected from colitis with comparable efficiency**

The colonic Foxp3+ T cells adoptively transferred into Rag mice to prevent colitis were composed of two subsets distinguished by their capacity to express IL-10. Experiments ascertained whether the Foxp3+/IL-10− and Foxp3+/IL-10+ T cell subsets, obtained from the colon of H. polygyrus bakeri–infected mice, would provide similar levels of colitis protection. Experiments showed that both subsets afforded comparable protection (Fig. 5), and reduced constitutive and OVA-stimulated cytokine release from the colonic LPMC isolated from the colitic mice (Fig. 5).

**Transfer of colonic Foxp3+/IL-10− T cells from H. polygyrus bakeri–infected reporter mice into Rag recipients resulted in accumulation of both Foxp3+/IL-10− and Foxp3+/IL-10+ T cells in the colon and MLN of the recipient animals**

The above observation that Foxp3+/IL-10− T cells can protect mice from colitis was unexpected, because previous studies showed that Foxp3+ T cells that make IL-10 are important for controlling immune responses in the intestinal mucosa (13). Thus, further studies determined whether transfer of Foxp3+ T cells that cannot produce IL-10 will result in the accumulation of both Foxp3+ T cell subsets in the colon of our Rag recipients.

Rag mice that received colonic Foxp3+/IL-10− T cells from H. polygyrus bakeri–infected reporter mice readily acquired large numbers of fluorescent CD4+ Foxp3+/IL-10− and Foxp3+/IL-10+ T cells in the colon and MLN of the recipient animals (Table II). The relative number of fluorescent Foxp3+ T cells (Fig. 4, H. polygyrus bakeri).

We also ascertained whether transfer of Foxp3+/IL-10− T cells would yield similar results. A surprising outcome was that Rag recipients of colonic Foxp3+/IL-10− T cells, examined 4 wk after transfer, displayed essentially no fluorescent Foxp3+ T cells in the colon and MLN (Fig. 6). They were absent from the terminal ileum also. Examination of colons at earlier time points after cell transfer (weeks 1 and 2) revealed such cells, but in small numbers (~1% of CD4+ T cells). Thus, transferring just colonic Foxp3+/IL-10− T cells failed to stably reconstitute the Foxp3 compartment.
assayed for IFN-γ reporter mice were infected with *H. polygyrus bakeri* (three separate experiments. Each experiment used five mice/group. For severity on a 4-point scale in stained histological sections. The animals were sacrificed 1 wk after stopping the piroxicam in food for 2 wk to stimulate colitis. LPMC isolated from Rag colons were analyzed for Foxp3 and IL-10 expression using FACS. The FACS plot shows the results from a representative experiment gated on colonic CD4+ T cells. This study made several important observations. As revealed by the double-reporter mice, the LP of WT mouse colon contains large numbers of CD4+ T cells that express Foxp3, and the majority of the Foxp3+ T cells also express IL-10. (Mean percentage of CD4+ T cells expressing Foxp3 eGFP: colon, 6.8 ± 1.8 and MLN, 3.4 ± 1.7. Data are means of three experiments ± SE.)

**Discussion**

This study made several important observations. As revealed by the double-reporter mice, the LP of WT mouse colon contains large numbers of CD4+ T cells that express Foxp3, and the majority of the Foxp3+ T cells also express IL-10. Relatively few Foxp3+ T cells in the colon express IL-10, suggesting that colonic Treg are the major source of this cytokine in this tissue. This was confirmed by RT-PCR analysis. Whereas *H. polygyrus bakeri* infection modestly expanded the number of CD4+ Foxp3+ T cells in the colon and MLN, the major effect of this infection was to activate or modulate colonic and MLN Treg, making them more capable at preventing colitis. Foxp3+ T cells that make IL-10 appear to be critically important for protection from colitis. Our study showed that colonic Foxp3+ T cells from *H. polygyrus bakeri*-infected mice fail to restrain colitis in our adoptive transfer model of IBD. Other studies using a similar animal model of IBD also support this prevention.
contention (21, 22). Also, mice bearing a conditional deletion of the IL-10 allele limited to Foxp3-expressing T cells develop spontaneous colitis (13). In our experiments, the transfer of colonic or MLN Foxp3+ T cells from H. polygyrus bakeri–infected reporter mice resulted in a much greater accumulation of Foxp3+/IL-10+ T cells in the colon and MLN of the recipient animals. Thus, in our colitis model, failure to adequately reconstitute Foxp3+/IL-10+ T cells in the colon and MLN of recipient colitis-induced Rag animals could be one of the reasons colonic and MLN Foxp3+ T cells from uninfected WT mice failed to control colitis. In a recent report, homing and expansion of Foxp3+ Treg within the LP were required for oral tolerance (23). It is tempting to speculate that, in our IBD model, the failure of GATA-3 in Treg differentiation and function may be quite complex. The DNA-binding inhibitor Id3 also has a role in Treg generation through enriching the binding of the transcription factor E2A to the Foxp3 promoter region perhaps through relief of GATA-3 inhibition (27). Many of these factors appeared to be integrated into the TGF-β signaling pathway, which is important for T cell differentiation (28). Foxo proteins can serve as co-activators downstream of the TGF-β signaling pathway and affect the differentiation of Foxp3+ Treg (29). Loss of Foxo protein expression in lymphocytes of mice with Gimap5 gene mutation may be the mechanism promoting spontaneous colitis in these animals (30). ICOS is a member of the CD28 superfamily, and stimulation via ICOS can enhance IL-10 secretion. ICOS signaling has been reported to be an important factor in promoting Treg function and survival (31). Further studies will be required to determine whether these or other regulatory pathways mediate H. polygyrus bakeri–induced Treg activation.

It is not known how H. polygyrus bakeri interfaces with colonic Treg to enhance their regulatory function. H. polygyrus bakeri can release soluble factors that elicit the expansion of regulatory-type T cells (9, 32, 33). Dendritic cell (DC) subsets are important for driving T cell differentiation in the gut and MLN, and can promote Treg development (34). Undefined H. polygyrus bakeri products have been shown to modulate DC function, rendering them capable of driving the CD4+ T cells into the Treg phenotype (32). Natural infection is associated with expansion within the MLN of a CD11cint/CD103+ DC subset associated with induction of Treg responses in vitro and in vivo (35). Because Foxp3+ T cells and other Treg subsets can ameliorate colitis in murine models of IBD, and tolerogenic DC can expand Treg in vitro and protect mice from colitis (36), it is reasonable to assume that tolerogenic DC/T cell interactions are an important part of the process.
H. polygyrus bakeri inhabits mostly the proximal small bowel, whereas it acts distally on the colon to limit disease. The process of protection most likely requires some form of communication between the parasite and the host. H. polygyrus bakeri releases factors that could have immune modulatory functions (37, 38). For instance, H. polygyrus bakeri secretions contain homologs to migration inhibition factor and C-type lectin receptors. Such molecules could interact with intestinal epithelial cells and DC that penetrate the epithelial barrier locally near the infection or far distally within the gut. Also, H. polygyrus bakeri infection affects the composition of the intestinal flora with unknown consequences (39). The factors produced by H. polygyrus bakeri that alter DC function and how these factors reach their cellular targets are not yet explored.

**Disclosures**

The authors have no financial conflicts of interest.

**References**