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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.

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

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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: jweinstock@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. Fuhlbirgge, Brigham and Women’s Hospital, Boston, MA) and IL-10 knockout (KO) Foxp3 mice (gift from breast cancer research). For some experiments, the animals also received unfractionated colonic content from wild-type mice or Foxp3+/IL-10 double-reporter mice. Some of these reporter mice were infected with the various manipulations of this model are provided in inflammatory drug (NSAID), mixed into their feed for 2 wk (42 mg/day). A proportion of 10% CD4+CD25+ T cells were labeled with FITC-CD4 and PE-CD25 mAbs (eBioscience, San Diego, CA). Then cells were sorted using FACS MoFlo equipment (Cytofam, Fort Collins, CO) with Summit V4.3 software. Viability was determined using exclusion of trypan blue dye.

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 FACSMerit multiparameter system (Cytofam, 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 (LPMCs) 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 LPMCs 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 with similar age (usually 5-6 wk old) were reconstituted i.p. with 1 × 107 C57BL/6 WT CD4+CD25+ splenic T cells and 3 × 107 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 (42 mg piroxicam/250 g chow, week 1; 62 mg piroxicam/250 g chow, week 2). The piroxicam (Sigma-Aldrich) was stopped, and colitis was studied 1 wk later. Thus, it was 4 wk from the day of cell transfer until animal sacrifice. Half of the colon divided longitudinally were fixed, sectioned, and stained with HE for microscopic examination to score the severity of colitis. The other half was dissociated with collagenase to isolate LPMCs, which were analyzed by flow cytometry and cultured in vitro. More details regarding the various manipulations of this model are provided in Results.

Cell culture

Colonic LPMC from Rag mice reconstituted with C57BL/6 WT CD4+CD25+ and OT2 CD4+CD25+ T cells were cultured (2.5 × 105 cells/well) for 48 h in 96-well flat-bottom plates. Cells were cultured alone or with OVA (10 μg/ml) (Sigma-Aldrich). The culture medium was RPMI 1640 containing 10% FCS, 25 mM HEPES buffer, 2 mM l-glutamine, 5 × 10−5 M 2-ME, 1 mM sodium pyruvate, 100 U/ml penicillin, 5 mg/ml gentamicin, and 100 mg/ml streptomycin (complete medium) (all Life Technologies, Gaithersburg, MD). To measure TGF-β, cells were cultured in RPMI 1640 only containing 1% FCS and 0.1% BSA (Sigma-Aldrich). After culture, the supernatants were assayed for IFN-γ, IL-17A, or IL-8 using ELISA (described below).

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 used the 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 supernatants. IFN-γ was detected with polyclonal rabbit anti–IFN-γ (gift of M. Wilson, University of Iowa), followed by biotinylated goat anti-rabbit IgG (eBioscience) to block nonspecific binding of conjugated Abs to FCs. 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–CD8–allophycocyanin, and anti–γ/δ–allophycocyanin (all from eBioscience). Usually, 105 cells were stained, and 105 cells were analyzed.

Flow cytometry analysis

Isolated LPMC were washed twice, adjusted to 106 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 non-specific binding of conjugated Abs to FCs. 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–CD8–allophycocyanin, 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 tissues were then 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 was then 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; OOS00005W) and rabbit anti-RFP (Life Technologies, Carlsbad, CA; R10367) polyclonal Abs. Secondary Abs were donkey anti-sheep Alexa fluor 488 (713545-147) (green) and donkey anti-rabbit Alexa fluor 594 (711585-152) (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 coveredslipped using Fluoromount G (Southern Biotech, Birmingham, AL) and viewed using a fluorescence microscope with appropriate filters for the two Alexa fluor in the Tufts Center for Neuroscience Research P30 NS047243.

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, 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(Ct GAPDH − Ct gene of interest) × 10−1 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.

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 ignore this piece of information.
The Rag mice were given piroxicam and sacrificed 4 wk after cell isolation from WT mice with regard to their capacity to prevent infection. This condition (19, 20) showed that the Rag mice were protected from 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-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 adoptively transferred into Rag mice along with 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 CD45.1 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 LPMC 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 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+ 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.
To further explore the importance of IL-10 in prevention of colitis, IL-10<sup>−/−</sup> Foxp3<sup>+</sup> eGFP reporter mouse were colonized for 2 wk with <i>H. polygyrus bakeri</i>. Foxp3<sup>+</sup> 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<sup>−/−</sup> Foxp3<sup>+</sup> 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<sup>−/−</sup> Foxp3<sup>+</sup> T cells, colon and MLN were examined for the presence of Foxp3<sup>+</sup> eGFP<sup>+</sup> CD4<sup>+</sup> T cells at the 4-wk time of sacrifice. Dispersed colonic LPMC and MLN cells were examined by flow cytometry. Fluorescent IL-10<sup>−/−</sup> Foxp3<sup>+</sup> CD4<sup>+</sup> T cells were numerous in both tissues. (Mean percentage of CD4<sup>+</sup> T cells expressing Foxp3<sup>+</sup> 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<sup>+</sup> T cells that express Foxp3, and the majority of the Foxp3<sup>+</sup> T cells also express IL-10. Relatively few Foxp3<sup>+</sup> 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 <i>H. polygyrus bakeri</i> infection modestly expanded the number of CD4<sup>+</sup> Foxp3<sup>+</sup> 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<sup>+</sup> T cells that make IL-10 appear to be critically important for protection from colitis. Our study showed that colonic Foxp3<sup>+</sup> T cells from <i>H. polygyrus bakeri</i>–infected IL-10<sup>−/−</sup> 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 Foxp3+ T cells and other Treg subsets to 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.

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 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 and function and survival (31). Further studies will be required to determine whether these or other regulatory pathways mediate H. polygyrus bakeri–induced Treg activation.
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, the factors produced by H. polygyrus bakeri could interact with intestinal epithelial cells and DC that 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

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