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Role of T Cell TGF- β Signaling in Intestinal Cytokine Responses and Helminthic Immune Modulation

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Abstract

Colonization with helminthic parasites induces mucosal regulatory cytokines, like IL-10 or TGF- β that are important in suppressing colitis. Helminths induce mucosal T cell IL-10 secretion and regulate lamina propria mononuclear cell Th1 cytokine generation in an IL-10 dependent manner in wild-type mice. Helminths also stimulate mucosal TGF- β release. As TGF- β exerts major regulatory effects on T lymphocytes, we investigated the role of T lymphocyte TGF- β signaling in helminthic modulation of intestinal immunity. T cell TGF- β signaling is interrupted in TGF- β RII DN mice by T cell-specific over-expression of a dominant negative TGF- β receptor II. We studied lamina propria mononuclear cell responses in wild-type and TGF- β RII DN mice that were uninfected or colonized with the nematode, *Heligmosomoides polygyrus*. Our results indicate an essential role of T cell TGF- β signaling in limiting mucosal Th1 and Th2 responses. Furthermore, we demonstrate that helminthic induction of intestinal T cell IL-10 secretion requires intact T cell TGF- β signaling pathway. Helminths fail to curtail robust, dysregulated intestinal Th1 cytokine production and chronic colitis in TGF- β RII DN mice. Thus, T cell TGF- β signaling is essential for helminthic stimulation of mucosal IL-10 production, helminthic modulation of intestinal interferon- γ generation and *H. polygyrus*-mediated suppression of chronic colitis.

Keywords

TGF- β ; IL-10; helminths; lamina propria mononuclear cells; regulatory T cells

Introduction

Helminth exposure is associated with immune modulation in the human or murine host and decreased immune reactivity to antigens unrelated to the parasite [1]. This response may be

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useful in the treatment of autoimmune and immunological diseases, like inflammatory bowel disease (IBD) [2;3]. Helminths stimulate the host to produce Th2 (IL-4, IL-5, IL-9, IL-13) or regulatory (IL-10, TGF- β) cytokines, while blocking Th1 cytokine responses (IL-12, IFN- γ) [4;5].

Helminthic parasites limit disease activity in various animal models of IBD. For example, rectal TNBS administration causes a T cell cytokine-driven colitis that is prevented by systemic administration of *Schistosoma mansoni* eggs or duodenal colonization with *Heligmosomoides polygyrus* larvae [6;7]. *H. polygyrus*-mediated regulation of IFN- γ , IL-12/23 (p40) production and protection from colitis is in part blocked by inhibiting IL10-signaling in this TNBS model.

IL-10 is a major immune regulatory cytokine [8] that helps prevent intestinal inflammation. Accelerated, severe colitis is triggered in IL-10 deficient and not wild-type mice by *Helicobacter hepaticus* infection or treatment of the animals with nonsteroidal antiinflammatory drugs [9-11]. While different cells can produce IL-10 and regulate immune responses, animal studies implicate CD4 T lymphocyte derived IL-10 as a non-redundant regulator of intestinal immune balance [12]. IL-10 is involved in helminthic regulation of mucosal Th1 cytokine responses where T cells constitute the major source of intestinal IL-10 [13;14]. The mechanism of how helminths induce IL-10 producing T cells is unknown. Recent evidence suggests that TGF- β is involved in the induction of IL-10 producing T cells [15;16].

TGF- β is an immune regulatory cytokine [17] that exerts major effects on other T cells. Cellular effect of TGF- β on intestinal T lymphocytes can be blocked by T cell-specific over-expression of a truncated dominant negative TGF- β receptor on T cells (TGF- β RII DN) [18]. We used TGF- β RII DN mice to test the hypothesis whether helminthic induction of intestinal T cell IL-10 is TGF- β -dependent. Our results show that T cell TGF- β signaling is essential for helminthic stimulation of mucosal IL-10 secretion. Moreover, helminths fail to regulate robust Th1 pathway and chronic colitis in the absence host T cell TGF- β signaling pathway.

Results

Helminths enhance TCR-triggered TGF- β responses from LPMC

We recently showed that LP T cells from *H. polygyrus* -colonized mice produce TGF- β in response to LPS stimulation [19]. Next, we tested whether 2-wk helminth colonization enhances TCR-triggered TGF- β responses in intestinal mucosa. Lamina propria mononuclear cells (LPMC) were isolated from *H. polygyrus*-colonized and uninfected control C57BL/6 mice, and stimulated with anti-CD3 and anti-CD28 mAb. Unstimulated LPMC from *H. polygyrus*-infected and uninfected WT mice secreted TGF- β at similar levels (Figure 1). However, anti-CD3/CD28 stimulated LPMC from *H. polygyrus* -colonized mice secreted substantially more TGF- β compared to LPMC from uninfected animals.

Next we tested whether intact TGF- β signaling was required for helminthic induction of T cell TGF- β production. TGF- β RII DN mice whose T cells over-express a truncated TGF- β receptor and are rendered unresponsive to TGF- β stimulation develop severe spontaneous colitis and wasting disease after 10 wk of age [18]. Colonization of TGF- β RII DN mice with *H. polygyrus* at 5-6 wk of age and isolation of their LPMC 2 wk later permits analysis of cytokine secretion from LPMC before colitis can be detected by histology (data not shown). Unstimulated or anti-CD3/CD28 stimulated TGF- β secretion was undetectable in LPMC of uninfected control and TGF- β RII DN mice (Figure 1). *H. polygyrus* colonization did not prime LPMC from TGF- β RII DN mice to make TGF- β constitutively or after anti-CD3/CD28 stimulation (Figure 1). *H. polygyrus* colonization also did not induce LPMC TGF- β secretion after LPS stimulation in TGF- β RII DN mice (data not shown), although *H. polygyrus* promotes LPMC TGF- β secretion in response to LPS in WT animals [19].

Helminthic modulation of LPMC IFN- γ production requires T cell TGF- β signaling

TGF- β might exert its greatest impact on immune regulation through interaction with T cells [17;18]. To investigate the role of TGF- β regulation on T cell cytokine secretion and helminthic immune modulation, LPMC from *H. polygyrus*-infected and uninfected control C57BL/6 WT or TGF- β RII DN mice were studied. *In vitro* anti-CD3/CD28 stimulated LPMC of TGF- β RII DN mice produced about 25-fold more IFN- γ compared to LPMC from WT animals (Figure 2). Helminth colonization had no regulatory effect on the LPMC IFN- γ generation from TGF- β RII DN mice, although worm colonization completely abrogated the IFN- γ response in LPMC from WT animals (Figure 2).

Th2 cytokine production and cell frequency in LPMC of TGF- β RII DN mice is elevated irrespective of helminth infection

Induction of Th2 cytokines with helminth infection may help limit Th1 cytokine production. Therefore, we tested to see if helminth colonization would promote secretion of the Th2 cytokines from LPMC of TGF- β RII DN mice. As expected, anti-CD3/CD28-stimulated LPMC from WT animals only produced IL-4 and IL-5 after helminth infection (Figure 2). However, unlike uninfected WT mice, strong IL-4 and IL-5 responses were observed from LPMC of uninfected TGF- β RII DN mice. Helminth colonization only modestly enhanced these responses (Figure 2).

Deletion of effector T cells is a possible mechanism through which helminths could regulate T cell function. To test for this possibility, we used intracytoplasmic staining and flow analysis to enumerate the number of IFN- γ and IL-4 expressing T cells before and after worm colonization. By flow cytometry, unstimulated LP T cells from uninfected WT mice expressed little or no IFN- γ or IL-4 (Figure 3A). After anti-CD3/CD28 stimulation, a small percentage of LPMC from uninfected WT mice stained positive for IFN- γ and essentially none expressed IL-4. In LPMC from helminth-infected animals, anti-CD3/CD28 stimulation induced T cells to express IL-4 (Figure 3B and E). Interestingly, although helminth infection strongly down-regulated IFN- γ secretion in WT mice (Figure 2), the number of IFN- γ + LP T cells did not change significantly after worm colonization (Figure 3B and E).

Similar to WT mice, few LPMC from uninfected and worm-infected TGF- β RII DN mice constitutively expressed IFN- γ or IL-4 (Figure 3C and D). Following anti-CD3/CD28 stimulation, the LPMC of uninfected TGF- β RII DN mice displayed a 4 to 5-fold increase in the percentage of T cells staining positive for IFN- γ compared to LP T cells from uninfected WT mice (Figure 3A, C and E). *H. polygyrus* infection did not significantly change the numbers of IFN- γ + positive T cells in the lamina propria from TGF- β RII DN animals. In contrast to LPMC from WT mice, some LPMC from uninfected TGF- β RII DN animals stained for IL-4 after T cell stimulation. Helminth infection increased the percentage of IL-4 producing Th1.2 + LPMC in TGF- β RII DN mice from $3.7 \pm 2.2\%$ to $6.3 \pm 0.5\%$ ($p < 0.05$) (Figure 3E). The percentage of IL-4 staining LPMC after anti-CD3/CD28 stimulation in helminth-infected WT or TGF- β RII DN mice was not statistically different (Figure 3E).

Worms fail to induce IL-10 production in LPMC of TGF- β RII DN mice

T cells produce IL-10 that can down-regulate Th1 responses [7]. Therefore, we studied whether helminths stimulate IL-10 secretion from LPMC of TGF- β RII animals where helminths fail to regulate IFN- γ secretion. LPMC from uninfected WT mice produced little IL-10 after anti-CD3/CD28 mAb stimulation (Figure 4). As expected, helminths promoted strong IL-10 production in LPMC of WT mice. IL-10 secretion was negligible in LPMC of TGF- β RII DN mice, and this response was not enhanced during worm colonization (Figure 4).

Effect of exogenous IL-10 on LPMC IFN- γ production from WT and TGF- β RII DN mice

To further test the role of IL-10 in down-regulating LPMC IFN- γ responses, we cultured LPMC from uninfected WT and TGF- β RII DN animals with exogenous recombinant IL-10. In cultures containing 5×10^5 cells/well in a 96 well-plate, exogenous IL-10 reduced anti-CD3/CD28-stimulated IFN- γ production by almost 50% in LPMC of WT mice, but did not affect IFN- γ production from TGF- β RII DN animals (Figure 5A).

The LPMC isolates from TGF- β RII DN mice contained more total T cells and the proportion of Th1 cells was also increased in TGF- β RII DN mice. These changes were associated with 25-fold increased IFN- γ output in LPMC from TGF- β RII DN compared to WT animals. Therefore, we repeated the experiments using fewer LPMC and T cells per well. Incubations in U-bottom shaped 96 well plates allowed us to culture LPMC from TGF- β RII DN mice, utilizing serial 2-fold dilutions in cell number and going as low as 30,000 cells per well with detectable IFN- γ output (Figure 5B). IL-10 mediated regulation of IFN- γ secretion became evident at cell densities of 120,000 cells/well and became comparable to IL-10 mediated regulation in WT LPMC in lower cell densities (Figure 5B and C). Anti-CD3/28 stimulated IFN- γ secretion was detectable in LPMC cultures from WT mice containing corresponding decreased cell numbers. IL-10-mediated regulation of IFN- γ generation in LPMC from WT mice did not change in these parallel cultures (Figure 5C).

The reduction in worm population in WT and TGF- β RII DN mice is similar

Th2 cytokines, whose secretion is enhanced during helminth infection, may help the host reduce the number of worms by either increasing intestinal mucus secretion or gut motility [4]. Because TGF- β RII DN mice show augmented Th2 production, it is possible that they rapidly expel helminths. To exclude the possibility that the failure of *H. polygyrus* colonization to regulate intestinal cytokine secretion is related to decreased helminth counts from the intestine of TGF- β RII DN mice, the number of worms in the intestine was counted up to 6 wk after initiation of the infection. TGF- β RII DN mice displayed a similar kinetics of decrease in worm population compared to their WT counterparts (Figure 6).

Worms have no effect on colitis development in TGF- β RII DN mice

To study the role of T cell TGF- β signaling in helminthic regulation of colitis, large intestines of 12-14 wk old uninfected or helminth-infected TGF- β RII DN animals were analyzed by histology. These animals develop severe chronic colitis after week 10. The colons of uninfected control mice exhibit severe inflammation by this age (Figure 7). Colitis is characterized by deep mononuclear cell infiltration, expansion of LP, widening of epithelium with loss of mucin, mucosal ulcerations and thickening of the muscle layer. Similar histologic changes were seen in colons of *H. polygyrus*-infected TGF- β RII DN mice (Figure 7). No colitis was seen in 12-14 wk old uninfected or worm-infected WT mice (data not shown). Chronic colitis in TGF- β RII DN mice was not associated with colon shortening (Figure 7), unlike other models of chronic intestinal injury [20]

Discussion

In this study, we demonstrated that helminths living in the duodenum promote TCR-triggered TGF- β production by LPMC isolated from the distal intestine. This study also showed the essential role of TGF- β in regulating intestinal cytokine synthesis, as mice with a T cell-specific TGF- β signaling defect fail to control intestinal Th1 or Th2 cytokine production. Loss of T cell TGF- β signaling abolished helminth-associated priming of immune regulatory mucosal cytokine (TGF- β and IL-10) production. The negligible amount of IL-10 production in LPMC from helminth-infected TGF- β RII DN mice was insufficient to control the robust LP T cells

IFN- γ output. Interference with the TGF- β signaling to T cells also abrogated worm-induced regulation of colitis.

Intact TGF- β signaling is required to limit intestinal mucosa IFN- γ production in uninfected mice, as the number of IFN- γ + LP T cells was increased by 4 to 5-fold and intestinal IFN- γ production by 25-fold in TGF- β RII DN animals compared to WT mice. The IFN- γ signal intensity in cytokine staining Thy1.2+ cells from TGF- β RII DN mice is increased compared to the IFN- γ signal intensity in cytokine staining Thy1.2+ LPMC from their WT counterparts (Figure 3). The overall IFN- γ cytokine fluorescence intensity by flow cytometry was 2.5-fold increased in Thy1.2+ cells from TGF- β RII DN mice compared to WT animals (Figure 3). This discrepancy between 4 to 5-fold increase in IFN- γ producing cells and 25-fold increase in IFN- γ output suggests that T cells from the TGF- β RII DN mice release more IFN- γ per cell compared to WT T cells. Similar correlations between cytokine fluorescence intensity by flow cytometry, the spot size by ELISPOT and the amount of cytokine secretion by ELISA have been reported previously [21;22]. Helminth colonization did not suppress IFN- γ synthesis in TGF- β RII DN mice, although helminth infection almost completely shut down LP T cell IFN- γ generation from WT animals. Thus, helminthic modulation of intestinal mucosal IFN- γ production requires intact T cell TGF- β signaling.

Regulation of IFN- γ responses could be mediated through several mechanisms. Helminths may induce various Th2 cytokines (e.g. IL-4, IL-13) and other regulatory agents (e.g. IL-10, TGF- β) that may limit or modulate Th1-type activity [23;24]. Another possibility is that worms may induce deletion of IFN- γ producing cells from the intestinal mucosa. We found by flow cytometry that IFN- γ -expressing T cells remain in the lamina propria of colonized WT mice. LP T cell IFN- γ production from helminth-infected WT mice could be restored by blocking IL-10 *in vitro* [7]. These data imply that Th1 cell deletion is not the mechanism that prevented TCR-stimulated IFN- γ release in helminth-colonized WT mice.

Strong Th2 cytokine production (IL-4 and IL-5) did not appear to limit mucosal IFN- γ secretion in TGF- β RII DN mice. We found strong Th2 responses in the mucosa of uninfected TGF- β RII DN mice, almost as strong as after helminth colonization in WT mice. T cell TGF- β signaling curtails Th2 cell differentiation presumably by inhibiting T cell GATA3 expression [25]. In this paper, we demonstrate that T cell TGF- β signaling is essential for limiting either the development or activity of Th2 cells in the normal worm-naïve gut. LPMC IL-4, IL-5 and TGF- β secretion were augmented in helminth-infected WT mice. This suggests that helminthic induction of intestinal IL-4 and IL-5 are not inhibited by the concurrent induction of TGF- β , since abolition of TGF- β -signaling did not result in hyper-expression of IL-4 or IL-5 after worm colonization. It is possible that redundant mechanisms beyond TGF- β signaling limit excessive Th2 responses after helminth colonization.

Helminth-induced Th2 responses can enhance intestinal motility that helps worm elimination [4]. Although the Th2 cytokine responses were robust in intestinal mucosa of TGF- β RII DN mice, the worm counts at different time points after infection were not significantly altered compared to WT animals. Thus, failure to achieve helminthic regulation of mucosal T cell IFN- γ production could not be attributed to differences in worm numbers between WT and TGF- β RII DN mice.

IL-10 is a master regulator of Th1 cells [26]. TGF- β is also a strong inhibitor of Th1 differentiation [17]. In the absence of TGF- β signaling to T cells, LP T cell IFN- γ secretion increases dramatically. LPMC Th1 cytokine production in TGF- β RII DN mice can be regulated by exogenous IL-10, when cells are cultured at lower densities, as seen in WT mice (Figure 5). T cells are a major source of IL-10 during helminth infection [7;14;27] and helminthic induction of IL-10 by T lymphocytes correlates with down-regulation of T cell IFN- γ output

[27]. As we show here, helminthic induction of intestinal IL-10 requires intact TGF- β signaling to T cells. Thus, T cell TGF- β pathway controls intestinal Th1 responses at multiple levels: TGF- β interferes with Th1 differentiation, thereby curtailing robust Th1 responses and TGF- β signaling to T cells is essential for the induction of intestinal T cell IL-10 production, which is important in suppressing Th1 cell IFN- γ output.

IL-10 producing T cells are also named Tr1 and they can originate from various effector T cell subsets, such as Th1, Th2, Th17 or CD8 T cells [12]. IL-10 may also originate from FoxP3 positive or FoxP3 negative regulatory T lymphocytes [28;29]. TGF- β has been shown to potentiate IL-10 production from Th1, Th17 effector, FoxP3 positive or FoxP3 negative regulatory T cells [15;16;28;29]. TGF- β may exert its dominant control on LP T cell IL-10 gene expression by activating Smad4 binding to the IL-10 gene promoter [30]. Helminth colonization results in enhanced TGF- β or Th2 cytokine production, increased T cell FoxP3 gene expression and novel regulatory CD8 T cells in the intestine, while helminths suppress intestinal Th1 and Th17 cytokine output [7;31-33]. Currently it is unclear, which intestinal T lymphocyte subset plays a major role in IL-10 production during worm infection.

Although helminthic regulation of mucosal inflammation or IFN- γ production is in part IL-10-mediated [7;13;14], *H. polygyrus* colonization can still regulate IFN- γ production in IL-10 deficient mice [31]. These results suggest that additional immune regulatory mechanisms besides IL-10 induction are employed by helminths. These mechanisms may include helminthic induction of intestinal TGF- β production. *H. polygyrus* stimulates LP T cell TGF- β release in IL-10 deficient animals (unpublished observations) similar to WT mice (Figure 1). T cell generated TGF- β is an important regulator of intestinal immune balance [34]. LPMC TGF- β production is diminished in TGF- β RII DN mice. Whether T cell produced TGF- β is important in *H. polygyrus*-mediated immune regulation and induction of IL-10 producing T cells remains to be established.

In summary, we demonstrate here that TGF- β signaling to T cells is essential in helminthic mucosal immune regulation. Although helminths regulate mucosal Th1 cytokines and colitis in WT mice [7], absence of T cell-specific TGF- β signaling prevents helminthic, and in part IL-10-mediated, regulation of IFN- γ secretion. Moreover, absence of T cell-specific TGF- β signaling disallows helminthic regulation of colitis.

Materials and Methods

Mice and *H. polygyrus* infection

This study used wild type (WT) C57BL/6 animals (Jackson Laboratory, Bar Harbor, ME) and syngeneic mice with T cell-specific defects in TGF- β signaling (TGF- β RII DN)[18]. 5-6 wk old mice were colonized with 150 *H. polygyrus* third stage larvae (L3) by oral gavage. Infective, ensheathed *H. polygyrus* larvae (U.S. National Helminthological Collection no. 81930) were obtained from mouse fecal cultures of eggs by the modified Baermann method and stored at 4°C until used. Animals were housed and handled appropriately following national guidelines and as approved by our Animal Review Committee. For LPMC cytokine analysis, mice were sacrificed 2 wk after helminth infection. For helminth counts, mice were sacrificed 2, 4 or 6 wk after helminth infection. The number of adult worms in the intestine was counted as described before [35;36].

LPMC isolation and culture

Distal small intestine from mice was washed extensively with RPMI, and all visible Peyer's patches were removed with scissors. The intestine was opened longitudinally, cut into 5 mm pieces and then incubated in 0.5 mM EDTA in calcium and magnesium free Hanks' for 20 min

at 37°C with shaking to release intraepithelial lymphocytes and epithelial cells. This was repeated after thorough washing. Tissue then was incubated 20 min at 37°C in 20 ml RPMI containing 10% FCS, 25 mM HEPES buffer, 2 mM L-glutamine, 5×10^{-5} M β -mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 5 mg/ml gentamycin, and 100 mg/ml streptomycin (all GIBCO) and 1 mg/ml collagenase (Sigma). At the end of the incubation, the tissue was subjected to further mechanical disruption using a 1 ml syringe. To remove debris, the LPMC preparations were sieved through gauze layered in a funnel and a 2 cm nylon wool column. After washing, cells (up to 2×10^7) were layered onto a column of Percoll with a 30:70% gradient. Cells were spun at 2200xG at room temperature for 20 min. Subsequent cell viability was >90% as determined by trypan blue exclusion.

After isolation, cells were cultured for 48h in 96 well flat bottom microtiter plates (Corning, Cambridge, MA) with 200 μ l of medium (5×10^5 cells/well) at 37°C. In certain experiments, LPMC were cultured at lesser cell densities ($0.3 \times 10^5 - 2.4 \times 10^5$ cells/well) for 48h in 96 well U-bottom microtiter plates with 200 μ l of medium. The culture medium was RPMI containing 10% FCS, 25 mM HEPES buffer, 2 mM L-glutamine, 5×10^{-5} M β -mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 5 mg/ml gentamycin, and 100 mg/ml streptomycin (all GIBCO). For most experiments, the cells were cultured alone or with anti-CD3 (2C11, ATCC) and anti-CD28 mAb (PharMingen, San Diego, CA) (each at 1 μ g/ml). Culture medium with 1% FCS was used for TGF- β ELISA[19]. Recombinant mouse IL-10 was added at 20 or 100 ng/ml end-concentration to block IFN- γ production.

Cytokine analysis

ELISA was used to measure the concentrations of various cytokines in the supernatants. To measure IFN- γ , plates were coated with a mAb to IFN- γ (HB170, ATCC) and incubated with supernatant. IFN- γ was detected with polyclonal rabbit anti-IFN- γ (gift from Dr. Mary Wilson, University of Iowa) followed by biotinylated goat anti-rabbit IgG (Accurate Chemical Co., Westbury, NY). Color development used streptavidin-HRP (Zymed, San Francisco, CA) and TMB substrate (Endogen, Woburn, MA), and plates were read at 490nm. IL-4 was captured with 11B11 (HB191, DNAX Research Institute, Palo Alto, CA) and detected with biotinylated BVD6 (provided by Kevin Moore and John Abrams, DNAX). IL-5 titers in supernatants were detected using mAb TRFK4 for capture and biotinylated TRFK5 for detection (Dr. Robert Coffman, DNAX). IL-10 was captured with anti-IL-10 mAb (MAB417, R&D Systems) and detected with biotinylated mAb (BAF417, R&D Systems). Total TGF- β was measured using acid-treated supernatant and antibody pairs from R&D Systems.

Flow cytometry

For IFN- γ and IL-4 intracellular cytokine staining, LPMC from control or *H. polygyrus*-infected mice were stimulated with anti-CD3 and anti-CD28 mAb for 14 hours with brefeldin A (Golgi Plug, BD PharMingen) added at 1 μ g/ml end-concentration during the last 12 hours of cell culture. After culture, cells were suspended as 2×10^7 cells/ml in FACS buffer (2% FCS in PBS) and Fc receptors were blocked with 2.4G2 mAb. Surface staining was performed with anti-Thy1.2 FITC (BD PharMingen). To identify cytokine-secreting cells, cells were co-stained with anti-IL-4-PE and anti-IFN- γ PE-Cy7 (BD PharMingen) using Cytotfix/Cytoperm Kit (BD PharMingen) according to manufacturer's instructions. Cells in lymphoid gate were analyzed for Thy1.2, IFN- γ and IL-4 protein expression.

Histologic analysis

5-6 wk old TGF- β R2 DN mice were colonized with *H. polygyrus* larvae. 6-8 wk later mice were sacrificed, their colons were rolled up onto glass slides after they were opened longitudinally. Tissues were fixed in 4% neutral buffered formalin, processed and 6 μ m sections were stained with hematoxylin and eosin. Each sample was given a colitis score under light

microscopy based on following criteria: *grade 0*, no change from normal tissue; *grade 1*, patchy mononuclear cell infiltrate in LP; *grade 2*, uniform mononuclear cell infiltration with slight epithelial hyperplasia; *grade 3*, epithelial and muscle hypertrophy with mononuclear cell infiltrate extending into muscle layer, mucus depletion, crypt abscesses, epithelial erosions and ulcerations; *grade 4*, lesions involving most of the colon.

Statistical analysis

Data are means $\pm$ SD of multiple determinations. Difference between two groups was compared using Student's t-test. P values <0.05 were considered significant.

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Abbreviations

1. <i>H. polygyrus</i>	Heligmosomoides polygyrus
2. LPMC	Lamina propria mononuclear cell
3. TGF- β RII DN	Transforming growth factor- β receptor II dominant negative
4. TNBS	Trinitrobenzene sulfonic acid
5. Treg	Regulatory T cell

References

1. Elliott DE, Summers RW, Weinstock JV. Helminths as governors of immune-mediated inflammation. *Int.J.Parasitol* 2007;37:457–464. [PubMed: 17313951]
2. Summers RW, Elliott DE, Urban JF Jr. Thompson R, Weinstock JV. Trichuris suis therapy in Crohn's disease. *Gut* 2005;54:87–90. [PubMed: 15591509]
3. Summers RW, Elliott DE, Urban JF Jr. Thompson RA, Weinstock JV. Trichuris suis therapy for active ulcerative colitis: a randomized controlled trial. *Gastroenterology* 2005;128:825–832. [PubMed: 15825065]
4. Finkelman FD, Urban JF Jr. The other side of the coin: the protective role of the TH2 cytokines. *J.Allergy Clin.Immunol* 2001;107:772–780. [PubMed: 11344341]
5. Sacco R, Hagen M, Sandor M, Weinstock JV, Lynch RG. Established T(H1) granulomatous responses induced by active Mycobacterium avium infection switch to T(H2) following challenge with Schistosoma mansoni. *Clin.Immunol* 2002;104:274–281. [PubMed: 12217338]
6. Elliott DE, Li J, Blum A, Metwali A, Qadir K, Urban JF Jr. Weinstock JV. Exposure to schistosome eggs protects mice from TNBS-induced colitis. *Am.J.Physiol Gastrointest.Liver Physiol* 2003;284:G385–G391. [PubMed: 12431903]
7. Setiawan T, Metwali A, Blum AM, Ince MN, Urban JF Jr. Elliott DE, Weinstock JV. Heligmosomoides polygyrus promotes regulatory T-cell cytokine production in the murine normal distal intestine. *Infect.Immun* 2007;75:4655–4663. [PubMed: 17606601]
8. Pestka S, Krause CD, Sarkar D, Walter MR, Shi Y, Fisher PB. Interleukin-10 and related cytokines and receptors. *Annu.Rev.Immunol* 2004;22:929–979. [PubMed: 15032600]
9. Burich A, Hershberg R, Waggle K, Zeng W, Brabb T, Westrich G, Viney JL, Maggio-Price L. Helicobacter-induced inflammatory bowel disease in IL-10- and T cell-deficient mice. *Am.J.Physiol Gastrointest.Liver Physiol* 2001;281:G764–G778. [PubMed: 11518689]
10. Kullberg MC, Ward JM, Gorelick PL, Caspar P, Hieny S, Cheever A, Jankovic D, Sher A. Helicobacter hepaticus triggers colitis in specific-pathogen-free interleukin-10 (IL-10)-deficient mice through an IL-12- and gamma interferon-dependent mechanism. *Infect.Immun* 1998;66:5157–5166. [PubMed: 9784517]

11. Berg DJ, Zhang J, Weinstock JV, Ismail HF, Earle KA, Alila H, Pamukcu R, Moore S, Lynch RG. Rapid development of colitis in NSAID-treated IL-10-deficient mice. *Gastroenterology* 2002;123:1527–1542. [PubMed: 12404228]
12. Maynard CL, Weaver CT. Diversity in the contribution of interleukin-10 to T-cell-mediated immune regulation. *Immunol.Rev* 2008;226:219–233. [PubMed: 19161427]
13. Hunter MM, Wang A, Hirota CL, McKay DM. Neutralizing anti-IL-10 antibody blocks the protective effect of tapeworm infection in a murine model of chemically induced colitis. *J.Immunol* 2005;174:7368–7375. [PubMed: 15905584]
14. Kitagaki K, Businga TR, Racila D, Elliott DE, Weinstock JV, Kline JN. Intestinal helminths protect in a murine model of asthma. *J.Immunol* 2006;177:1628–1635. [PubMed: 16849471]
15. Awasthi A, Carrier Y, Peron JP, Bettelli E, Kamanaka M, Flavell RA, Kuchroo VK, Oukka M, Weiner HL. A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. *Nat.Immunol* 2007;8:1380–1389. [PubMed: 17994022]
16. McGeachy MJ, Bak-Jensen KS, Chen Y, Tato CM, Blumenschein W, McClanahan T, Cua DJ. TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nat.Immunol* 2007;8:1390–1397. [PubMed: 17994024]
17. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. Transforming growth factor-beta regulation of immune responses. *Annu.Rev.Immunol* 2006;24:99–146. [PubMed: 16551245]
18. Gorelik L, Flavell RA. Abrogation of TGF-beta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 2000;12:171–181. [PubMed: 10714683]
19. Ince MN, Elliott DE, Setiawan T, Blum A, Metwali A, Wang Y, Urban JF Jr. Weinstock JV. Heligmosomoides polygyrus induces TLR4 on murine mucosal T cells that produce TGF-beta after lipopolysaccharide stimulation. *J.Immunol* 2006;176:726–729. [PubMed: 16393954]
20. Rakoff-Nahoum S, Hao L, Medzhitov R. Role of toll-like receptors in spontaneous commensal-dependent colitis. *Immunity* 2006;25:319–329. [PubMed: 16879997]
21. Hesse MD, Karulin AY, Boehm BO, Lehmann PV, Tary-Lehmann M. A T cell clone's avidity is a function of its activation state. *J.Immunol* 2001;167:1353–1361. [PubMed: 11466353]
22. Openshaw P, Murphy EE, Hosken NA, Maino V, Davis K, Murphy K, O'Garra A. Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarized T helper 1 and T helper 2 populations. *J.Exp.Med* 1995;182:1357–1367. [PubMed: 7595206]
23. Elliott DE, Urban JF Jr. Argo CK, Weinstock JV. Does the failure to acquire helminthic parasites predispose to Crohn's disease? *FASEB J* 2000;14:1848–1855. [PubMed: 10973934]
24. Maizels RM, Yazdanbakhsh M. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nat.Rev.Immunol* 2003;3:733–744. [PubMed: 12949497]
25. Gorelik L, Fields PE, Flavell RA. Cutting edge: TGF-beta inhibits Th type 2 development through inhibition of GATA-3 expression. *J.Immunol* 2000;165:4773–4777. [PubMed: 11045997]
26. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. *J.Immunol* 2008;180:5771–5777. [PubMed: 18424693]
27. Sher A, Fiorentino D, Caspar P, Pearce E, Mosmann T. Production of IL-10 by CD4+ T lymphocytes correlates with down-regulation of Th1 cytokine synthesis in helminth infection. *J.Immunol* 1991;147:2713–2716. [PubMed: 1680917]
28. Maynard CL, Harrington LE, Janowski KM, Oliver JR, Zindl CL, Rudensky AY, Weaver CT. Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. *Nat.Immunol* 2007;8:931–941. [PubMed: 17694059]
29. Maynard CL, Hatton RD, Helms WS, Oliver JR, Stephensen CB, Weaver CT. Contrasting roles for all-trans retinoic acid in TGF- β -mediated induction of Foxp3 and Il10 genes in developing regulatory T cells. *J.Exp.Med.* 2009
30. Kitani A, Fuss I, Nakamura K, Kumaki F, Usui T, Strober W. Transforming growth factor (TGF)-beta1-producing regulatory T cells induce Smad-mediated interleukin 10 secretion that facilitates coordinated immunoregulatory activity and amelioration of TGF-beta1-mediated fibrosis. *J.Exp.Med* 2003;198:1179–1188. [PubMed: 14557415]
31. Elliott DE, Setiawan T, Metwali A, Blum A, Urban JF Jr. Weinstock JV. Heligmosomoides polygyrus inhibits established colitis in IL-10-deficient mice. *Eur.J.Immunol* 2004;34:2690–2698. [PubMed: 15368285]

32. Metwali A, Setiawan T, Blum AM, Urban J, Elliott DE, Hang L, Weinstock JV. Induction of CD8+ regulatory T cells in the intestine by *Heligmosomoides polygyrus* infection. *Am.J.Physiol Gastrointest.Liver Physiol* 2006;291:G253–G259. [PubMed: 16825660]
33. Elliott DE, Metwali A, Leung J, Setiawan T, Blum AM, Ince MN, Bazzone LE, Stadecker MJ, Urban JF Jr. Weinstock JV. Colonization with *Heligmosomoides polygyrus* suppresses mucosal IL-17 production. *J.Immunol* 2008;181:2414–2419. [PubMed: 18684931]
34. Li MO, Wan YY, Flavell RA. T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. *Immunity* 2007;26:579–591. [PubMed: 17481928]
35. Urban JF Jr. Katona IM, Paul WE, Finkelman FD. Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice. *Proc.Natl.Acad.Sci.U.S.A* 1991;88:5513–5517. [PubMed: 2062833]
36. Urban JF Jr. Katona IM, Finkelman FD. *Heligmosomoides polygyrus*: CD4+ but not CD8+ T cells regulate the IgE response and protective immunity in mice. *Exp.Parasitol* 1991;73:500–511. [PubMed: 1683629]

Figure 1.

TCR-triggered TGF- β production in LPMC from uninfected control or *H. polygyrus*-infected WT and TGF- β RII DN mice. LPMC from uninfected or helminth-infected WT or TGF- β RII DN mice were cultured *in vitro* alone (Cells) or with anti-CD3/CD28 mAb (Cells + α CD3/28) for 48 h in TGF- β culture medium. Supernatants then were analyzed for TGF- β by ELISA. Data show mean $\pm$ SD from three independent experiments with each experiment containing multiple determinations. (WT: unstimulated LPMC Uninfected vs. Infected, $p > 0.05$, NS; anti-CD3/CD28 stimulated Uninfected vs. Infected, $**p < 0.01$).

Figure 2.

Helminths cannot modulate the Th1 response and only weakly promote the Th2 response in LPMC from TGF- β RII DN mice. LPMC from uninfected or helminth-infected WT or TGF- β RII DN mice were stimulated *in vitro* with anti-CD3/CD28 mAb for 48 h. Supernatants were harvested and analyzed for IFN- γ , IL-4 and IL-5 by ELISA. Data show mean $\pm$ SD from at least three independent experiments with each experiment containing multiple determinations. (IFN- γ : WT Uninfected vs. Infected, *** p <0.001; TGF- β RII DN Uninfected vs. Infected, p >0.05, NS. IL-4: WT Uninfected vs. Infected, *** p <0.001; TGF- β RII DN Uninfected vs. Infected, * p <0.05. IL-5: WT Uninfected vs. Infected, *** p <0.001; TGF- β RII DN Uninfected vs. Infected, * p <0.05).

Figure 3.

Helminth-associated changes in cytokine producing LP T cells from WT and TGF-βRII DN mice. Representative dot plots of LPMC from (A) uninfected WT, (B) helminth-infected WT, (C) uninfected TGF-βRII DN or (D) helminth-infected TGF-βRII DN mice that were stimulated overnight *in vitro* with anti-CD3/28. Brefeldin A was added to culture supernatants for the last 12 hours. Subsequently, cells were harvested and stained for Thy1.2, IFN-γ and IL-4 for FACS analysis. (E) Data show mean ± SD of the percentage IFN-γ (upper panel) or IL-4 (lower panel) producing Thy1.2 positive LP cells from four (WT) and three (TGFβ RII DN) independent experiments. (IFN-γ: WT Uninfected vs. Infected, $p > 0.05$, NS; TGF-βRII DN Uninfected vs. Infected, $p > 0.05$, NS. IFN-γ: WT Uninfected vs. TGFβ RII DN Uninfected, $*p < 0.05$; WT Infected vs. TGFβ RII DN Infected, $*p < 0.05$. IL-4: WT Uninfected vs. Infected, $*p < 0.05$; TGF-βRII DN Uninfected vs. Infected, $p < 0.05$; WT Uninfected vs. TGFβ RII DN Uninfected $*p < 0.05$; WT Infected vs. TGFβ RII DN Infected, $p > 0.05$, NS).

Figure 4.

Helminthic induction of IL-10 in LPMC is dependent on T cell TGF- β signaling. LPMC from uninfected or helminth-infected WT or TGF- β R DN mice were stimulated *in vitro* with anti-CD3/CD28 mAb for 48 hr. Supernatants were harvested and analyzed for IL-10 by ELISA. Data show mean $\pm$ SD from three independent experiments with each experiment containing multiple determinations. (WT Uninfected vs. Infected, *** p <0.001; TGF- β RII DN Uninfected vs. Infected, p >0.05, NS).

Figure 5.

Effect of exogenous IL-10 on LPMC IFN- γ production from WT and TGF- β RII DN mice. LPMC from uninfected WT (**A and C**) or TGF- β RII DN (**A and B**) mice were stimulated *in vitro* with anti-CD3/CD28 (white bars) for 48 hr, in indicated cell numbers per well, with parallel cultures also containing 20 ng/ml (gray bars) or 100 ng/ml recombinant IL-10 (black bars). Supernatants were harvested and analyzed for IFN- γ by ELISA. Data show mean $\pm$ SD of (**A**) IFN γ secretion (two independent experiments with each experiment containing multiple determinations) or (**B-C**) the percentile ratio of LPMC IFN γ secretion in each condition to IFN- γ output of anti-CD3/28-stimulated culture (three independent experiments with each experiment containing multiple determinations). ((**A**) WT Uninfected vs. Infected,

*** $p < 0.001$; TGF- β RII DN Uninfected vs. Infected, $p > 0.05$, NS). **(B-C)** (* $p < 0.05$ for IFN- γ secretion between IL-10 containing and IL-10 naïve cultures in incubations with the corresponding cell numbers).

Figure 6.

The reduction in worm population in WT and TGF-βRII DN mice is similar. The number of duodenal helminths was counted in 6 WT and 6 TGF-βRII DN mice at each time point, 2, 4 and 6 weeks after worm colonization. Data from 18 WT and 18 TGF-βRII DN mice in 3 different time points were accumulated in 3 independent experiments. Data show mean ± SD at each time point. (2 wk: WT vs. TGF-βRII DN, $p > 0.05$; 4 wk: WT vs. TGF-βRII DN, $p > 0.05$; 6 wk: WT vs. TGF-βRII DN, $p > 0.05$, NS).

Figure 7.

H. polygyrus colonization does not reverse spontaneous colitis in TGF-βRII DN mice. (A) Representative example of dissected colons from 12-14 wk old WT, uninfected control TGF-βRII DN and *H. polygyrus*-infected TGF-βRII DN mouse. (B-D) Representative example of colonic histology of 12-14 wk old WT (B), uninfected control TGF-βRII DN (C) and *H. polygyrus*-infected TGF-βRII DN (D) mouse (4x magnified). (E) Colonic tissues of uninfected or helminth-infected TGF-βRII DN mice were analyzed by histopathology. Data show mean ± SD of histologic colitis scores from 10 uninfected and 13 *H. polygyrus*-infected mice in 3 independent experiments (TGF-βRII DN Uninfected vs. Infected, $p > 0.05$, NS).