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HELIGMOSOMOIDES BAKERI INFECTION DECREASES SMAD7 EXPRESSION IN INTESTINAL CD4+ T CELLS WHICH ALLOWS TGFβ TO INDUCE IL10-PRODUCING REGULATORY T CELLS THAT BLOCK COLITIS

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Abstract

Helminthic infections modulate host immunity and may protect their hosts from developing immunological diseases like inflammatory bowel disease (IBD). Induction of regulatory T cells (Tregs) may be an important part of this protective process. *Heligmosomoides polygyrus bakeri (Hpb)* infection also promotes the production of the regulatory cytokines TGFβ and IL10 in the gut. In the intestines, TGFβ helps induce regulatory T cells. This study used Foxp3/IL10 double reporter mice to investigate the effect of TGFβ on the differentiation of colon and MLN-derived, murine Foxp3$^-$ IL10$^-$ CD4$^+$ T cells into their regulatory phenotypes. Foxp3$^-$ IL10$^-$ CD4$^+$ T cells from *Hpb*-infected mice, as opposed to T cells from uninfected animals, cultured *in vitro* with TGFβ and anti-CD3/CD28 mAb differentiated into Foxp3$^+$ and/or IL10$^+$ T cells. The IL10 producing T cells nearly all displayed CD25. Smad7 is a natural inhibitor of TGFβ signaling. In contrast to gut T cells from uninfected mice, Foxp3$^-$ IL10$^-$ CD4$^+$ T cells from *Hpb*-infected mice displayed reduced Smad7 expression and responded to TGFβ with Smad2/3 phosphorylation. The TGFβ-induced Tregs that express IL10 blocked colitis when transferred into the Rag/CD25$^-$ CD4$^+$ T cell transfer model of IBD. TGFβ had a greatly diminished capacity to induce Tregs in *Hpb*-infected transgenic mice with constitutively high T-cell specific Smad7 expression. Thus, infection with *Hpb* causes down-modulation in Smad7 expression in intestinal CD4$^+$ T cells, which allows the TGFβ produced in response to the infection to induce the Tregs that prevent colitis.

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Keywords

helminths; Smad7; T cells; Colitis; IL-10

INTRODUCTION

Inflammatory bowel disease (IBD) is a problem of increasing prevalence around the world. Improving hygiene with an associated alteration in the composition of the intestinal microbiota is postulated to be a major cause for the spread of these diseases (1). Loss of helminthic infections could be a contributing factor for this rise, which is supported by clinical and epidemiologic studies (2). Helminths, like *Heligmosomoides polygyrus bakeri* (*Hpb*), are strong inducers of regulatory T cells (3–5), macrophages (6–10) and dendritic cells (11,12) in their host, which block colitis in several different murine models of IBD. These experimental observations provide mechanistic support for the concept.

Regulatory-type T cells help maintain mucosal immune homeostasis (13). *Hpb* infection stimulates T cells in the gut and mesenteric lymph nodes (MLN) to express Foxp3 (3–5). Colonic and MLN Tregs from *Hpb* infected mice, as opposed to Tregs from uninfected animals, have a strongly enhanced capacity to block colitis in the Rag/$CD4^+CD25^-$ T cell transfer model of IBD (5). This is due in part through induction of GATA3 within these cells (14,15) (Weinstock, JV, unpublished). GATA3 also helps to control IL10 expression (16).

Hpb infection induces the gut to produce immune regulatory cytokines like TGFβ and IL10 (17). T cells expressing TGFβ receptors that cannot respond to TGFβ ligation develop colitis showing the importance of the interaction of TGFβ with T cells in preventing disease (18,19). T cells that make IL10 also have a critically role in protecting mice from colitis (20). IL10-producing, $Foxp3^+$ T cells are of particular importance (5,21–23). TGFβ, via inducing phosphorylation of Smad2 and Smad3 (24), stimulates T cells to differentiate into Tregs in the gut microenvironment (25,26). Thus, it is conceivable that *Hpb* protects mice from colitis through stimulating TGFβ production in the intestine, which then promotes the development of regulatory T cells that make IL10.

To further test this hypothesis, this study examined the effect of TGFβ on the differentiation of colon or MLN-derived, $Foxp3^-$ $IL10^-$ $CD4^+$ T cells cultured *in vitro*. It was expected that TGFβ would readily induce gut $CD4^+$ T cells from both *Hpb*-infected and uninfected mice to differentiate into Tregs. It was discovered that only T cells from *Hpb*-infected mice readily changed into their various regulatory phenotypes upon TGFβ stimulation. With regard to T cells derived from the colon, the most commonly induced regulatory $CD4^+$ T cell subsets displayed either Foxp3 and IL10, or just IL10. Both of these subsets expressed CD25 and readily blocked colitis when transferred into the Rag/$CD4^+$ $CD25^-$ T cell transfer model of IBD. The expression of Smad7, a negative regulator of TGFβ receptor signaling, was strongly inhibited in the gut- and MLN-derived T cells of *Hpb*-infected mice. This allowed TGFβ to trigger its receptor on T cells permitting phosphorylation of Smad2/3, an event that did not occur in colonic T cells from uninfected animals. Thus, down-modulation of Smad7 expression appears to be an important mechanism through which helminths promote regulatory T cell development in the gut.

MATERIALS AND METHODS

Mice:

This study used Foxp3 mRFP/IL10 eGFP double reporter mice that were produced by cross breeding Foxp3 mRFP and IL10 eGFP single reporter mice obtained from (Dr. Richard Flavell, Yale University, CT). Rag1 and wild-type (WT) and OT2 CD4.1mice were from Jackson Laboratory, Bar Harbor, ME. The CD2-Smad7 high expression transgenic mice were obtained from Dr. Massimo C. Fantini, Dept. of Systems Medicine, University of Rome "Tor Vergata", Rome, Italy. The heterozygote C57BL/6 background CD2-Smad7 female mice were bred with C57BL/6 WT. Transgenic integration was confirmed by a PCR of genomic DNA. All mice were on the C57BL6 background. Breeding colonies were maintained in SPF facilities at Tufts University. Animals were housed and handled following national guidelines and as approved by our Animal Review Committee.

Hpb infection:

The 5- to 6-wk-old mice were colonized with or without 125 *Hpb* third stage larvae by oral gavage, and infected mice were used after two weeks. Infective, ensheathed *Hpb* L3 (U.S. National Helminthological Collection no. 81930) were obtained from fecal cultures of eggs by the modified Baermann method and stored at 4°C.

Dispersion of splenocytes and mesenteric lymph node (MLN) cells, and isolation of splenic T cells:

Single cell suspensions of splenocytes and MLN cells were prepared by gentle teasing in RPMI 1640 medium (RPMI) (GIBCO, Grand Island, NY). The cells were washed three times in RPMI. Splenic T cells were isolated by negative selection using the EasySep mouse T cell enrichment kit as outlined by the manufacturer (Stemcell Technologies, #19751, Vancouver, Canada). Viability was determined using exclusion of trypan blue dye.

Lamina propria mononuclear cells (LPMC) isolation and LP cell fractionation:

LPMCs were isolated from the colon as described (27). Cell viability was 90% as determined by trypan blue exclusion. Both for the dispersed LPMC and MLN cells, the $Foxp3^{-}IL10^{-}$ $CD4^{+}$ T cells were stained and isolated by FACS. Anti-Thy1.2 mAb and FACS confirmed that the isolated cells were >90% T cells.

Real-time PCR (rt-PCR):

Total RNA was isolated from individual samples using Quick-RNA Mini Prep (Zymo Research, Irvine, CA) as per manufacturer's instructions. RNA quality and quantity was determined using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA was converted to cDNA using qScript cDNA SuperMix (Quanta Bioscience, Gaithersburg, MD). Rt-PCR was performed using the Eco rt-PCR System (Illumina, San Diego, CA). GAPDH levels were used to normalize the data. Taqman real time primers for Smad2, 4, 7, GADPH were obtained from Applied Biosystems (Grand Island, NY).

Sandwich ELISAs:

ELISAs were performed using paired antibodies. To measure IFNγ, plates were coated with a mAb to IFNγ (HB170, ATCC) and incubated with supernatant. IFNγ was detected with polyclonal rabbit anti-IFNγ (gift from Dr. Mary Wilson, University of Iowa) followed by biotinylated goat anti-rabbit IgG (Axcell, Westbury, NY). IL17 ELISA was done using primary capture mAb and biotinylated anti-IL17A mAb (R & D Systems, Minneapolis, MN).

Flow cytometry:

LPMC or MLN cells were washed twice and adjusted to 1×10^6 cells/ml in the RPMI medium 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 from Life Technologies, Gaithersburg, MD) and stained with saturating amounts of conjugated mAb for 30 min at 4°C. Following staining, cells were washed twice and re-suspended in complete medium for analysis on the BDLSRii Flow cytometer using FACSDIVA V6.1.1 software (BD, eBioscience, Mountain View, CA). Before adding labeled mAb, each tube received 1 μg of anti-Fc mAb to block nonspecific binding of conjugated Abs to FcRs. The mAbs used for staining or cell sorting were anti-CD4-APC, anti-Thy1.2-APC and anti-CD25-APC (all above from eBioscience, San Diego, CA). The PE-labeled anti-TGFβ receptor 1 and receptor 2 mAbs were from R&D Systems, Minneapolis, MN.

Intracellular staining for phosphorylated Smad2/3:

Isolated colonic $Foxp3^-$ $IL10^-$ $CD4^+$ T cells were cultured in RPMI complete medium for 45 min at 37°C. Some cultures also contained TGFβ (10ng/ml). Cells then were fixed, permeabilized and intracellularly stained for phosphorylated Smad2/3 following the eBioscience intracellular (nuclear) staining protocol using anti- phosphorylated Smad2/3-PE (from BD Bioscience, Cat#: 562586). Briefly, after washing with Fixation/Permeabilization buffer (eBioscience, Cat#, 00–5521), fix cells in 1 ml Fixation/Permeabilization buffer were maintained overnight in the dark at 4°C. Next day, cells were washed with Permeabilization buffer (eBioscience, Cat#, 00–8333), and 5 ul anti-phosphorylated Smad2/3 mAb-PE was added to T cells in 100 ul Permeabilization buffer for 1 h at room temperature in the dark. Then, the cells were washed with Permeabilization buffer followed by washes with lymphocyte growth medium (LGM) to remove the unbound mAb. Finally, the cells were placed in LGM for cytometric analysis.

Intracellular staining for IL-10

Mouse colonic $CD4^+$ cells were staining with Alexa 647-CD25 (Biolegand Inc., San Diego, CA) for 30 min in the dark. They then were washing once, fixed, permeabilized and intracellular stained for IL-10 following the eBioscience intracellular staining protocol using anti-IL10-PE (from eBioscience).

Production of *Hpb* excretory and secretory product (HES)(28):

HES was from Dr. Mary Stevenson, McGill University, Canada or made in our laboratory. The *Hpb* were collected from the gut of infected mice and cultured in serum free RPMI with 2% glucose and antibiotics (Pen, Strep, Gentamycin, polymyxin). The supernatant was collected after 36 hrs of culture and concentrated using a 3KDa concentrator.

Induction of colonic Foxp3$^+$ and/or IL-10$^+$ CD4$^+$ T cells:

Dispersed colonic LPMC or MLN cells from either *Hpb*-infected or uninfected Foxp3mRFP/IL10eGFP double reporter mice were labeled with anti-CD4-APC. Foxp3- IL10- CD4$^+$ T cells were isolated using FACS to select anti-CD4-APC positive T cells, excluding cells displaying endogenous fluorescence for Foxp3 and IL10. Foxp3$^-$ IL10$^-$ CD4$^+$ T cells (5×10^4) were placed in complete medium, and cultured in 96-well round-bottom plates for 72 h at 37°C with or without anti-CD3 (5 ug/ml) and anti-CD28 (2.5 ug/ml, eBioscience, #14–0281) mAbs. Anti-CD3 mAb was derived from ATCC cell line #145–2C11. Some wells also contained rTGFβ (10ng/ml, R&D Systems, #240-B). Some cultures also contained *Hpb* excretory and secretory product (HES). Then, the cells were harvested and analysis by BD cytometer for the presence of Foxp3$^-$ IL10$^-$, Foxp3$^+$ IL10$^-$, Foxp3$^-$ IL10$^+$ and Foxp3$^+$ IL10$^+$ CD4$^+$ T cells.

For the T cell transfer experiments outlined below, the CD4$^+$ T cells were cultured with rTGFβ for 7 days to obtain enough T cells for this experiment. In 7-day cultures, the medium was exchanged at day-4. The T cell phenotypes were present in similar proportions in 3- and 7-day T cells cultures.

Modulation of colitis and cell culture experiments:

Rag mice of similar age (5- to 6-wks-old) were reconstituted i.p. with 2×10^5 CD25$^-$ CD4$^+$ splenic T cells from 57BL/6 WT mice. Some mice also received 1×10^5 colonic Foxp3$^-$ IL10$^+$ or Foxp3$^+$ IL10$^+$ CD4$^+$ T cells, from *Hpb*-infected Foxp3mRFP/IL10eGFP reporter mice, that were induced to express Foxp3 or IL10 by *in vitro* exposure to TGFβ. Other mice received the IL10$^-$ Foxp3$^-$ CD4$^+$ T cells that kept their original phenotype after TGFβ exposure. One week after T cell transfer, Rag mice received piroxicam mixed into their feed for 2 weeks (42 mg piroxicam /250g chow week 1, and 62 mg piroxicam/250g chow week 2). The piroxicam (Sigma) was stopped, and colitis was studied 1 week later. Thus, it was 4 weeks from the day of cell transfer until animal sacrifice. The colons were divided longitudinally. One half was fixed, sectioned and stained for microscopic examination to score the severity of colitis. An investigator blinded to the experimental conditions graded the severity of the colonic inflammation using a well-described 4-point scale (29). The other half of the colon was dissociated with collagenase to isolate LPMC that were cultured *in vitro*.

LP cell culture:

Dispersed Colonic LPMC from T cell-reconstituted, Rag mice were cultured (2×10^5 cells per well) in RPMI complete medium with splenic OT2 T cells (10^5 cells/well) for 48h in 96-well round-bottomed plates. Cells were cultured alone or without OVA (50 ug/ml,

Worthington Biochemicals #3054 Lakewood, NJ) to stimulate cytokine secretion. After culture, the supernatants were assayed for IFNγ or IL-17A using ELISAs.

Statistical analysis:

Data are means ±SE of multiple determinations. Difference between two groups was compared using two-tailed Student's t-test. Analyses were comparisons between the means of independent experiments done 3 or more times as indicated in the legends. P values <0.05 were considered significant. We used the t-test, as is standard in the field. The t-test (a parametric test) is appropriate because the data points are averages from a series of experiments with different mice and at times multiple tissue sections per mice. The group means are therefore averages of a large number of observations and can thus be assumed to be normally distributed.

RESULTS

Colonic LP CD4$^+$ T cells from Hpb-infected mice more readily convert into their regulatory phenotypes after exposure to TGFβ.

In the gut, *Hpb* can protect mice from colitis by stimulating the production of TGFβ (18,30) and by inducing the expression of IL10-producing Tregs (5). In the intestines, TGFβ helps induce regulatory T cells (31). In this study, experiments determined if infection with *Hpb* rendered intestinal T cells more susceptible to conversion into their regulatory phenotypes through the action of TGFβ.

Using Foxp3/IL10 double reporter mice, it is possible to isolate Foxp3$^-$ IL10$^-$ CD4$^+$ T cells from the colonic LP. Foxp3$^-$ IL10$^-$ CD4$^+$ T cells from uninfected mice were cultured for 3 days with TGFβ and anti-CD3/CD28. This resulted in the appearance of a minimal number of CD4$^+$ T cells expressing Foxp3 and/or IL10 (5.2%) (Figure 1A, **No *Hpb***). The most common regulatory T cell phenotype that appeared was Foxp3$^+$ IL10$^-$ CD4$^+$ (3.5%).

However, colonic Foxp3$^-$ IL10$^-$ CD4$^+$ T cells from *Hpb*-infected mice cultured with anti-CD3/CD28 and TGFβ readily converted to the regulatory phenotypes (30.2%) (Figure 1A, ***Hpb***). Foxp3$^-$ IL10$^+$ CD4$^+$ and Foxp3$^+$ IL10$^+$ CD4$^+$ were the most common regulatory T cell phenotypes in the cell cultures (22.7 and 5.9%, respectively).

In the absence of TGFβ, anti-CD3/CD28 stimulation induced few T cells with a regulatory phenotype. Induction was mostly limited to the Foxp3$^-$ IL10$^+$ CD4$^+$ T cell subset derived from colonic Foxp3$^-$ IL10$^-$ CD4$^+$ T cells isolated from *Hpb*-infected mice (6.6%) (Figure 1B). The appearance of other regulatory phenotypes was ≤1%. These data show that TGFβ was important for induction of these regulatory T cell phenotypes in these cell cultures. Gut Foxp3$^-$ IL10$^-$ CD4$^+$ T cells cultured alone or only with TGFβ did not alter their phenotype (data not shown).

Also examined was the effect of TGFβ and anti-CD3/CD28 stimulation on Foxp3$^-$ IL10$^-$ CD4$^+$ T cells isolated from the MLN of infected and uninfected mice. Compared to Foxp3$^-$ IL10$^-$ CD4$^+$ T cells from uninfected mice, Foxp3$^-$ IL10$^-$ CD4$^+$ T cells from *Hpb*-infected mice cultured with anti-CD3/CD28 and TGFβ more readily converted to Foxp3$^+$ IL10$^-$

$CD4^+$ T cells, and to a lesser degree $Foxp3^-$ $IL10^+$ $CD4^+$ and $Foxp3^+$ $IL10^+$ $CD4^+$ T cells (Figure 1C, D).

Helminths can produce molecules with activity homologues to TGFβ (32). Therefore, some experiments used HES (50 ug/ml) in placed of TGFβ in the cell culture experiments. Three separate HES preparations displayed little ability to induce expression of the regulatory T cell phenotypes (please see Supplemental Fig. 1).

Hpb infections inhibits Smad7 expression in $Foxp3^-$ $IL10^-$ $CD4^+$ T cells isolated from the colon or MLN

In T cells, TGFβ binds to its receptors and initiates signaling through the Smad signaling pathway. The activated TGFβ R1 directly phosphorylates Smad2 and Smad3, which then associates with Smad4. The Smad protein complex translocates to the nucleus, where it transcriptionally controls gene expression. Smad7 is an inhibitory molecule that infers with phosphorylation of Smad2 and Smad3, and thus can impede the signaling of TGFβ.

It was determined if *Hpb* infection effected Smad2, 4 or 7 expression in gut or MLN $Foxp3^-$ $IL10^-$ $CD4^+$ T cells. $Foxp3^-$ $IL10^-$ $CD4^+$ T cells freshly isolated from the colon of *Hpb*-infected mice displayed about an 85% reduction in Smad7 mRNA expression compared to similar T cells isolated from uninfected mice (Figure 2). $Foxp3^-$ $IL10^-$ $CD4^+$ T cells isolated from the MLN showed about a 70% decrease in Smad7 expression. *Hpb* infection did not affect Smad2/4 expression in colonic $Foxp3^-$ $IL10^-$ $CD4^+$ T cells, but did depress the expression of Smad2/4 in MLN $Foxp3^-$ $IL10^-$ $CD4^+$ T cells.

TGFβ readily induces Smad2/3 phosphorylation only in colonic $Foxp3^-$ $IL10^-$ $CD4^+$ T cells from Hpb-infection mice.

TGFβ engagement with its receptor induces phosphorylation of Smad2/3. Experiments ascertained if infection with *Hpb* enhanced the capacity of TGFβ to phosphorylate Smad2/3 in colonic $Foxp3^-$ $IL10^-$ $CD4^+$ T cells. The number of freshly isolated colonic $Foxp3^-$ $IL10^-$ $CD4^+$ T cells from *Hpb*-infected mice constitutively displaying phosphorylated Smad2/3 was nearly 3-fold higher than colonic $Foxp3^-$ $IL10^-$ $CD4^+$ T cells isolated from uninfected mice (Figure 3A, B). Culturing colonic $Foxp3^-$ $IL10^-$ $CD4^+$ T cells from *Hpb*-infected mice with TGFβ greatly increased the number of cells displaying Smad2/3 phosphorylation. However, exposing $Foxp3^-$ $IL10^-$ $CD4^+$ T cells from uninfected mice to TGFβ did not affect T cell Smad phosphorylation (Figure 3).

Colonic $CD4^+$ T cells induced to express Foxp3 and/or IL10 prominently display CD25.

Most regulatory T cells express CD25. It was determined if colonic $Foxp3^-$ $IL10^-$ $CD4^+$ T cells from *Hpb*-infected mice, when induced to express Foxp3 and/or IL10, also expressed CD25. Nearly 100% of the TGFβ-induced, Foxp3+ $IL10^+$ and $Foxp3^-$ $IL10^+$ $CD4^+$ T cells expressed CD25 (Figure 4). After exposure to TGFβ, T cells from infected mice that retained the $Foxp3^-$ $IL10^-$ $CD4^+$ T cell phenotype were much less likely to display CD25 as were T cells that were $Foxp3^+$ $IL10^-$.

Colonic Foxp3^{-} IL10^{-} CD4^{+} T cells induced to express Foxp3 and/or IL10 block colitis in the Rag/CD25^{-} CD4^{+} T cell transfer model of IBD.

The above experiments showed that many colonic Foxp3^{-} IL10^{-} CD4^{+} T cells from *Hpb*-infected mice incubated with TGFβ will begin to express Foxp3 and/or IL10. The next series of experiments determined if these newly generated regulatory-type T cells could function in the Rag/CD25^{-} CD4^{+} T cell transfer model of IBD to prevent colitis. In this IBD model, Rag−/− mice, deficient in T and B cells, are reconstituted with splenic CD25^{-} CD4^{+} T cells. These mice subsequently develop colitis because they remain deficient in regulatory T cells.

Rag mice received splenic CD25^{-} CD4^{+} T cells administered i.p. Some mice also received the Foxp3^{-} IL10^{+} CD4^{+} T cells, Foxp3^{+} IL10^{+} CD4^{+} T cells or Foxp3^{-} IL10^{-} CD4^{+} T cells that developed from the freshly isolated colonic Foxp3^{-} IL10^{-} CD4^{+} T cells cultured 7 days with TGFβ and anti-CD3/CD28. The cultures contained too few Foxp3^{+} IL10^{-} CD4^{+} T cells to conduct adoptive transfer experiments with this T cell subset. After NSAID administration to induce colitis, the animals were sacrificed four weeks later to assess severity of colitis and the responsiveness of isolated colitis LPMC to OVA stimulation after OT2 T cell supplementation (Figure 5A, **experimental design**).

It has been shown that isolated LPMC cultured *in vitro* with OT2 cells will vigorous secrete IFNγ and IL17 when stimulated with OVA. However, the amount of IFNγ and IL17 produced will be greatly reduced if the isolated LPMC contain Treg that can make IL10. Thus, this feature of this experimental colitis model adds an objective and reproducible measurement of Treg activity in the cultures (5).

Figure 5B, C show, as expected, that severe colitis developed in Rag mice receiving only splenic CD25^{-} CD4^{+} T cells (**none transferred**). Mice receiving additional Foxp3^{-} IL10^{-} CD4^{+} T cells fared no better. Recipients of Foxp3^{+} IL10+ or Foxp3^{-} IL10^{+} CD4^{+} T cells displayed essentially no colitis.

LPMC isolated from Rag mice that received only splenic CD25^{-} CD4^{+} T cells, or splenic CD25^{-} CD4^{+} T cells and cultured Foxp3^{-} IL10^{-} CD4^{+} T cells produced substantial amounts of IFNγ and IL17 when cultured *in vitro* with OVA. However, LPMC from mice receiving splenic CD25^{-} CD4^{+} T cells and Foxp3^{+} IL10^{+} T cells or Foxp3^{-} IL10^{+} CD4^{+} T cells showed blunted responsiveness to OVA stimulation (Figure 2D).

Colonic Foxp3^{-} IL10^{-} CD4^{+} T cells from transgenic mice, displaying T cell-specific high SMAD7 expression, infected with Hpb do not readily convert into their regulatory phenotypes after exposure to TGFβ.

The above experiments showed that colonic Foxp3^{-} IL10^{-} CD4^{+} T cells from *Hpb*-infected mice cultured with TGFβ readily converted into their regulatory phenotypes when cultured *in vitro* with TGFβ. This was not the case using similar colonic T cells from uninfected animals. Also, nearly all of these regulatory phenotypes expressed CD25. It also was shown that Foxp3^{-} IL10^{-} CD4^{+} T cells from *Hpb*-infected mice displayed low Smad7 expression compared to T cells from uninfected controls. Since Smad7 is an important inhibitor of TGFβ signaling in T cells, experiments determined if Smad7 regulation by *Hpb* infection

was likely an important factor in allowing these cells to differentiate into their regulatory phenotypes.

Experiments used transgenic mice that constitutively express Smad7 at high levels in T cells to determine if Smad7 modulation was important for *Hpb* enhancement of regulatory T cell production. It first was shown that freshly isolated colonic $CD25^{-}$ $CD4^{+}$ T cells from these transgenic animals expressed similar levels of Smad7 in the presence or absence of the usual two-week *Hpb* infection. Also, the magnitude of Smad7 mRNA expression was much high then that in colonic T cells from the WT control animals (Figure 6A).

These isolated cells then were cultured *in vitro* for 3 days with TGFβ and anti-CD3/CD28 similar to the other experiments. Following incubation, the cells were examined for the expression of CD25, a marker of the regulatory T cells (see Figure 4), and IL10. IL10 expression was determined using intracytoplasmic staining for this cytokine.

The data show that colonic $CD25^{-}$ $IL10^{-}$ CD4+ T cells from *Hpb*-infected control mice more readily convert into the key $CD25^{+}$ $IL10^{+}$ T cell subset important for controlling colitis. This is not the case for colonic $CD25^{-}$ $IL10^{-}$ $CD4^{+}$ T cells from the Smad7-high expressing transgenic mice. Moreover, very few cells converted to regulatory phenotype when Smad7 was hyper expressed (Figure 6B, C).

Infection with Hpb did not alter the expression of TGFβR1 or R2 on freshly isolated colonic $Foxp3^{-}$ $IL10^{-}$ $CD4^{+}$ T cells

TGFβ initiates signaling via ligand-dependent activation of TGFβ receptors R1 and R2. It was determined if *Hpb* infection altered the expression of TGFβR1 and R2 on colonic $Foxp3^{-}$ $IL10^{-}$ $CD4^{+}$ T cells. Figure 7 shows that freshly isolated colonic $Foxp3^{-}$ $IL10^{-}$ $CD4^{+}$ T cells from *Hpb*-infected and uninfected mice displayed TGFβR1 and R2 similarly.

DISCUSSION

Previous studies revealed that infection with *Hpb* can protect mice from colitis through the induction of regulatory T cells that make IL10 (5). Regulatory T cells are the major source of this cytokine in the gut. *Hpb* also induces the gut mucosa to make TGFβ (18,30), and *Hpb* produces a molecule(s) displaying TGFβ-like activity (33). TGFβ signaling via TGFβRs expressed on T cells is needed for *Hpb* prevention of colitis (18). TGFβ has a major role in inducing intestinal T cells to differentiate into their regulatory phenotypes (31). Thus, it seemed likely that *Hpb* induces the gut to make TGFβ, which in turn, promotes the development of gut IL10-producing, regulatory T cells that blocked the intestinal inflammation.

It was unexpected to find that TGFβ had limited capacity to induce colonic $Foxp3^{-}$ $IL10^{-}$ $CD4^{+}$ T cells from healthy uninfected mice to express Foxp3 or IL10. However, *Hpb* infection rendered gut T cells highly responsive to TGFβ-induced conversion to $Foxp3^{+}$ and/or 10^{+} T cells. The above described results implied that *Hpb* infection effected mucosal T cells rendering them more responsive to TGFβ-induced conversion to regulatory T cells, revealing perhaps another important *Hpb*-induced regulatory process.

It also was shown that colonic T cells that were induced to express Foxp3 and/or IL10 can block colitis in the murine Rag/$CD4^+$ $CD25^-$ T cell transfer model of IBD. This is a well-established system for studying Treg cell-mediated suppression *in vivo* (34). Many such models inconsistently develop colitis. To enhance expression of disease, mice were fed a NSAID (piroxicam) for a two-week interval. This causes all the mice to rapidly develop severe colitis after the NSAID is stopped. The NSAID disrupts production of protective arachadonic acid metabolites in the mucosa (29) making the animals more prone to colitis. Many types of NSAIDs worsen IBD (35) (36) making this model relevant to human disease.

In this model, T cells that make IL10 are important for protecting mice from the colitis (5,21,22) at least in part through the inhibit of IFNγ production in the mucosa. Also, regulatory T cells that inhibit colitis usually express CD25 (37). In our experiments, nearly all the intestinal T cells induced by TGFβ to express IL10 displayed CD25.

TGFβ signals via activation of two distinct receptors. TGFβ binds directly to the type 2 receptor that draws the type 1 receptor into the ligand-receptor complex. It also transphosphorylates and activates the type I receptor. The latter has a major role in downstream signaling via phosphorylation of Smad2 and Smad3 intracellular proteins, which then complex with Smad4. Smad7 is a key negative regulatory element of TGFβ receptor signaling (38).

Thus, it was reasonable to assume, and we indeed found, that *Hpb* infection influenced the T cell Smad signaling pathway. A notable observation was that *Hpb* infection resulted in a marked down-modulation in Smad7 expression in colonic and MLN $Foxp3^-$ $IL10^-$ $CD4^+$ T cells. The decrease in Smad7 was shown to be of critical importance for the heightened TGFβ responsiveness in our T cell cultures, since *Hpb* infection no longer enhanced TGFβ-responsive regulatory T cell expression if the T cells came from the transgenic mice displaying sustained heightened Smad7 expression. Moreover, it was interesting to note that Smad7 transgenic T cells from uninfected mice expressed Smad7 at levels higher than that of T cells from the uninfected WT control animals. In the absence of infection, TGFβ could induce some T cells to convert to their regulatory phenotypes, but much less so in transgenic T cells. The strain of mice used in the Smad7 transgenic mouse experiments came from a different source and could differ in gut microbiota, considered by some a confounding factor in these particular studies.

Since Smad7 inhibits TGFβ receptor signaling, it was anticipated and found that TGFβ much more readily phosphorylated Smad2/3 in colonic $Foxp3^-$ $IL10^-$ $CD4^+$ T cells from *Hpb*-infected mice as opposed to T cells from uninfected animals. Infection with *Hpb* did not influence the expression of TGFβR1 or R2 on the colonic $Foxp3^-$ $IL10^-$ $CD4^+$ T cells making it unlikely that changes in the relative number of T cells expressing TGFβ receptors was responsible for the enhancement of Smad2/3 phosphorylation.

The Smad regulatory pathway appears to have importance in IBD. Smad7 is over-expressed in mucosal T cells of patients with IBD interfering with TGFβ signaling (39). The relevance of Smad7 was demonstrated in murine models of colitis (40) in which Smad7 blockage protects mice from the disease. A clinical trial suggested that an orally active antisense

oligonucleotide inhibitor of Smad7 expression has at least short-term benefit in patients with Crohn's disease (41) although a larger trial failed to support this observation.

It was interesting to note that with reduction in the expression of Smad7 in T cells, TGFβ could induce intestinal and MLN CD4+ T cells to differentiate into an array of Foxp3+ and/or $IL10^+$ $CD4^+$ T cells. Foxp3 expression is controlled by a promoter and several enhancers, one of which is called CNS1. TGFβ helps covert T cells into $Foxp3^+$ regulatory T cells at sites peripheral from the thymus via phosphorylation of Smad3, which then binds to the CNS1 enhancer (26).

TGFβ also has a role in inducing intestinal T cells to express IL10 as shown by the abrogation of IL10 production in transgenic mice having T cells that cannot signal in response to TGFβ binding (18). Smad2 can function as a transcription co-activator of STAT3(42), which can promote induction of T cells that make IL10 (43,44).

TGFβ only converted colonic and MLN Foxp3- IL10- CD4+ T cells into their regulatory phenotypes if there was concomitant TCR stimulation. Many intestinal Tregs recognize local antigens derived from commensal bacteria (45). It is speculated that naïve colonic T cells express these TCRs. In the right setting, it appears that engagement of these TCRs with bacteria-derived foreign antigens allows differentiation into Tregs, which prevent development of effector cells that drive colitis. Certain intestinal bacteria are particularly adept at inducing Tregs that make IL10 (25,46).

Hpb infected also induced a statistically significant reduction in SMAD2 and SMAD4 expression in $CD4^+$ T cells isolated from the MLNs of *Hpb* infected mice. This decrease in SMAD2 and SMAD4 was not seen in CD4+ T cells isolated from the colon. TGFβ, as in the colon, was more effective at induction of Tregs in $Foxp3^-$ $IL10^-$ $CD4^+$ T cells from MLN of *Hpb*-infected mice. However, we observed many more $Foxp3^+$ $IL10^-$ $CD4^+$ T cells and fewer of the other phenotypes then that seen using colonic T cells. Since Smad2/4 are important components of the TGFβ signaling pathway, perhaps this reduction in SMAD2/4 expression is responsible for the differential and weaker response of *Hpb* MLN T cells to TGFβ.

Helminths express homologues to TGFβ that modulate host immunity (32). *Hpb* produces several such molecules, immunologically distinct from murine TGFβ, capable of binding to the TGFβ receptor complex to induce Foxp3 (4). Culture supernatants from *Hpb* maintained *in vitro* contain worm excretory and secretory products (HES) that display TGFβ activity. In our *in vitro* T cell cultures, three separate lots of HES had minimal capacity to induce intestinal $Foxp3^-$ $IL10^-$ $CD4^+$ T cells to display regulatory phenotypes with or without concomitant TCR stimulation. Thus, it is conceivable that *Hpb* TGFβ homologues do not display all the biological activities of murine TGFβ or that intestinal T cells behave differently form T cells derived from different tissues.

In conclusion, we showed, perhaps for the first time, that an organism (helminth) living in the gut can enhance the ability of TGFβ to induce regulatory T cells in colonic LPMC that are capable of blocking colitis. Also, since Tregs limit adaptive immune responses, this also could be a survival advantage for the worm. Moreover, we identified the mechanism through

which the helminth enhanced Treg development, which is the down modulation of Smad7 expression in the gut $CD4^+$ T cells. Future work will attempt to determine how *Hpb* modulates Smad7.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

Hpb	Heligmosomoides polygyrus bakeri
HES	*Hpb* excretory and secretory product
IBD	Inflammatory bowel disease
LP	Lamina propria
LPMC	Lamina propria mononuclear cells
LGM	Lymphocyte growth medium
MLN	Mesenteric lymph node
RPMI	RPMI 1640 medium

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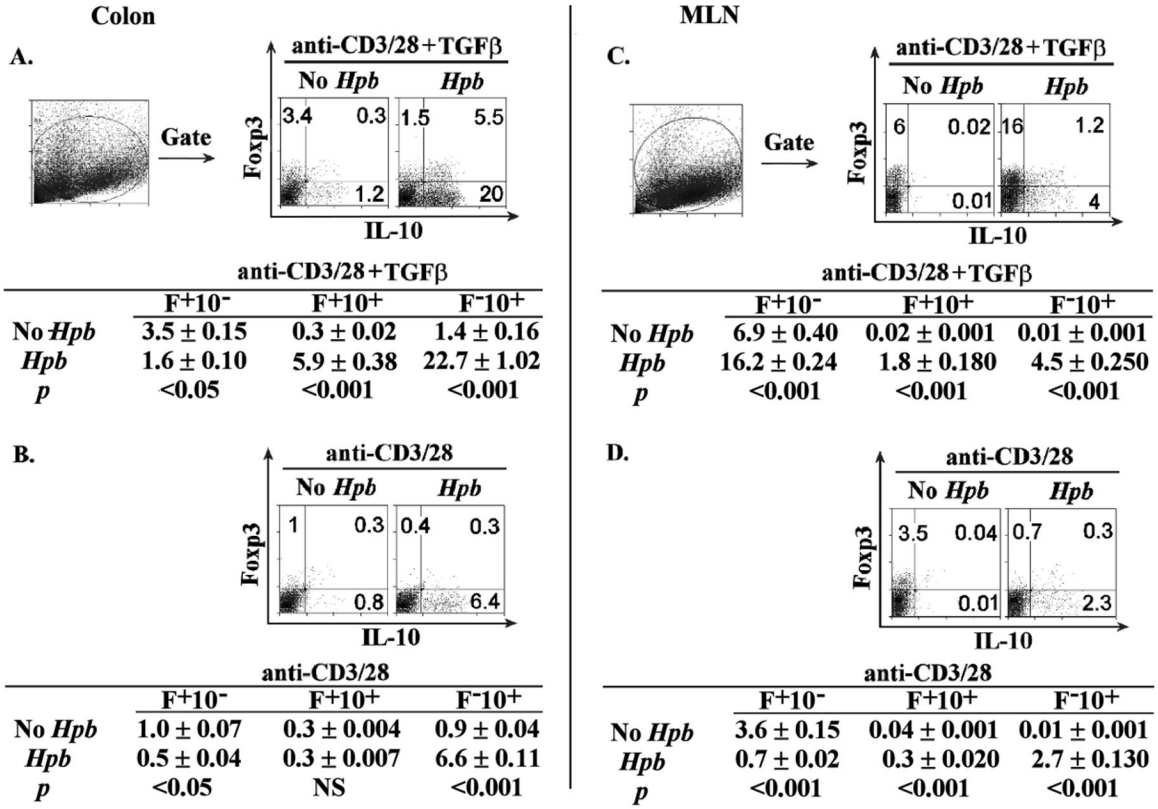


Fig. 1.

TGFβ readily converts Foxp3^{-} IL10^{-} CD4^{+} T cells from LP or MLN of *Hpb*-infected mice, as opposed to Foxp3^{-} IL10^{-} CD4^{+} T cells from uninfected mice, to Foxp3^{+} IL10^{+} CD4^{+} and Foxp3^{-} IL10^{+} CD4^{+} T cells.

Foxp3^{-} IL10^{-} CD4^{+} T cells were isolated from dispersed colonic LPMC or MLN of *Hpb*-infected (*Hpb*) and uninfected (no *Hpb*) mice using FACS. These cells were cultured *in vitro* in complete medium for 72h with anti-CD3/CD28 in the presence or absence of TGFβ (10ng/ml). The T cells then were subject to flow analysis to determine the percentage of the T cells that converted to Foxp3^{+} IL10^{-} CD4^{+}, Foxp3^{+} IL10^{+} CD4^{+} or Foxp3^{-} IL10^{+} CD4^{+}. Panel A and B show results using colonic LP T cells, whereas panels C and D show results using MLN T cells. Data in the tables are mean percentage ± SE calculated from the means of five independent experiments containing three or four mice per group. Also shown are representative FACS plots for each experimental condition.

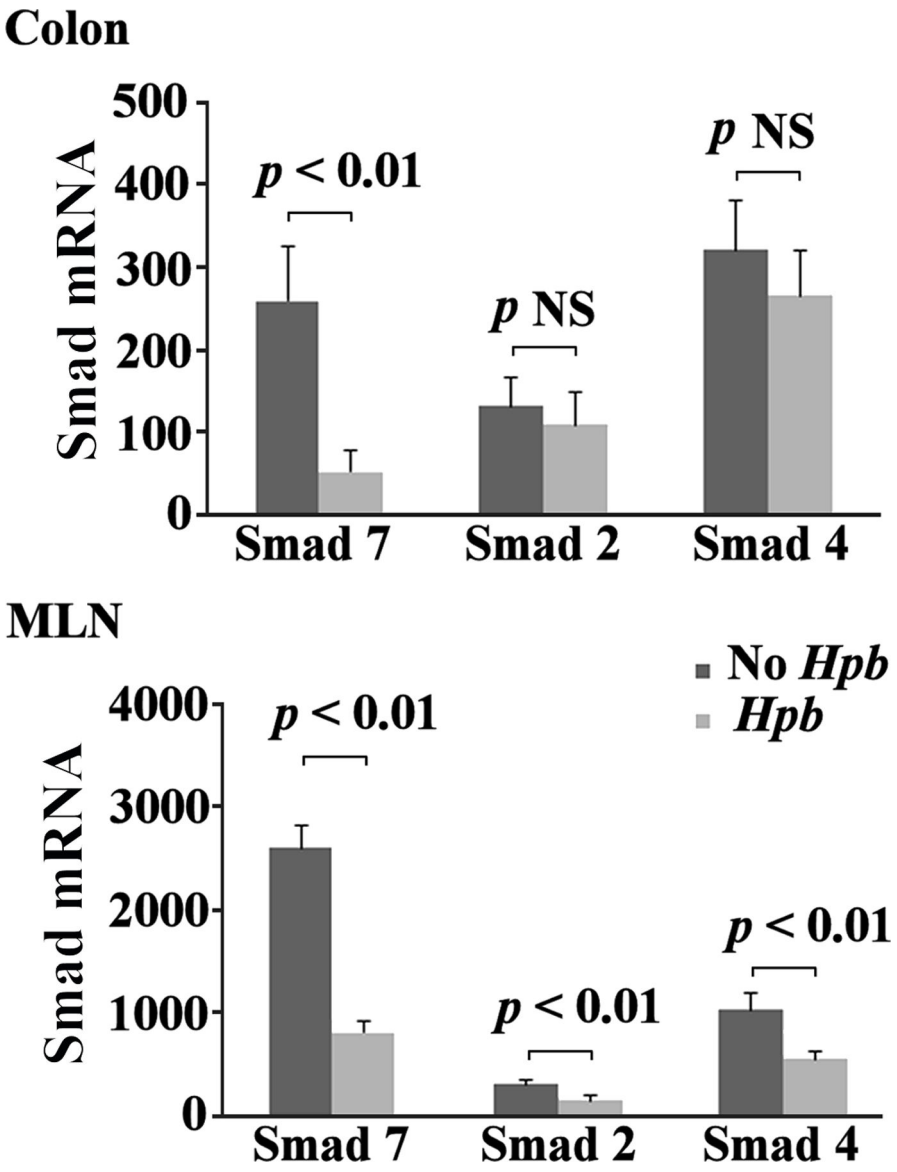
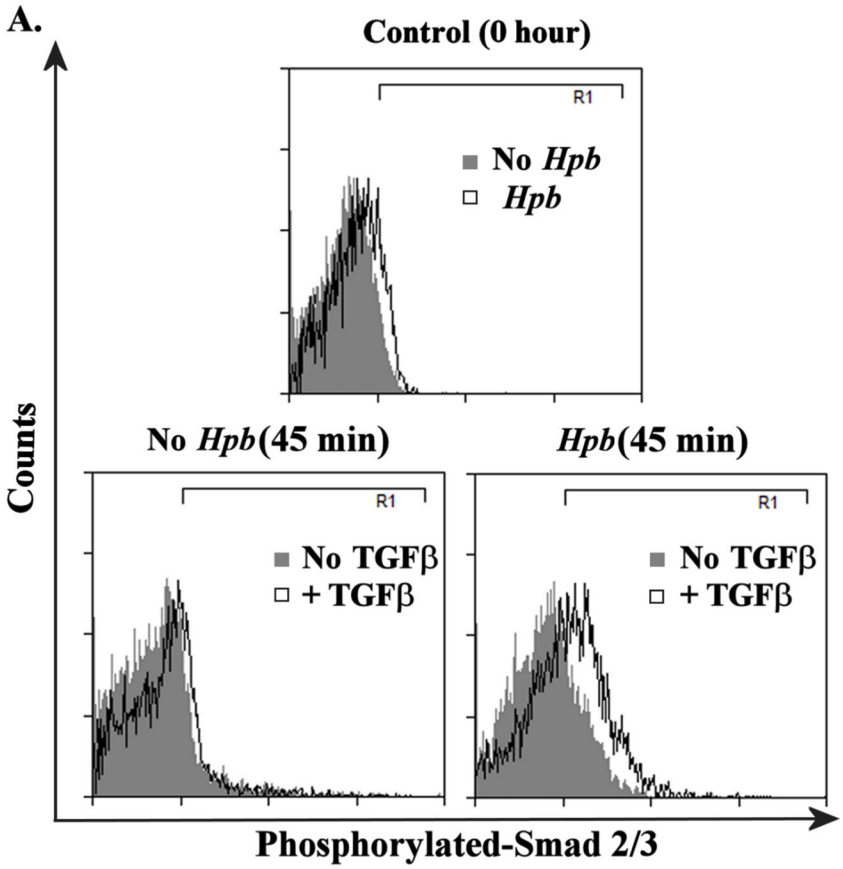


Fig. 2.

Hpb infection inhibits Smad7 mRNA expression in colonic and MLN Foxp3$^-$ IL10$^-$CD4$^+$ T cells.

Mice were infected with *Hpb* (*Hpb*). Control mice of similar age were not infected (No *Hpb*). Two weeks later, Foxp3$^-$ IL10$^-$ CD4$^+$ T cells were isolated from colon or MLN. The RNA was extracted and converted to cDNA. Quantitative rt-PCR was used to assess the level of Smad 7, 2 and 4 mRNA expressed. Data were normalized relative to the expression of the housekeeping gene GAPDH. Both colonic and MLN Foxp3$^-$ IL10$^-$ CD4$^+$ T cells from *Hpb* infected mice expressed less Smad7 mRNA compared with cells from uninfected mice ($p < 0.01$). MLN T cells also displayed a relatively small, but significant decrease in expression of Smad2 and 4, not seen in colonic T cells. Data are mean percentage ± SE calculated from the means of three independent experiments each using three or four mice per experimental group to obtain Smad mRNA.



B.

	No *Hpb*	*Hpb*	
Control (0 h)	9.0 ± 0.3	26.0 ± 0.3	$p < 0.01$
No TGFβ (45 min)	10.0 ± 0.6	27.3 ± 0.4	$p < 0.01$
TGFβ (45 min)	12.7 ± 2.4	49.1 ± 2.7	$p < 0.01$
	p NS	$p < 0.01$	

Fig. 3.

Colonic LP Foxp3$^-$ IL10$^-$ CD4$^+$ T cells isolated from *Hpb*-infected mice, compared to T cells from uninfected animals, displayed a higher degree of constitutive Smad2/3 phosphorylation, which increased further upon exposure to TGFβ.

Foxp3$^-$ IL10$^-$ CD4$^+$ T cells were isolated from dispersed colonic LPMC of *Hpb*-infected (*Hpb*) and uninfected (no *Hpb*) mice using FACS. Using immunostaining and flow cytometry, the isolated cells were analyzed for intracellular Smad2/3 phosphorylation immediately after cell isolation or after 45 min incubation in complete medium with or without TGFβ (10ng/ml).

Panel (A) shows three representative overlapping flow cytometry graphs depicting the degree of Smad2/3 phosphorylation in colonic Foxp3- IL10- CD4+ T cells. The bracket in each FACS plot indicates the window of phosphorylated Smad2/3-specific staining. The upper graph shows that colonic Foxp3$^-$ IL10$^-$ CD4$^+$ T cells from *Hpb* infected mice (*Hpb*)

are more likely to constitutively express phosphorylated Smad2/3 at the time of isolation (0 time) than similar T cells from uninfected mice (No *Hpb*). TGFβ induces Smad2/3 phosphorylation in colonic Foxp3^{-} IL10^{-} CD4^{+} T cells from *Hpb* infected mice (*Hpb*) (lower right panel), but fails to do so in the T cells from uninfected mice (No *Hpb*) (lower left panel).

Panel (B) is a table showing mean percentage of colonic Foxp3^{-} IL10^{-} CD4^{+} T cells ± SE expressing phosphorylated Smad2/3 freshly after isolation (time 0) or after a 45 min incubation with or without TGFβ. The cells came from either *Hpb*-infected or uninfected control mice. These data are calculated from the means of three independent experiments. Each used LP cells pooled from three or four mice for each experimental group.

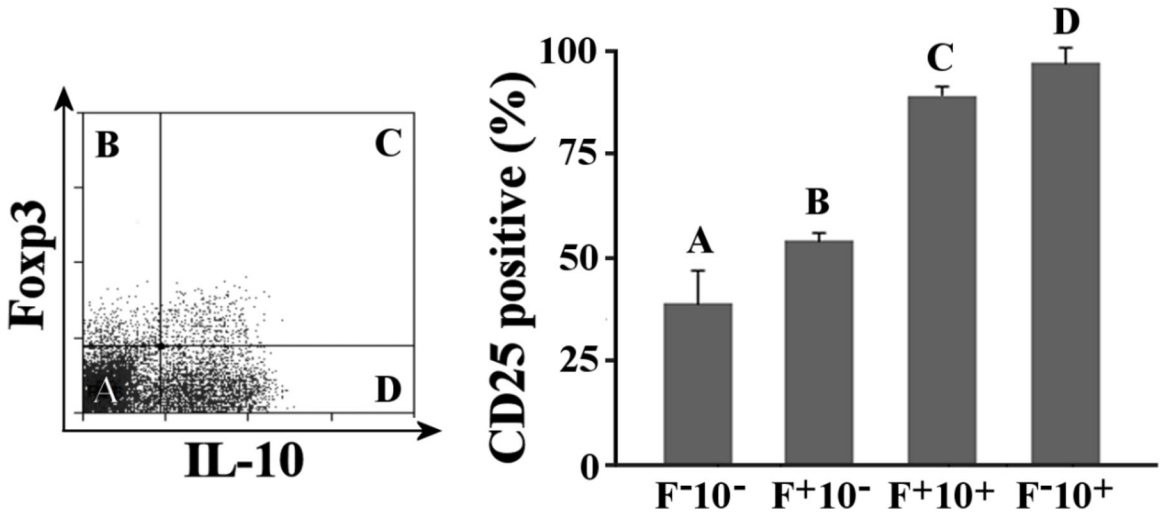


Fig. 4.

CD25 is expressed on nearly all colonic LP CD4$^+$ T cells, isolated from *Hpb*-infected mice, induced to express IL10 by exposure to TGFβ.

Colonic Foxp3$^-$ IL10$^-$ CD4$^+$ T cells from *Hpb*-infected (*Hpb*) mice were cultured *in vitro* in complete medium for 72h with anti-CD3/CD28 in the presence of TGFβ (10 ng/ml). The T cells then were subjected to flow analysis to determine the percentage of the T cells that displayed CD25 after conversion to Foxp3$^+$ IL10$^-$ CD4$^+$, Foxp3$^+$ IL10$^+$ CD4$^+$, or Foxp3$^-$ IL10$^+$ CD4$^+$. The representative FACS plot shows the various subsets of colonic T cells induced after TGFβ exposure (left panel). The figure on the right shows that the percentage of cells expressing CD25 was highest on the Foxp3$^+$ IL10$^+$ CD4$^+$ and Foxp3$^-$ IL10$^+$ CD4$^+$ T cell subsets. Data are means ± SE calculated from the means of three independent experiments each using pooled T cells from three or four mice. A or B vs. C or D, $p<0.01$; A vs. B, $p<0.05$

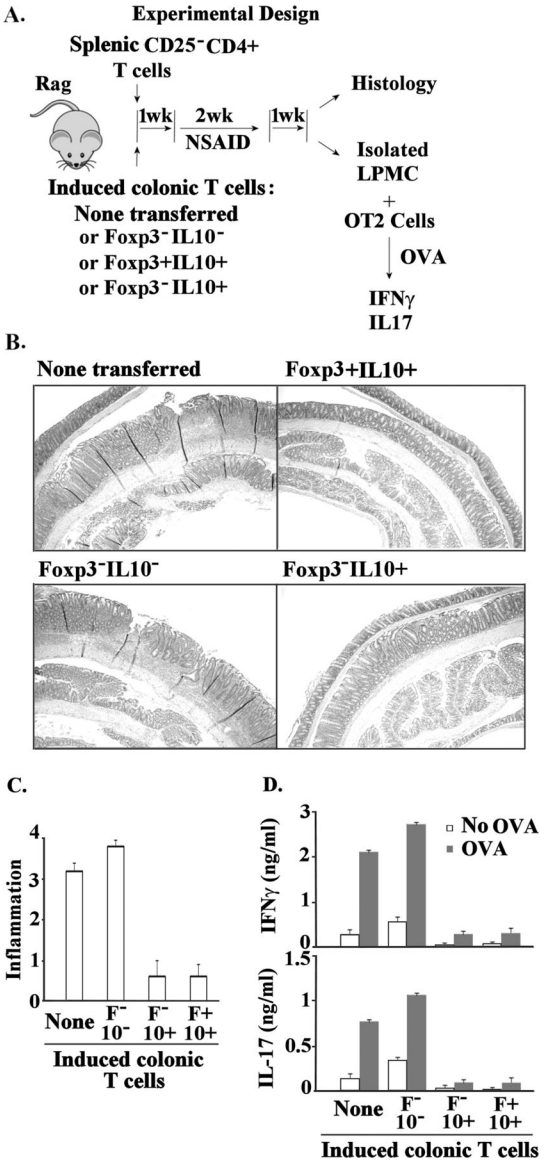


Fig. 5.

Colonic LP CD4$^+$ T cells from *Hpb*-infected mice induced to express IL10 by incubation with TGFβ will inhibit colitis when transferred into the Rag CD25$^-$ CD4$^+$ T cell transfer model of IBD.

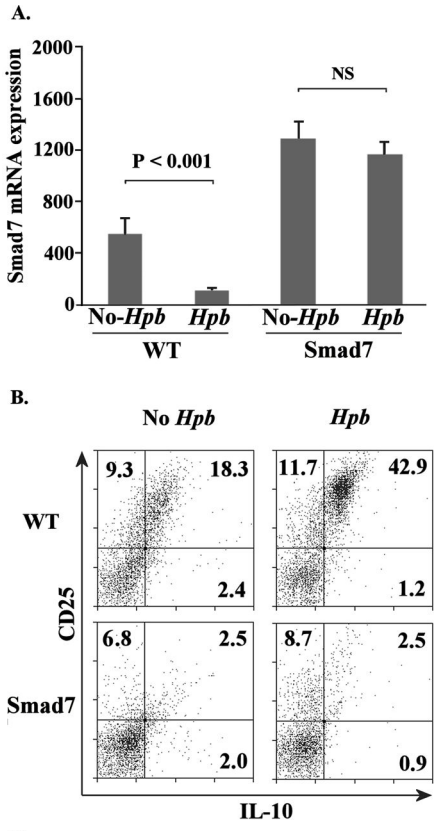
The experimental design is outlined in **panel A**. Colonic Foxp3$^-$ IL10$^-$ CD4$^+$ T cells from *Hpb*-infected mice were cultured *in vitro* in complete medium for 72h with anti-CD3/CD28 and TGFβ to induce putative regulatory T cell subsets. The T cells then were subject to flow cytometry to isolate the T cells subsets that converted to Foxp3$^+$ IL10$^+$ or Foxp3- IL10+. Also, isolated was the Foxp3$^-$ IL10$^-$ T cell subset. Foxp3$^-$ IL10$^-$, Foxp3$^+$ IL10$^+$ or Foxp3$^-$ IL10$^+$ CD4$^+$ T cells were transferred (10^5/mouse) by ip injection into Rag mice that also received splenic CD25$^-$ CD4$^+$ T cells (2×10^5/mouse) from WT mice to promote colitis. A fourth group of Rag mice was reconstituted just with splenic CD25$^-$ CD4$^+$ T cells. Animals then were treated with piroxicam as described to induce colitis. At the end of the

experiment, colonic tissue was sectioned and examined microscopically to score the severity of the colitis using a 4-point scale. **B** and **C** show severity of colitis in mice receiving just splenic $CD25^{-}$ $CD4^{+}$ T cells (No colonic T cell transfer) or $CD25^{-}$ $CD4^{+}$ T cells and one additional colonic T cell subset.

B) As depicted in these representative images stained with H&E and photographed at x40, an intense mucosa lymphocytic infiltration developed in mice receiving just splenic $CD25^{-}$ $CD4^{+}$ T cells (no colonic T cell transfer). This inflammation failed to develop in recipients of splenic $CD25^{-}$ $CD4^{+}$ T cells and TGFβ-induced, colonic $Foxp3^{+}$ $IL10^{+}$ or $Foxp3^{-}$ $IL10^{+}$ T cells.

C) The table shows that recipients of splenic $CD25^{-}$ $CD4^{+}$ T cells along with TGFβ-induced, colonic $Foxp3^{+}$ $IL10^{+}$ or $Foxp3^{-}$ $IL10^{+}$ $CD4^{+}$ T cells developed little colitis. Data are means ± SE from 3 separate experiments each containing four to five mice per group. No colonic T cell transfer or colonic $Foxp3^{-}$ $IL10^{-}$ recipients vs. the colonic $Foxp3^{+}$ $IL10^{+}$ or $Foxp3^{-}$ $IL10^{+}$ T cells recipients, $p<0.01$.

Also studied was the effect of T cell transfer on the capacity of colonic LPMC to support antigen-induced T cell cytokine responses (panel **D**). LPMC were isolated from strips of colon at the time of sacrifice for histological analysis. These cells were cultured *in vitro* at 2×10^{5} cells per well in RPMI complete medium for 48h with OT2 T cells (10^{5} cells/well) and OVA (50 ug/ml) to stimulate cytokine secretion. Cell culture supernatants were assayed for IFNγ and IL17 content using ELISAs after the 48h culture period. LPMC from Rag mice reconstituted with splenic $CD25^{-}$ $CD4^{+}$ T cells and TGFβ-induced, colonic $Foxp3^{+}$ $IL10^{+}$ or $Foxp3^{-}$ $IL10^{+}$ T cells secreted less cytokines in response to OVA as compared to LPMC from Rag mice that received only splenic $CD25^{-}$ $CD4^{+}$ T cells or splenic $CD25^{-}$ $CD4^{+}$ T cells and colonic $Foxp3^{-}$ $IL10^{-}$ T cells. Data are means ± SE calculated from the means of 3 independent experiments each comprised of triplicate determinations. LPMC cytokine response of mice receiving no colonic T cell transfer or colonic Foxp3- IL10- T cells vs. that of mice receiving colonic $Foxp3^{+}$ $IL10^{+}$ or $Foxp3^{-}$ $IL10^{+}$ T cells, $p<0.01$.



C.

	WT		
	CD25+/IL10-	CD25+/IL10+	CD25-/IL10+
No-*Hpb*	7.9 ± 0.8	19.2 ± 0.5	5.7 ± 2.3
Hpb	14.5 ± 2.1	41.1 ± 1.6	3.2 ± 1.3
	P < 0.05	P < 0.001	NS

	Smad7		
	CD25+/IL10-	CD25+/IL10+	CD25-/IL10+
No-*Hpb*	6.7 ± 0.5	3.2 ± 0.5	2.0 ± 0.1
Hpb	8.4 ± 2.6	3.3 ± 0.8	2.0 ± 0.6
	NS	NS	NS

Fig. 6.

Colonic CD25^{-} CD4^{+} cells from Smad7 high expressing transgenic mice infected with *Hpb* do not readily convert to their regulatory phenotypes after TGFβ stimulation *in vitro*.

(Panel A) Colonic T cells were isolated from *Hpb*-infected or uninfected C57BL6 SMAD7 high expression transgenic mice or from control WT mice. The CD4^{+}CD25^{-}T cells were sorted by FACS, and mRNA was extracted and converted to cDNA. Quantitative rt-PCR was used to assess the level of Smad7 mRNA expressed in these cells. Data were normalized relative to the expression of the housekeeping gene GAPDH. Smad7 mRNA expression was high in the Smad7 transgenic T cells relative to that of the control T cells. Also, *Hpb* infection did no decrease Smad7 expression in the transgenic T cells as it did in the WT T cells.

(Panel B and C) Colonic CD25^{-} CD4+ T cells isolated from either uninfected or *Hpb*-infected Smad7 transgenic mice or WT control animals, were cultured for 72h with anti-

CD3/CD28 and TGFβ. The cultured cells then were stained for Alexa Fluor 647-labeled CD25, and then fixed, permeabilized and stained for intracellular IL-10. Flow analysis shows that T cells from *Hpb*-infection WT mice are more prone to convert to their $CD25^{+}/IL10^{-}$ and $CD25^{+}/IL10^{+}$ regulatory phenotypes after stimulation with TGFβ compared to T cells from their uninfected controls. This is not the case with T cells from Smad7 transgenic mice. Data in **panel A and C** are mean ± SE calculated from the means of three independent experiments each containing three or four mice per group. The numbers in the **panel B and C** are the percentage of cells positive for the particular T cell phenotype.

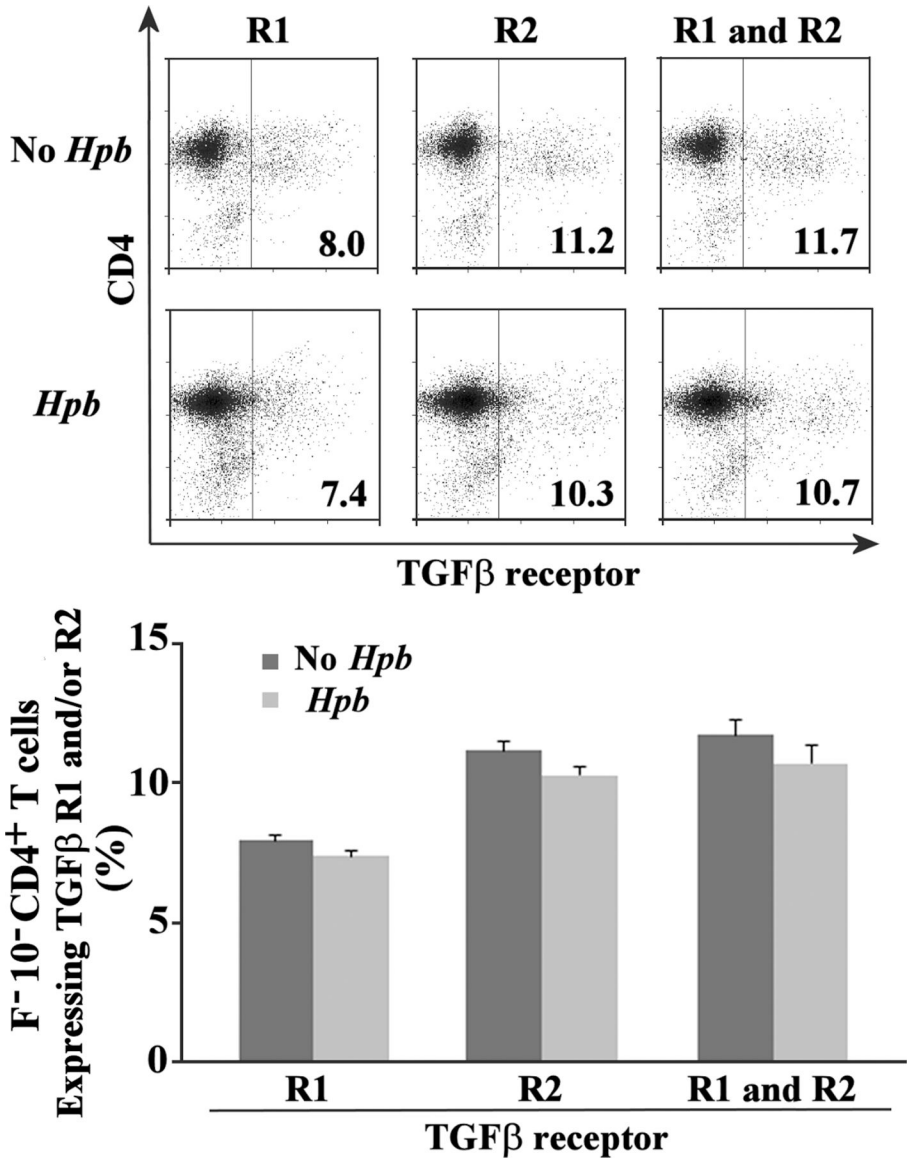


Fig. 7.

Percentage of colonic LP Foxp3^{-} IL10^{-} CD4^{+} T cells from either *Hpb*-infected or uninfected mice expressing TGFβ receptor 1 and/or 2 are similar.

Colonic Foxp3^{-} IL10^{-} CD4^{+} T cells, freshly isolated from mice infected with *Hpb* (*Hpb*) or who were uninfected (No *Hpb*), were stained with PE-labeled, anti-TGFβ receptor 1 and/or 2 mAb. The cells then were analyzed by flow cytometry for expression of these receptors. The data show that *Hpb*-infection did not affect the proportion of freshly isolated T cells expression TGFβ receptor 1 or 2. Data in the lower panel are means calculated from the means of four independent experiments, each using four mice/group. *Hpb* vs. No *Hpb*, p=NS