

CD4⁺CD25⁺ T_R Cells Suppress Innate Immune Pathology Through Cytokine-dependent Mechanisms

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Abstract

CD4⁺CD25⁺ regulatory T (T_R) cells can inhibit a variety of autoimmune and inflammatory diseases, but the precise mechanisms by which they suppress immune responses in vivo remain unresolved. Here, we have used *Helicobacter hepaticus* infection of T cell-reconstituted recombination-activating gene (RAG)^{-/-} mice as a model to study the ability of CD4⁺CD25⁺ T_R cells to inhibit bacterially triggered intestinal inflammation. *H. hepaticus* infection elicited both T cell-mediated and T cell-independent intestinal inflammation, both of which were inhibited by adoptively transferred CD4⁺CD25⁺ T_R cells. T cell-independent pathology was accompanied by activation of the innate immune system that was also inhibited by CD4⁺CD25⁺ T_R cells. Suppression of innate immune pathology was dependent on T cell-derived interleukin 10 and also on the production of transforming growth factor β. Thus, CD4⁺CD25⁺ T_R cells do not only suppress adaptive T cell responses, but are also able to control pathology mediated by innate immune mechanisms.

Key words: regulatory T cells • *Helicobacter* • immune tolerance • mucosal immunity • IL-10

Introduction

The key role that the immune system plays in regulating intestinal integrity has been demonstrated by the development of multiple rodent models of mucosal inflammation that are associated with immunological defects, particularly those that affect T cells (1). Growing evidence indicates that T cell responses toward intestinal antigens are normally of a tolerogenic nature and that dysregulation of mucosal T cell responses may lead to a loss of tolerance and the development of harmful intestinal inflammation similar to that observed in human inflammatory bowel disease (IBD)* (1–3).

A well characterized murine IBD model is the intestinal inflammation that occurs after adoptive transfusion of naive CD4⁺CD45RB^{high} T cells from normal mice into immunodeficient recipients (4, 5). The resultant pathology is due to an excessive Th1-driven inflammatory response in the intestine and cotransfer of CD4⁺CD45RB^{low}CD25⁺ T cells from normal mice inhibits the colitis (6, 7). Additional studies in a variety of experimental models of au-

toimmune and inflammatory diseases have confirmed that regulatory T (T_R) cells capable of controlling immune pathology are an integral part of the T cell repertoire in normal animals (for reviews, see references 8 and 9) and that their activity is enriched within the minor subpopulation of CD4⁺CD45RB^{low} T cells that express the CD25 marker (10).

Although the precise cellular and molecular pathways involved remain to be defined, current evidence indicates that T_R cells are able to utilize multiple mechanisms to suppress immune responses. In vitro studies have demonstrated that T_R cells can inhibit the activation of other T cells either directly via a cell contact-dependent mechanism (11–13), or indirectly by down-modulating the activity of antigen-presenting cells (14, 15). In addition, studies in animal models have provided strong evidence of a role for cytokines in the effector function of T_R cells in vivo, and the inhibition of intestinal inflammation appeared to be crucially dependent on the presence of the immunosuppressive cytokines IL-10 and TGF-β (7, 16, 17). These molecules can mediate multiple suppressive effects on both T cells and on cells of the innate immune system (18, 19). Although these studies raise the possibility that T_R cells also inhibit innate immune activation in vivo, the ability of T_R

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*Abbreviations used in this paper: IBD, inflammatory bowel disease; LPL, lamina propria leukocyte; MLN, mesenteric lymph node; T_R, regulatory T.

cells to suppress non-T cell-mediated immune pathology has not yet been investigated.

The intestinal microflora are essential for the development of intestinal inflammation and a number of different bacterial species have been identified as being able to trigger disease in rodent models of IBD (20–23). One such organism is *Helicobacter hepaticus*, a Gram negative spiral bacterium that colonizes the intestinal crypts of the cecum and colon, establishing a life-long infection (24). Although *H. hepaticus* does not usually cause disease in immunocompetent mice, infection is associated with hepatitis in susceptible inbred strains (25) and it can cause colitis and typhlitis in immunodeficient mice (26, 27). *H. hepaticus* infection has also been shown to exacerbate colitis in specific pathogen-free (SPF) IL-10^{-/-} mice and in RAG^{-/-} mice reconstituted with naive CD45RB^{high} CD4⁺ T cells (22, 23). In this study, we have used *H. hepaticus* infection of immunodeficient RAG^{-/-} mice as a model to study the ability of CD4⁺CD25⁺ T_R cells to inhibit bacterially driven T cell-dependent and T cell-independent intestinal inflammation.

Materials and Methods

Mice. 129SvEv, 129SvEvRAG2^{-/-}, and 129SvEvIL-10^{-/-} mice were bred under SPF conditions and maintained in micro-isolator cages with filtered air in the Pathology Services Building at the University of Oxford. Mice were routinely screened for the presence of *Helicobacter spp.* and were 6–10 wk old when first used.

Bacteria. *Helicobacter hepaticus* was isolated from infected 129SvEvRAG2^{-/-} mice as described previously (24), by culturing cecal scrapings from infected mice on blood agar plates containing trimethoprim, vancomycin and polymixin B (all from Oxoid) under microaerophilic conditions (CampyPak; Oxoid). *H. hepaticus* were characterized morphologically using phase-contrast microscopy and were Gram negative, and urease, catalase, and oxidase positive. Identity was confirmed by PCRs specific for *H. hepaticus* 16sRNA (27), urease (28), and cytolethal distending toxin (CDT; reference 29). Cultures were expanded for 3–4 d in TSB (Oxoid) containing 10% FCS (GIBCO BRL) and *Helicobacter*-free 129SvEvRAG2^{-/-} mice were inoculated by oral gavage of 2×10^8 cfu *H. hepaticus*.

Purification of T Cell Subsets. CD4⁺ T cell subsets were isolated from spleen cells from *Helicobacter*-free 129SvEv mice using magnetic bead based separation techniques and FACS[®] sorting, essentially as described previously (7, 30). Briefly, single cell suspensions were depleted of CD8⁺, MHC class II⁺, Mac-1⁺, and B220⁺ cells by negative selection using a panel of rat mAb, followed by sheep anti-rat-coated Dynabeads (Dyna). CD4⁺CD45RB^{low}CD25⁺ T cells were purified by staining with biotinylated-anti-CD25 (BD Biosciences) followed by streptavidin-conjugated microbeads (Miltenyi Biotec) and positively selected using AutoMACS (Miltenyi Biotec), according to the manufacturer's instructions. CD4⁺CD45RB^{high} T cells were similarly isolated using AutoMACS, following staining with FITC-anti-CD45RB (BD Biosciences), followed by anti-FITC-conjugated microbeads (Miltenyi Biotec). Alternatively, after staining with Cy-Chrome-conjugated anti-CD4, PE-conjugated anti-CD25 and FITC-anti-CD45RB (all from BD Biosciences), CD4⁺ T cell subsets were purified by cell sorting, us-

ing a FACS[®]Vantage[™] (BD Biosciences). Purity of the CD4⁺ T cell subsets was assessed using flow cytometry and ranged from 92–99%.

T Cell Reconstitution and Antibody Treatments. 129SvEv RAG2^{-/-} mice were reconstituted by intraperitoneal injection of 4×10^5 CD4⁺CD45RB^{high} T cells and/or 10^5 CD4⁺CD45RB^{low}CD25⁺ T cells and infected with *H. hepaticus* 24 h later. Antibody treatments were commenced the day after T cell reconstitution. Where indicated, mice were treated with 1 mg/wk anti-IL-12p40 (C17.8.20) for the first 2 wk after infection, or weekly throughout the duration of the experiment with 2 mg anti-TNF- α (XT22), 2 mg anti-TGF- β (1D11), or 0.5 mg anti-IL10R (1B1.2) or isotype control by intraperitoneal injection.

Assessment of Intestinal Inflammation. Mice were killed 8–12 wk after *H. hepaticus* infection and samples of cecum, proximal, mid, and distal colon were isolated and fixed in buffered 10% formalin. 5 μ m paraffin-embedded sections were cut and stained with hematoxylin and eosin. Inflammation was assessed as previously described (7, 17), each sample was graded semiquantitatively from 0–4 and typical features of each grade are: 0 = normal; 1 = mild epithelial hyperplasia; 2 = pronounced hyperplasia and significant inflammatory infiltrates; 3 = severe hyperplasia and infiltration with significant decrease in goblet cells; 4 = severe hyperplasia, severe transmural inflammation, ulceration, crypt abscesses, and substantial depletion of goblet cells. Cecum and colons were assessed separately and at least three separate sections from each sample were scored blind.

Flow Cytometry. Aliquots of $1-5 \times 10^5$ cells were stained in FACS[®] buffer (PBS, 0.1% BSA, 5 mM EDTA; both from Sigma-Aldrich) using the following panel of mAb to murine cell surface molecules (all from BD Biosciences); biotinylated anti-NK cells (DX5), biotinylated anti-Gr1 (Ly6G), allophycocyanin (APC)-conjugated anti-CD11b, PE-anti-CD11c, peridinin chlorophyll protein (PerCP)-conjugated anti-CD4, FITC-conjugated anti-class II MHC, PE-anti-mouse TCR β . Biotinylated antibodies were detected using PE-, APC-, or FITC-conjugated streptavidin (SA; all from BD Biosciences). Staining steps were performed for 30 min on ice and, after washing with FACS[®] buffer, samples were fixed in FACS[®] buffer containing 2% paraformaldehyde (BDH) and analyzed using a FACSCalibur[™] with CELLQuest[™] software (Becton Dickinson). For intracellular cytokine analysis, spleen cells (2.5×10^6 cells/ml) were incubated in complete RPMI 1640 (10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin and streptomycin [GIBCO BRL], and 0.05 mM 2-mercaptoethanol [Sigma-Aldrich]) supplemented with 1,000 U/ml rhIL-2 (Sigma), for 18 h at 37°C in a humidified incubator with 5% CO₂. Brefeldin A (BFA, 10 mg/ml; Sigma-Aldrich) was added for the final 4 h of incubation. Cell surface staining was performed as described above and after fixing, the samples were incubated in permeabilization (Perm) buffer (PBS, 0.1% BSA, 0.5% saponin; Sigma-Aldrich) containing PE-anti-mouse IFN- γ or isotype control (BD Biosciences) for 30 min at room temperature, washed in Perm buffer, and analyzed as above.

Isolation of Lamina Propria Leukocytes. Lamina propria leukocytes (LPLs) were purified from ceca as described (6). Briefly, ceca were pooled from 4–6 mice per group, contents were removed, and ceca were rinsed in PBS. Ceca were cut into 0.3–0.5-cm pieces and repeatedly incubated in Ca- and Mg-free PBS containing 10% FCS and 5 mM EDTA to release intestinal epithelial cells. The remaining tissue was further digested with collagenase/dispase (100 U/ml; Sigma-Aldrich) and the LPL were then layered on a discontinuous Percoll gradient (Amersham Biosciences). After centrifugation at 600 g for 20 min, the LPL popu-

lation was recovered at the 40/75% interface, washed twice in PBS/BSA, and analyzed using flow cytometry as detailed above.

Quantitation of *H. hepaticus* Using Real-time PCR. DNA was purified from cecal or fecal scrapings taken from *H. hepaticus*-infected mice using the DNA Stool kit (QIAGEN). *H. hepaticus* DNA was quantified as described (29) using a real-time PCR method based on the *cdtB* gene, performed with the ABI prism Taqman 7700 sequence detection system (PE Biosystems). Standard curves were constructed using *H. hepaticus* DNA that was purified from bacterial cultures using the DNeasy kit (QIAGEN).

Statistics. The nonparametric Mann-Whitney test was used for comparing pathology scores and cell counts and differences were considered statistically significant when $P < 0.05$.

Results

CD4⁺CD25⁺ T_R Cells Protect Against both T Cell-dependent and T Cell-independent Intestinal Inflammation Triggered by Infection with *H. hepaticus*. To examine whether T_R cells could prevent intestinal inflammation induced by the bacterial pathogen *H. hepaticus*, we isolated CD4⁺CD45RB^{low}CD25⁺ T cells and CD4⁺CD45RB^{low}CD25⁻ T cells from normal mice and coinjected these subpopulations with CD4⁺CD45RB^{high} T cells into 129SvEvRAG2^{-/-} recipients that were then infected with *H. hepaticus*. Although CD4⁺CD45RB^{high} T cell reconstitution of RAG2^{-/-} mice induced only minimal changes in intestinal architecture (Figs. 1 and 2 D), concomitant infection of these mice with *H. hepaticus* led to the development of severe intestinal inflammation, characterized by marked epithelial cell hyperplasia, extensive inflammatory infiltrates, and goblet cell depletion (Figs. 1 and 2 E). While cotransfer of CD4⁺CD45RB^{low}CD25⁺ T cells completely inhibited the development of disease,

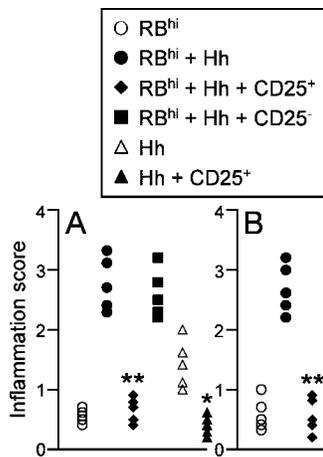


Figure 1. CD4⁺CD25⁺ T cells prevent both T cell-dependent and T cell-independent intestinal inflammation. 129SvEvRAG2^{-/-} mice were reconstituted with purified CD4⁺ T cell subsets as indicated (4×10^5 CD45RB^{high} T cells and/or 10^5 CD4⁺CD45RB^{low}CD25⁺ T cells or CD4⁺CD45RB^{low}CD25⁻ T cells) and infected 24 h later by oral gavage with 2×10^8 cfu *H. hepaticus*. Mice were killed 8–12 wk later and pathology in the colon (A) and cecum (B) was assessed histologically. Each symbol represents a single animal and results are representative of two similar experiments. ** $P < 0.01$ vs. RB^{hi} + Hh; * $P < 0.05$ vs. Hh.

CD4⁺CD45RB^{low}CD25⁻ T cells were unable to prevent *H. hepaticus*-triggered intestinal inflammation (Figs. 1 and 2, F and G).

H. hepaticus infection of RAG2^{-/-} mice led to the development of significant intestinal inflammation, even in the absence of T cell reconstitution (Figs. 1 and 2 B). This T cell-independent pathology was generally less severe than the T cell-mediated disease, again involving marked epithelial cell hyperplasia, but with little depletion of mucin-producing cells (compare Fig. 2, B and E). Inflammatory infiltrates comprised of both polymorphonuclear cells and mononuclear cells were also present, although markedly less dense than those present in the T cell-mediated form of disease (compare Fig. 2, B and E). Consistent with previous reports of *H. hepaticus*-induced intestinal disease (23, 27), the most severe T cell-independent inflammation was observed in the cecum (Figs. 1 and 2 I). Importantly, reconstitution of RAG2^{-/-} mice with CD4⁺

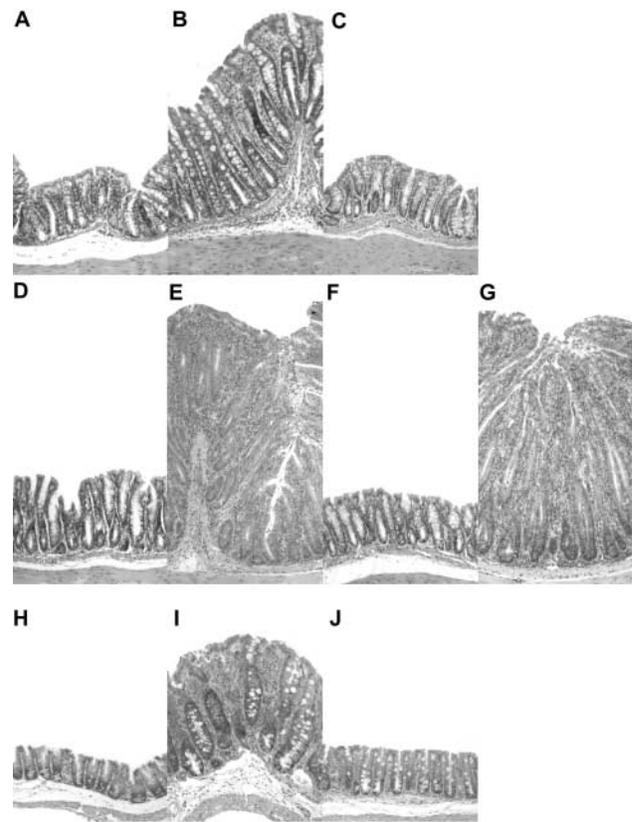


Figure 2. Representative photomicrographs of hematoxylin and eosin stained intestinal tissues from 129SvEvRAG2^{-/-} mice that were reconstituted with purified CD4⁺ T cell subsets and/or infected with *H. hepaticus* as outlined in Fig. 1. (A–G) Colon sections from a control RAG2^{-/-} mouse (A) and from mice that received; *H. hepaticus* only (B), *H. hepaticus* and CD4⁺CD45RB^{low}CD25⁺ T cells (C), CD4⁺CD45RB^{high} T cells only (D), CD4⁺CD45RB^{high} T cells and *H. hepaticus* (E), CD45RB^{high}CD4⁺ T cells plus CD4⁺CD45RB^{low}CD25⁺ T cells and *H. hepaticus* (F), or CD45RB^{high}CD4⁺ T cells plus CD4⁺CD45RB^{low}CD25⁻ T cells and *H. hepaticus* (G). (H–J) Cecum sections from a control RAG2^{-/-} mouse (H) and from mice that received; *H. hepaticus* only (I), or *H. hepaticus* and CD4⁺CD45RB^{low}CD25⁺ T cells (J). Original magnification: $\times 50$.

CD45RB^{low}CD25⁺ T cells also inhibited the development of *H. hepaticus*-induced T cell-independent intestinal inflammation in both the cecum and colon (Figs. 1 and 2, C and J). Conversely, reconstitution with CD4⁺CD45RB^{low}CD25⁻ T cells did not prevent *H. hepaticus*-triggered T cell-independent intestinal pathology (unpublished data). Together, these results demonstrate that *H. hepaticus* is able to induce significant intestinal inflammation in RAG2^{-/-} mice, that this inflammation is exacerbated when the RAG2^{-/-} mice are reconstituted with CD4⁺CD45RB^{high} T cells and, most importantly, that both the T cell-dependent and T cell-independent forms of disease are inhibited by CD4⁺CD45RB^{low}CD25⁺ T_R cells.

T Cell-independent Immune Pathology Triggered by Infection with *H. hepaticus* Is Driven by Proinflammatory Cytokines and Is Associated with Local and Systemic Activation of Innate Immunity that Is Inhibited by CD4⁺CD25⁺ T_R Cells. To examine the role of proinflammatory cytokines in the T cell-independent intestinal inflammation we treated *H. hepaticus*-infected RAG2^{-/-} mice with monoclonal antibodies to IL-12p40 or TNF- α , from the onset of infection. As shown in Fig. 3, treatment with anti-IL-12p40 or anti-TNF- α significantly inhibited the development of intestinal inflammation, suggesting that the intestinal pathology was driven by the activation of host innate immune responses.

In addition to the marked intestinal changes, we also observed significant splenomegaly in RAG2^{-/-} mice that were infected with *H. hepaticus* (Fig. 4 A). Flow cytometric analyses (31) revealed that this was primarily due to substantial increases in the numbers of neutrophils (Gr1^{high}CD11b^{high}, 10 to 20-fold; Fig. 4 B) and monocytes/macrophages (Gr1^{low}CD11b^{high}, 5 to 10-fold; Fig. 4 C) present in the spleens of *H. hepaticus*-infected RAG2^{-/-} mice, with only modest increases in the number of dendritic cells (CD11c^{high}MHC II^{high}, twofold; Fig. 4 D). Although there were only similarly modest increases in the total number of NK cells (twofold; Fig. 4 E), there was also a twofold increase in the proportion of NK cells producing IFN- γ (control 5.6 \pm 1.3%; *Hh* 10.4 \pm 2.8%; *Hh* + CD25⁺T 4.1 \pm 1.9%), which together resulted in substantial increases in the number of IFN- γ ⁺ NK cells (fourfold; Fig. 4 F), confirming that *H. hepaticus* infection led to extensive activation of innate immune cells in RAG2^{-/-} mice. Importantly, adoptive transfer of CD4⁺

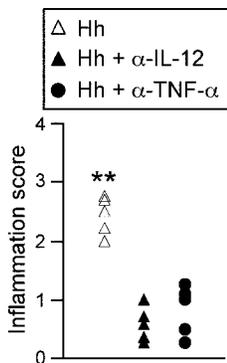


Figure 3. *H. hepaticus*-triggered T cell-independent intestinal inflammation is driven by proinflammatory cytokines. 129SvEvRAG2^{-/-} mice were infected by oral gavage with 2×10^8 cfu *H. hepaticus*. Where indicated, mice received 1 mg/wk anti-IL-12p40 for the first 2 wk after infection or 2 mg/wk anti-TNF- α throughout the course of the infection. Mice were killed 8–12 wk later and pathology in the cecum was assessed histologically. Each symbol represents a single animal and results are representative of two similar experiments. **P < 0.01 vs. anti-IL-12p40 or anti-TNF- α .

CD45RB^{low}CD25⁺ T_R cells significantly reduced splenomegaly, granulocyte and monocyte recruitment and NK cell activation observed in RAG2^{-/-} mice after *H. hepaticus* infection (Fig. 4).

To assess innate immune activation at the site of infection, we isolated leukocyte populations from the lamina propria of the cecum (LPL) and from the mesenteric lymph node (MLN) and characterized their composition using flow cytometry. Due to the small numbers of leukocytes present in these tissues, we analyzed pooled LPL or MLN populations from the different groups of mice (Table I). *H. hepaticus* infection of RAG^{-/-} mice elicited a marked increase in the number of LPL isolated from cecal tissue (eightfold, Table I). Strikingly, although they were not present in LPL populations isolated from control RAG^{-/-} mice, neutrophils constituted almost 20% of LPL isolated from inflamed ceca in *H. hepaticus*-infected RAG^{-/-} mice (Table I). By contrast, no marked differences in the relative proportions of monocytes or NK cells were observed in LPL populations isolated from control or *H. hepaticus*-infected RAG^{-/-} mice (Table I). Importantly, reconstitution of *H. hepaticus*-infected RAG^{-/-} mice with CD4⁺CD25⁺ T_R cells prevented the accumulation of neutrophils in the cecal lamina propria and reduced LPL numbers to those found in control RAG mice (Table I). Interestingly, CD4⁺

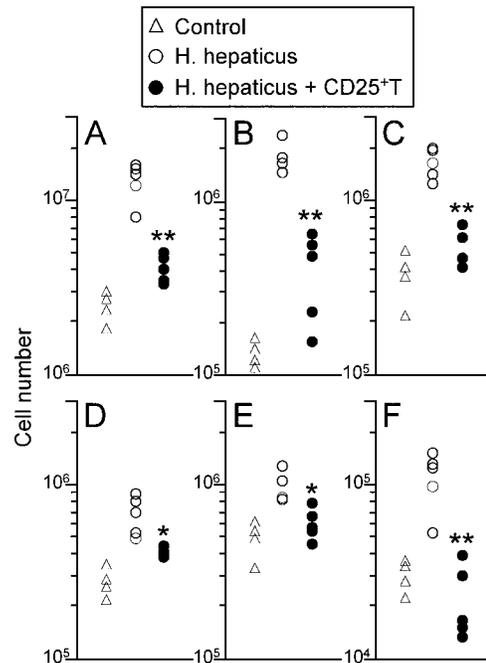


Figure 4. *H. hepaticus* infection of RAG2^{-/-} mice induces systemic activation of innate immunity that is inhibited by CD4⁺CD25⁺ T cells. 129SvEvRAG2^{-/-} mice were reconstituted with 10^5 CD4⁺CD45RB^{low}CD25⁺ T cells and/or infected by oral gavage with 2×10^8 cfu *H. hepaticus*. Mice were killed 8–12 wk later and spleen cell populations were enumerated using FACS[®] analyses. Graphs shown represent numbers of total spleen cells (A), neutrophils (B), monocytes/macrophages (C), dendritic cells (D), NK cells (E), and IFN- γ -producing NK cells (F). Each symbol represents a single animal and results are representative of two similar experiments. **P < 0.01 vs. Hh; *P < 0.05 vs. Hh.

T cells accounted for around 15% of the LPL isolated from *H. hepaticus*-infected RAG^{-/-} mice that had received CD4⁺CD25⁺ T_R cells (Table I). Although *H. hepaticus* infection also slightly increased the number of MLN cells when compared with control RAG^{-/-} mice (2–3-fold), this was primarily due to an increased proportion of monocytes, with no significant increase in the proportion of neutrophils and a marked decrease in the proportion of NK cells (Table I). However, in contrast to the spleen and LPL populations, fivefold higher numbers of MLN cells were present in *H. hepaticus*-infected RAG^{-/-} mice that had received CD4⁺CD25⁺ T_R cells, and this was primarily due to the high proportion (22%) of CD4⁺ T cells found in the MLN populations isolated from these mice (Table I).

Together, the results described above indicate that CD4⁺CD25⁺ T_R cells do not mediate their effects only on other T cells, but are also able to control chronic immune pathology mediated by sustained activation of innate immune mechanisms. In addition, they show that CD4⁺CD25⁺ T_R cells efficiently reconstitute both mucosal and systemic lymphoid compartments, suggesting that they may act at multiple sites to inhibit harmful immune pathology caused by the accumulation and activation of innate immune cells.

CD4⁺CD25⁺ T_R Cells Do Not Influence *H. hepaticus* Colonization Levels. To assess whether CD4⁺CD25⁺ T_R cells might have inhibited intestinal pathology by mediating protective immunity against *H. hepaticus*, we used a real time PCR assay (29) to quantitate *H. hepaticus* levels in cecum and colon scrapings from infected RAG2^{-/-} mice, in the presence or absence of CD4⁺CD25⁺ T_R cells. In infected RAG2^{-/-} mice we detected the highest levels of

Table I. Composition of Mucosal Lymphoid Populations in *H. hepaticus*-infected RAG^{-/-} Mice

Tissue	Group	Cell no.	Neuts				CD4 ⁺ T	
			%	%	%	%	%	
LPL	Control	2.8 × 10 ⁴	0.6	18.2	8.8	3.0	0.3	
	<i>Hh</i>	2.1 × 10 ⁵	17.5	13.0	6.0	8.1	0.3	
	<i>Hh</i> + 25 ⁺	2.5 × 10 ⁴	0.4	14.6	7.3	5.8	15.2	
MLN	Control	1.4 × 10 ⁵	1.8	15.1	4.5	47.7	0.2	
	<i>Hh</i>	3.6 × 10 ⁵	4.0	31.7	4.7	25.2	0.3	
	<i>Hh</i> + 25 ⁺	7.7 × 10 ⁵	0.5	10.2	5.6	15.8	22.1	

Groups of 129SvEvRAG2^{-/-} mice were left untreated (Control), or infected by oral gavage with 2 × 10⁸ cfu *H. hepaticus* (*Hh*), or infected with *H. hepaticus* and reconstituted with 1 × 10⁵ CD4⁺CD45RB^{low}CD25⁺ T cells (*Hh* + 25⁺). Mice were sacrificed 8–12 wk later and LPL and MLN populations (pooled from 4–6 mice per group) were characterized using FACS[®] analyses. Mean cell number for each tissue was calculated by dividing the total number of cells isolated by the number of mice pooled. Results shown are representative of two similar experiments.

H. hepaticus in the cecum, with 5 to 10-fold lower levels present in the colon (Fig. 5). Crucially, reconstitution of RAG2^{-/-} mice with CD4⁺CD25⁺ T_R cells had no measurable effect on the levels of *H. hepaticus* DNA detected in the cecum or colon (Fig. 5), indicating that the protection from *H. hepaticus*-triggered intestinal inflammation was not due to eradication of the bacteria or decreased levels of colonization.

CD4⁺CD25⁺ T_R Cell-mediated Protection against T Cell-independent Intestinal Inflammation Is Cytokine Dependent. Previous studies have highlighted the important roles of IL-10 and TGF-β in the regulation of intestinal inflammation (7, 16, 17, 32). In addition, *H. hepaticus* has also been shown to be able to trigger severe intestinal inflammation in IL-10^{-/-} mice (23, 33). We therefore examined whether these cytokines also played a role in the inhibition of T cell-independent intestinal inflammation induced by *H. hepaticus*. Thus, RAG2^{-/-} mice were reconstituted with CD4⁺CD25⁺ T_R cells, infected with *H. hepaticus* and treated throughout the course of infection with monoclonal antibodies reactive with IL-10R or TGF-β. As shown in Fig. 6, treatment with anti-IL-10R or anti-TGF-β completely ablated the ability of CD4⁺CD25⁺ T_R cells to inhibit *H. hepaticus*-induced intestinal inflammation. Furthermore, we also observed that CD4⁺CD25⁺ T cells from IL-10^{-/-} mice were unable to inhibit *H. hepaticus*-induced intestinal inflammation (Fig. 6), suggesting that T cell-derived IL-10 plays a crucial role in the control of innate immune pathology by CD4⁺CD25⁺ T_R cells. The inability to protect from T cell-independent intestinal inflammation was not due to ineffective T cell reconstitution, as approximately twofold more CD4⁺T cells were recovered from RAG2^{-/-} mice that received IL-10^{-/-} CD4⁺CD25⁺ T cells or anti-IL-10R or anti-TGF-β treatment, when compared with those that received wild-type CD4⁺CD25⁺ T cells only (unpublished data).

Discussion

Although the role of T cells in the induction of intestinal inflammation has received much attention, these results emphasize that innate immune mechanisms are also able to

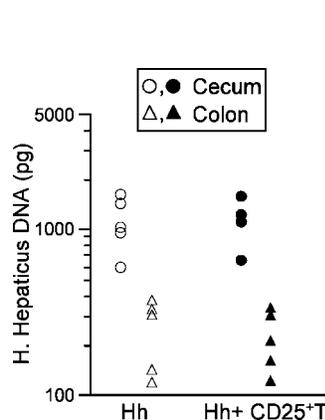


Figure 5. CD4⁺CD25⁺ T cell reconstitution does not affect *H. hepaticus* colonization levels in RAG2^{-/-} mice. 129SvEvRAG2^{-/-} mice were reconstituted with 10⁵ CD4⁺CD45RB^{low}CD25⁺ T cells and/or infected by oral gavage with 2 × 10⁸ cfu *H. hepaticus*. Mice were killed 10 wk later, DNA was isolated from cecum and colon samples and *H. hepaticus* DNA was quantified using a real-time PCR assay. Each symbol represents a single animal and results are representative of two similar experiments.

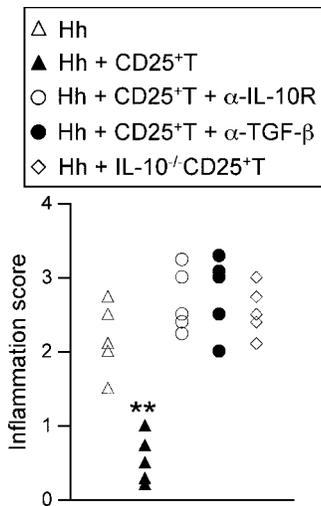


Figure 6. CD4⁺CD25⁺ T cell-mediated protection against T cell-independent intestinal inflammation is dependent on IL-10 and TGF- β . 129SvEvRAG2^{-/-} mice were reconstituted with 10⁵ CD4⁺CD45RB^{low}CD25⁺ T cells from wild-type or IL-10^{-/-} mice and/or infected by oral gavage with 2 \times 10⁸ cfu *H. hepaticus*. Where indicated mice received 0.5 mg/wk anti-IL-10R or 2 mg/wk anti-TGF- β . Mice were killed 8–12 wk later, ceca samples were taken for histological examination. Each symbol represents a single animal and results are representative of two similar experiments. **P < 0.01 vs. Hh.

mediate significant intestinal inflammation upon stimulation with pathogenic organisms like *H. hepaticus* (26, 27, 34, 35). In addition, we show here that T cell-independent intestinal inflammation triggered by *H. hepaticus* infection of RAG2^{-/-} mice was driven by proinflammatory cytokines, was accompanied by local and systemic innate immune activation and was completely inhibited by cotransfer of CD4⁺CD25⁺ T_R cells through cytokine-dependent mechanisms. These results represent the first clear evidence showing that the immune suppressive properties of CD4⁺CD25⁺ T_R cells are not limited to effects on other T cell responses, but also include inhibition of immune pathology mediated by cells of the innate immune system.

The mechanisms by which T_R cells mediate their suppressive functions are the subject of intensive debate. Different in vivo models of disease suppression show different patterns of dependency on various immune suppressive cytokines and there is good evidence that a cell contact-dependent mechanism is involved in the suppression of T cell responses in vitro (for reviews, see references 8 and 36). An alternative explanation that has been proposed for T_R cell phenomena is that regulatory activity is not the sole property of a defined T cell subpopulation, but rather can be accomplished by any kind of T cell that is able to compete for 'space' and survival factors with potentially pathogenic T cells (37). However, suppression of *H. hepaticus*-triggered innate immune pathology was a unique property of CD4⁺CD25⁺ T_R cells. In addition, our results illustrate that CD4⁺CD25⁺ T_R cells do not exclusively mediate their suppressive functions by inhibiting other T cell responses. Since in many inflammatory and autoimmune diseases pathology is associated with the activation and accumulation of innate immune effector cells, the inhibition of such responses by T_R cells extends the therapeutic potential of manipulations of T_R cell function. Nevertheless, it is important to emphasize the delicate balance that T_R cells must maintain between allowing protective immune responses to proceed efficiently, while inhibiting harmful immune pathology. This raises the possibility that excessive T_R cell

activity may actually impede protective immunity. One such instance was recently reported in *scid* mice infected with *Pneumocystis carinii*, where the elimination of *P. carinii* mediated by adoptively transferred CD4⁺CD25⁻ T cells was suppressed when CD4⁺CD25⁺ T cells were cotransferred (38).

The suppression of T cell-independent immune pathology by CD4⁺CD25⁺ T_R cells was dependent on the actions of IL-10 and TGF- β , as treatment with anti-IL-10R or anti-TGF- β completely abrogated protection against *H. hepaticus*-induced intestinal inflammation. This observation further confirms the central role of these immunosuppressive cytokines in preventing intestinal inflammation, highlighted by their key role in the suppression of T cell-mediated colitis by CD4⁺CD45RB^{low} T cells (6, 16, 17) and by the development of colitis in IL-10^{-/-} mice (32). Our findings are also consistent with a previous study showing that the development of T cell-independent intestinal inflammation in RAG2^{-/-} mice exposed to a flora that included *H. hepaticus* could be inhibited by treatment with rIL-10 (39). Importantly, our results suggest a crucial role for T cell-derived IL-10 in this process, as CD4⁺CD25⁺ T cells from IL-10^{-/-} mice were unable to inhibit the development of intestinal inflammation. There is clear evidence that IL-10 has inhibitory effects on cells of the innate immune system, including the suppression of pro-inflammatory cytokine and chemokine production by activated monocytes/macrophages and neutrophils (for a review, see reference 19). An excellent example highlighting the importance of the actions of IL-10 in controlling innate immune cells in the intestine is the observation that transgenic mice with a cell-type deletion in Stat-3, in which macrophages and neutrophils are unresponsive to IL-10, spontaneously develop intestinal inflammation (40).

We observed that *H. hepaticus* infection of 129SvEv RAG2^{-/-} mice triggered intestinal inflammation that was characterized by epithelial cell hyperplasia and by marked infiltration of both granulocytic and mononuclear cells. Immune pathology was most prominent in the cecum, where the highest colonization by *H. hepaticus* occurred. The immune pathology found in *H. hepaticus*-infected RAG2^{-/-} mice was driven by proinflammatory cytokines, as it was significantly inhibited by treatment with anti-IL-12p40 or anti-TNF- α antibodies. In addition, *Helicobacter*-triggered intestinal inflammation is associated with increased production of proinflammatory molecules, such as IL-1, TNF- α , iNOS, and IFN- γ (33, 35). The induction of innate immune pathology in the gastrointestinal tract has also been observed in immunodeficient mice infected with other bacteria, including *Campylobacter fetus* (41) and *Citrobacter rodentium* (42), suggesting that potentially pathogenic innate immune responses may occur commonly in the intestine after colonization with pathogenic organisms.

The present study demonstrates that the development of T cell-mediated colitis in 129SvEvRAG2^{-/-} mice requires *H. hepaticus* infection, whereas we have previously found that adoptive transfer of naive syngeneic CD4⁺ T cells into

CB17 *scid* or C57BL/6 RAG^{-/-} mice leads to severe colitis, even in the absence of *H. hepaticus* (7). While the reasons for these differences are not yet clear, they highlight the fact that the incidence and severity of colitis observed in immune deficient mice after T cell adoptive transfer will be heavily dependent on both environmental factors, especially the intestinal micro-flora, as well as genetic factors, such as mouse strain differences. Similarly, different immune deficient strains in our animal facilities exhibit different susceptibilities to T cell-independent intestinal inflammation induced by *H. hepaticus* infection, with C57BL/6 RAG^{-/-} mice and CB17 *scid* mice exhibiting minimal intestinal inflammation after infection with *H. hepaticus* (unpublished data). In our opinion, similar host environmental and genetic influences, in combination with bacterial strain differences, may account for the wide range of susceptibilities, kinetics, and severity of disease observed in different immunodeficient mouse strains upon infection with *H. hepaticus* (26, 27, 34, 35, 43).

The inflammatory changes found in *H. hepaticus*-infected 129SvEvRAG2^{-/-} mice were not limited to the intestine, they were accompanied by systemic innate immune activation as evidenced by pronounced splenomegaly that was associated with accumulation of neutrophils and monocytes and by the enhanced activation of NK cells. Importantly, we found that adoptively transferred CD4⁺CD25⁺ T_R cells efficiently reconstituted both systemic and mucosal lymphoid compartments, including the lamina propria of the large intestine and the mesenteric lymph nodes. Reconstitution with CD4⁺CD25⁺ T_R cells completely inhibited the accumulation and activation of innate immune cells observed in *H. hepaticus*-infected RAG^{-/-} mice, both in the intestinal lamina propria and also in the spleen. Our results show that, in addition to inhibiting innate immune pathology in the intestine, CD4⁺CD25⁺ T_R cells can also suppress the activation and accumulation of innate immune cells in systemic lymphoid tissues, suggesting that CD4⁺CD25⁺ T_R cells may operate both locally at the inflammatory site and systemically to prevent immune pathology.

It is not yet clear whether CD4⁺CD25⁺ T_R cells recognize self-peptides, peptides derived from foreign antigens or both. In this study we isolated CD4⁺CD25⁺ T_R cells from normal 129SvEv mice that were free from *Helicobacter* infection and demonstrated that they could inhibit intestinal inflammation triggered by *H. hepaticus*. These results are consistent with the finding that CD4⁺CD25⁺ T_R cells isolated from germ-free mice were able to inhibit intestinal pathology mediated by CD4⁺CD45RB^{high} T cells (44), confirming that T_R cells can suppress harmful immune pathology triggered by organisms to which they have not been previously exposed. The nature of CD4⁺ T cell responses directed against *H. hepaticus* has been examined by Kullberg and colleagues in the IL-10^{-/-} murine model of IBD (23, 33). *H. hepaticus*-infected IL-10^{-/-} mice developed severe colitis that was characterized by the presence of *H. hepaticus*-specific CD4⁺ Th1 cell responses in the MLN, while *H. hepaticus* infected wild-type mice did not develop

intestinal pathology and mounted an IL-10 dominated response (23, 33). Recently, Kullberg et al. have extended these findings using *H. hepaticus* infection of naive T cell-reconstituted C57BL/6 RAG^{-/-} mice as a model of bacterially driven T cell-mediated colitis (43). This study found that both CD25⁺ and CD25⁻CD4⁺CD45RB^{low} T cell populations from wild-type C57BL/6 mice could inhibit the development of colitis, but only when these cells were purified from *H. hepaticus*-infected donors (43). Protection from colitis was associated with IL-10 production by the CD4⁺CD45RB^{low} T cells, but was not dependent on TGF-β. These results suggest *H. hepaticus* infection of normal immune competent mice induces CD4⁺ T_R cells that prevent bacterially induced colitis. While it is difficult to reconcile the discrepancies between these findings and our own observations, we believe that a number of factors may contribute. First, as noted above, both genetic strain differences and environmental factors influence susceptibility to *H. hepaticus*-induced intestinal inflammation and it is possible that different *H. hepaticus* isolates may also exhibit differing pathogenic potentials. Second, the intestinal inflammation triggered by *H. hepaticus* in T cell-reconstituted C57BL/6 RAG^{-/-} mice is clearly more severe than the T cell-independent pathology we observed in 129SvEv RAG^{-/-} mice, therefore it is not surprising that the inhibition of T cell-mediated colitis may well require higher numbers of bacteria-specific T_R cells. Lastly, as CD25⁻CD4⁺ T cell populations have been shown to possess T_R cell activity in other experimental models (45–47) and because expression of CD25 by T_R cells is highly dynamic in vivo (48), it is not entirely surprising that *H. hepaticus* infection of normal immune competent mice may give rise to a heterogeneous population of CD45RB^{low}CD4⁺ T_R cells capable of inhibiting *H. hepaticus*-driven intestinal inflammation. Further studies will be required to more clearly define the roles of *H. hepaticus*-specific T_R cells, and of T_R cells recognizing self-antigens or other components of the intestinal flora, in the regulation of pathological innate and adaptive immune responses triggered after infection with *H. hepaticus*.

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