**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

*J Immunol* published online 12 July 2013

http://www.jimmunol.org/content/early/2013/07/12/jimmunol.1201457
**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: 000–000.

*Division of Gastroenterology-Hepatology, Department of Internal Medicine, Tufts Medical Center, Boston, MA 02111; and *Division of Genomics and Immunology Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service, United States Department of Agriculture, Beltsville, MD 20705

Received for publication May 25, 2012. Accepted for publication May 28, 2013.

This work was supported by National Institutes of Health Grants DK38327, DK058755, and DK091987; the Broad Foundation; the Schneider Family; the Friedman Family; and the Gilman Family.

Address correspondence and reprint requests to Dr. Joel V. Weinstock, Division of Gastroenterology (Box 233), Tufts Medical Center, 800 Washington Street, Boston, MA 02111. E-mail address: jweinstock2@tuftsmedicalcenter.org.

The online version of this article contains supplemental material.

Abbreviations used in this article: Ct, cycle threshold; DC, dendritic cell; eGFP, enhanced GFP; IBD, inflammatory bowel disease; KO, knockout; LP, lamina propria; LPMC, LP mononuclear cell; MLN, mesenteric lymph node; NSAID, nonsteroidal anti-inflammatory drug; Treg, regulatory T cell; WT, wild-type.
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. Fuhlbrigge, Brigham and Women’s Hospital, Boston, MA) and IL-10 knockout (KO) Foxp3 Chicago. Foxp3 mRFP/IL-10 eGFP double-reporter mice were produced by cross-breeding Foxp3 mRFP and IL-10 eGFP single-reporter mice 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-wk-old mice were colonized with 125 H. polygyrus bakeri third-stage larvae by oral gavage. Infected mice were used after 2 wk. Infective, ensheathed 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 isolated by FACS. The viability of the isolated cells always was >95%, as 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 1×107 C57BL/6 WT CD4+CD25+ splenic T cells and 3×105 OT2 CD45.1 splenic T cells. In some experiments, mice also received 1×105 Foxp3+, Foxp3+/IL-10+, Foxp3+/IL-10−, 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 unfractonated colonic Foxp3+ T cells, or colonic Foxp3−/IL-10− or Foxp3−/IL-10+ 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. Infected mice were used after 2 wk. Infective, ensheathed 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.

Results

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 LSR II 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 nonspecific binding of conjugated Abs to FcRs. The mAbs used for staining or cell sorting were anti-CD4-FITC, anti-CD4-allophycocyanin, or anti-CD4-PECy5; anti-CD25-PE; and anti-CD45.1-allophycocyanin, anti-CD8allophycocyanin, and anti-γ/δ-allophycocyanin (all from eBioscience). Usually, 105 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. Colon sections then were fixed in 4% paraformaldehyde (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, Torrence, 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; OSO00005WS) and rabbit anti-RFP (Life Technologies, Carlsbad, CA; R0367) polyclonal Abs. Secondary Abs were donkey anti-sheep Alexa fluor 488 (713-545-147) and donkey anti-rabbit Alexa fluor 594 (711-585-152) (red) affinity-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 fluoros in the Tufts Center for Neuroscience Research P30 NS047243.

Flow cytometry analysis

Flow cytometry analysis

Quantitative real-time RT-PCR

Total RNA was isolated from individual samples using Quick-RNA Miniprep (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 (Cθ) value for GAPDH and the gene of interest for each sample, the equation 2−(θCθ) was used to obtain normalized values.

Downloaded from http://www.jimmunol.org/ on April 17, 2017
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 LPMC 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.

Reporter mice were infected with H. polygyrus bakeri for 2 wk. As compared with age-matched uninfected control animals, there was a modest, but significant increase in the proportion of colonic LP CD4+ T cells that were Foxp3+/IL-10- or Foxp3+/IL-10+ (Fig. 1). Also, the proportion of Foxp3+ CD4+ T cells expressing IL-10 increased slightly in the colon and terminal ileum as well. In the MLN, the Foxp3+/IL-10- CD4+ T cell subset expanded relative to the total CD4+ T cell population, whereas there were no changes in Foxp3+/IL-10- or Foxp3+/IL-10+ CD4+ T cells.

The proportion of CD8+ and γδ T cells expressing Foxp3 did not change after H. polygyrus bakeri infection (data not shown). In the colon and TI, H. polygyrus bakeri infection did not induce inflammation or alter the composition of the LPMC isolated from these tissues. Thus, H. polygyrus bakeri infection induced a small, but significant increase in total CD4+ Foxp3+ T cell number in these tissues.

Foxp3 mRFP+ and IL-10 eGFP+ cells are mostly in the LP

Fluorescent immunohistochemistry was used to localize the Foxp3+ and IL-10+ T cells to specific regions of the colon. Sections of colon from uninfected mice were treated with anti-eGFP and anti-mRFP Abs to identify the cells expressing IL-10 (eGFP+) and Foxp3 (mRFP+). The LP contained many Foxp3+ T cells. IL-10+ cells were also present, and the IL-10+ staining was localized mostly to the Foxp3+ T cells. There were few such cells seen outside of the LP (data not shown). The distribution of Foxp3+ T cells in colon of H. polygyrus bakeri–infected mice appeared similar to that of uninfected animals (data not shown).

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

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. The low level of IL-4 detected in all three T cell subsets increased following H. polygyrus bakeri infection. IFN-γ and IL-17 were expressed at low levels in the three T cell subsets. H. polygyrus bakeri infection caused a decrease in the relative expression of IL-17 only in the Foxp3+ CD4+ T cells (Fig. 2).

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 a 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...
more prone to IBD. This is relevant to human IBD, because administration of many types of NSAIDs worsens the disease (17, 18). We also adaptively transferred CD4^+CD25^+ OT2 T cells into the Rag mice concomitantly with the other cells so that we could study an Ag-specific T cell response in the colonic LPMC. OT2 T cells are transgenic T cells that respond to OVA. 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 it is well appreciated that these cytokines help drive the disease in human IBD and in many animal models of this condition (19, 20).

Using this model, it was asked whether the _H. polygyrus bakeri_ infection affected the functionality of the colonic Foxp3^+ T cells isolated from WT mice with regard to their capacity to prevent IBD. Foxp3^+ T cells from the colon of _H. polygyrus bakeri_–infected or uninfected WT reporter mice were adaptively transferred into Rag mice along with the appropriate splenic CD4^+CD25^- T cells. Another group of Rag mice received the appropriate CD4^+CD25^- 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.

Colonic LPMC were isolated from the colitis-induced Rag mice 4 wk after cell transfer and cultured with or without OVA. Colonic 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 CD45.2. 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 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. 3IL-4 or IL-17, no _H. polygyrus bakeri_ versus _H. polygyrus bakeri_, p < 0.05.

**FIGURE 3.** Colonic 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 colonic 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 colonic 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.
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 colon 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+ 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 (Fig. 6) and MLN (Fig. 4) at the standard time of sacrifice (4 wk). In these tissues, the relative number of CD4+ T cells expressing Foxp3 with or without IL-10 was similar to that observed in Rag mice reconstituted with unfractionated colonic 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* or Foxp3+/IL-10 (three separate experiments. Each experiment used five mice/group. For severity on a 4-point scale in stained histological sections. (0.01. Foxp3+/IL-10–infected IL-10–infected reporter mice infected for 2 wk with *H. polygyrus bakeri*. These cells 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 food for 2 wk to stimulate colitis. LPMC isolated from Rag colon 4 wk after cell transfer 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. In Supplemental Table III, data from three experiments are expressed as mean percentages ± SE of colonic LP or MLN CD4+ T cells expressing Foxp3 and/or IL-10. Cells for each analysis were pooled from three to four individual mice.

FIGURE 5. Both colonic Foxp3+/IL-10– and Foxp3+/IL-10+ T cells from *H. polygyrus bakeri*–infected mice blocked colitis. (A) Foxp3/IL-10 reporter mice were infected with *H. polygyrus bakeri* of 2 wk. Then Foxp3+/IL-10– and Foxp3+/IL-10+ T cells were isolated from dispersed colonic LPMC using FACS. These cells 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 (diagram displays experimental design). (B) Colitis was scored for severity on a 4-point scale in stained histological sections. (C) Dispersed colonic 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. For (B), no transfer versus Foxp3+/IL-10– or Foxp3+/IL-10+, p < 0.01. Foxp3+/IL-10– versus Foxp3+/IL-10+, p = NS. For (C), no transfer versus Foxp3+/IL-10– or Foxp3+/IL-10+, unstimulated or OVA stimulated, p < 0.01. Foxp3+/IL-10– versus Foxp3+/IL-10+, IFN-γ, p < 0.05. Unstimulated versus OVA stimulated, p < 0.05.

Adoptive transfer of colonic Foxp3+ T cells from *H. polygyrus bakeri*–infected mice into Rag recipients resulted in Treg accumulating within their tissues, but failed to protect them from colitis

To further explore the importance of IL-10 in prevention of colitis, IL-10–Foxp3 eGFP reporter mouse were colonized for 2 wk with *H. polygyrus bakeri*. Foxp3+ T cells isolated from the colon of these animals adoptively transferred into Rag recipients did not prevent colitis (Fig. 7). Colonic LPMC, isolated from the Rag recipients of IL-10–Foxp3+ T cells, cultured in vitro produced amounts of IFN-γ and IL-17 without or with OVA stimulation similar to that of control mice.

In the Rag mice that received IL-10–Foxp3+ T cells, colon and MLN were examined for the presence of Foxp3 eGFP+ CD4+ T cells at the 4-wk time of sacrifice. Dispersed colonic LPMC and MLN cells were examined by flow cytometry. Fluorescent IL-10–Foxp3+ CD4+ T cells were numerous in both tissues. (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 expressed 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 IL-10– mice fail to restrain colitis in our adoptive transfer model of IBD. Other studies using a similar animal model of IBD also support this.
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 into colitis-induced Rag recipients 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 within the tissues of recipient 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 colonic Foxp3+/IL-10+ T regulatory activity, but are important for replenishing the less sustainable Foxp3+/IL-10+ Treg subset critical for colitis protection.

Adoptive transfer of the colonic Foxp3+/IL-10+ T cells from H. polygyrus bakeri–infected WT animals was sufficient to prevent colitis over the 4-wk interval of observation, although they could not reconstitute the Treg population long-term. However, colonic Foxp3+/IL-10+ T cells from uninfected WT mice could not mediate this protection. In recipient mice, colonic Foxp3+/IL-10+ T cells from uninfected mice, as opposed to similar cells from H. polygyrus bakeri–infected mice, were less able to reconstitute the Foxp3+/IL-10+ Treg subset or control colitis. This could infer that H. polygyrus bakeri infection affects the function of both Foxp3+/IL-10+ and Foxp3+/IL-10- T cell subsets.

What H. polygyrus bakeri does to colonic Treg to promote their function remains speculative. Only a limited number of molecular pathways involved in Treg activation has been characterized or proposed. GATA-3 is a transcription factor important for initiating and maintaining the expression of IL-4, IL-5, and IL-13. It also appears that GATA-3 helps to control IL-10 expression (24). Recent data suggested that GATA-3 plays an important role in promoting Treg function (25, 26). However, GATA-3 may also serve an inhibitory role in Treg differentiation (27), suggesting that the role of GATA-3 in Treg differentiation and function may be quite complex. The DNA-binding inhibitor Id3 also has a role in 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 anticolonogenic 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 CD11c+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