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

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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 uninjected 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.

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 underlyng 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 uninjected 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-100 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.

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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+ mice. For cell transfer, Rag mice were administered piroxicam, a nonsteroidal anti-inflammatory drug (NSAID), mixed into their feed for 2 wk (42 mg/kg body wt per day) (42). In some experiments, mice also received 10^5 C57BL/6 OT2 CD45.1 splenic T cells. In some experiments, mice also received 10^5 Foxp3/IL-10 double-reporter mice. Some of these reporter mice were infected with Heligmosomoides polygyrus bakeri and biotinylated anti-IL-17A mAb (BAF421) (R&D Systems). The IL-4 ELISA was performed using primary capture mAb (MAB165) and biotinylated anti-IL-4 mAb (BAF504) (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 from reporter cells. IFN-γ was detected with polyclonal goat anti-IFN-γ conjugated to horseradish peroxidase (from M. Wilson, University of Iowa), followed by biotinylated horse anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). ELISA reagents were from TechnoGenix (HB170; American Type Culture Collection) and incubated with primary Ab (BAF240), also from R&D Systems. Cells for the TGF-β ELISA were grown in low serum RPMI 1640 medium, which was acidified, neutralized, and then assayed.

Sandwich ELISAs

ELISAs were performed using paired Abs mostly from R&D Systems (Minneapolis, MN), according to manufacturer’s instructions. The IL-17 ELISA was done using primary capture mAb (MAB721) and biotinylated anti-IL-17A mAb (BAF421) (R&D Systems). The IL-4 ELISA was performed using primary capture mAb (MAB404) and biotinylated anti-IL-4 mAb (BAF404) (R&D Systems). To measure IFN-γ, plates were coated with a mAb to IFN-γ (HB170; American Type Culture Collection) and incubated with secondary Ab. IFN-γ was detected with polyclonal goat anti-IFN-γ conjugated to horseradish peroxidase (from M. Wilson, University of Iowa), followed by biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and incubated with secondary Ab. IFN-γ was detected with polyclonal goat anti-IFN-γ conjugated to horseradish peroxidase (from M. Wilson, University of Iowa), followed by biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and incubated with secondary Ab. IFN-γ was detected with polyclonal goat anti-IFN-γ conjugated to horseradish peroxidase (from M. Wilson, University of Iowa), followed by biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and incubated with secondary Ab. IFN-γ was detected with polyclonal goat anti-IFN-γ conjugated to horseradish peroxidase (from M. Wilson, University of Iowa), followed by biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and incubated with secondary Ab. IFN-γ was detected with polyclonal goat anti-IFN-γ conjugated to horseradish peroxidase (from M. Wilson, University of Iowa), followed by biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and incubated with secondary Ab. IFN-γ was detected with polyclonal goat anti-IFN-γ conjugated to horseradish peroxidase (from M. Wilson, University of Iowa), followed by biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and incubated with secondary Ab. IFN-γ was detected with polyclonal goat anti-IFN-γ conjugated to horseradish peroxidase (from M. Wilson, University of Iowa), followed by biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and incubated with secondary Ab. IFN-γ was detected with polyclonal goat anti-IFN-γ conjugated to horseradish peroxidase (from M. Wilson, University of Iowa), followed by biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and incubated with secondary Ab. IFN-γ was detected with polyclonal goat anti-IFN-γ conjugated to horseradish peroxidase (from M. Wilson, University of Iowa), followed by biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and incubated with secondary Ab. IFN-γ was detected with polyclonal goat ant
**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.

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 2−/−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 adoptively 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-\gamma 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 \textit{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 \textit{H. polygyrus bakeri}–infected or uninfected WT reporter mice were adoptively 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 give 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 \textit{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. Colonie LPMC cultured without Ag produced substantial amounts of IFN-\gamma and IL-17, but much more in the presence of OVA. Only adoptive transfer of colonic Foxp3^+ T cells from \textit{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-\beta, 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-\beta secretion (data not shown).

The loss of LPMC OVA responsiveness after adoptive transfer of colonic Treg from \textit{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 CD45.2. 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 CD45.2. 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 CD45.2. 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 CD45.2. 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 CD45.2. 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 CD45.2. 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 CD45.2. 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 CD45.2.

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...
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 also was determined whether colonic 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+ 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.

**FIGURE 4.** Colonic LPMC Foxp3+ T cells from H. polygyrus bakeri–infected mice readily accumulated in Rag intestine after adoptive transfer. Rag mice received colonic LP Foxp3+ T cells from Foxp3+/-10 reporter mice that were infected for 2 wk with H. polygyrus bakeri, or they received similar cells from age-matched, uninfected animals. Using FACS, colonic LPMC were analyzed for Foxp3 and IL-10 expression 4 wk after cell transfer. The FACS plot shows the results from a single representative experiment gated on the colonic CD4+ T cell subset. Data in Supplemental Table II are mean percentages ± SE of CD4+ colonic LP or MLN T cells expressing Foxp3 and/or IL-10 from three separate experiments. Each group in each individual experiment pooled cells from at least three individual mice.
assayed for IFN-

To further explore the importance of IL-10 in prevention of colitis, IL-10+/Foxp3+ T cells from H. polygyrus bakeri–infected mice 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 that express Foxp3 and IL-10. Colonic and MLN cells were examined by flow cytometry. Fluorescent staining showed that Foxp3+ eGFP+ 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 or Foxp3+/IL-10+ T cells derived from colons of Foxp3/IL-10–infected IL-10– mice fail to 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.

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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 or Foxp3+/IL-10+ T cells derived from colons of Foxp3/IL-10–infected IL-10– mice fail to 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 that express Foxp3 and IL-10. Colonic and MLN cells were examined by flow cytometry. Fluorescent staining showed that Foxp3+ eGFP+ 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.)

**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 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 IL-10−/− 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 that express Foxp3 and IL-10. Colonic and MLN cells were examined by flow cytometry. Fluorescent staining showed that Foxp3+ eGFP+ 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.)

**FIGURE 6.** In the Rag colitis model, the proportion of colonic and MLN CD4+ T cells that were Foxp3+/IL-10+ or Foxp3+/IL-10+ substantially increased after the transfer of colonic Foxp3+/IL-10− T cells from H. polygyrus bakeri–infected reporter mice. Rag mice received Foxp3+/IL-10− T cells or Foxp3+/IL-10+ T cells derived from colons of Foxp3/IL-10 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.
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 GATA-3 to replete the IL-10–expressing Treg in the colon was of most importance.

It was interesting to note that transfer of Foxp3+/IL-10+ T cells from H. polygyrus bakeri–infected WT mice reconstituted the Foxp3+/IL-10+ and the Foxp3+/IL-10+ CD4+ T cell subsets in the colon and MLN of recipient colitis-induced Rag animals. There even appeared a small subset of Foxp3+ T cells that expressed IL-10. However, donor WT colonic Foxp3+/IL-10+ T cells appeared briefly in recipient colon and MLN and then disappeared. Thus, it is possible that the colonic Foxp3+/IL-10+ T cell is a distinct Treg subset that retains the capacity to expand and persist in the recipient mice, whereas Foxp3+/IL-10+ T cells lack this capacity. This could signify that, in the colon, Foxp3+/IL-10+ T cells have limited 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 also may 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 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 Treg differentiation (28). Foxo proteins can serve as coactivators 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 anticolitogenic 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


