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Colitogenic Th1 Cells Are Present in the Antigen-Experienced T Cell Pool in Normal Mice: Control by CD4$^+$ Regulatory T Cells and IL-10[1]

Chrystelle Asseman,* Simon Read,[†] and Fiona Powrie[2†]

CD4$^+$ regulatory T cells have been shown to prevent intestinal inflammation; however, it is not known whether they act to prevent the priming of colitogenic T cells or actively control these cells as part of the memory T cell pool. In this study, we describe the presence of colitogenic Th1 cells within the CD4$^+$CD45RB^{low} population. These pathogenic cells enrich within the CD25$^-$ subset and are not recent thymic emigrants. CD4$^+$CD45RB^{low} cells from germfree mice were significantly reduced in their ability to transfer colitis to immune deficient recipients, suggesting the presence of commensal bacteria in the donor mice drives colitogenic T cells into the Ag-experienced/memory T cell pool. This potentially pathogenic population of Ag-experienced T cells is subject to T cell-mediated regulation in vivo by both CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells in an IL-10-dependent manner. Furthermore, administration of an anti-IL-10R mAb to unmanipulated adult mice was sufficient to induce the development of colitis. Taken together, these data indicate that colitogenic Th1 cells enter into the Ag-experienced pool in normal mice, but that their function is controlled by regulatory T cells and IL-10. Interestingly, IL-10 was not absolutely required for CD4$^+$CD25$^+$ T cell-mediated inhibition of colitis induced by transfer of naive CD4$^+$CD45RB^{high} cells, suggesting a differential requirement for IL-10 in the regulation of naive and Ag-experienced T cells. *The Journal of Immunology,* 2003, 171: 971–978.

I t is now appreciated that disruption of immune regulatory networks, including deletion of cytokine genes, cell signaling molecules, and alterations in T cell subsets, can lead to an inflammatory bowel disease (IBD[3])-like syndrome in mice (1, 2). In many of the IBD models, intestinal inflammation is the consequence of the development of a dysregulated Th1 response driven by resident bacteria (3–7). This is relevant to IBD in humans, as Th1 responses are elevated in the intestine of patients with Crohn's disease (8, 9). Furthermore, PBLs from patients with IBD proliferate in response to intestinal bacteria, whereas those from normal individuals do not (10), suggesting that IBD may develop as a consequence of a loss of tolerance to enteric bacteria.

Previous studies from this laboratory and others have shown that transfer of CD4$^+$CD45RB^{high} T cells, a predominantly naive population, from the periphery of normal mice to SCID recipients leads to the development of a Th1-mediated colitis with similarities to IBD in humans (3, 11–15). Intestinal inflammation is driven by resident bacteria, as colitis does not develop after T cell transfer to immune deficient recipients raised under germfree (GF) or restricted flora conditions (16, 17). T cells capable of responding to enteric bacteria are present in T cell-restored immune deficient

mice with colitis, and are most likely involved in the pathogenesis of the disease (7).

Colitis induced by transfer of CD4$^+$CD45RB^{high} T cells can be prevented by cotransfer of cells contained within the Ag-experienced CD4$^+$CD45RB^{low} population (11, 12). Regulatory T (T$_R$) cell function is enriched within the 30% of CD4$^+$CD45RB^{low} cells that express CD25 (18), the same population that has been found to suppress a number of T cell-mediated responses, including autoimmune disease (19, 20), allograft rejection (21), antitumor immunity (22), and T cell activation in vitro (23, 24). However, control of immune pathology is not restricted to CD4$^+$CD25$^+$ cells, and there is evidence that CD4$^+$CD25$^-$ T cells also possess some regulatory activity (18, 20, 25), although the relationship between these phenotypically distinct subsets of T$_R$ cells is not known.

The mechanisms by which T$_R$ cells regulate immune responses remain controversial (26, 27). Administration of anti-TGF-β (28) or anti-IL-10R mAb (29) abrogates the ability of CD4$^+$CD45RB^{low} cells to inhibit colitis, suggesting these cytokines are involved in the mechanism of immune suppression. TGF-β is also required for suppression of intestinal inflammation by CD4$^+$CD25$^+$ cells (18); however, it is not known whether the same is true of IL-10. Indeed, there is conflicting data on the requirement for IL-10 in suppression mediated by CD4$^+$CD25$^+$ cells. Although CD4$^+$CD25$^+$ cells from IL-10$^{-/-}$ mice fail to control the proliferation of the progeny of CD4$^+$CD45RB^{high} cells in vivo (25), inhibition of autoimmune gastritis (30) and T cell activation in vitro occurs independently of IL-10 (23, 24).

Although it is clear that thymocytes (31) and naive T cells can induce colitis, the colitogenic potential of cells within the Ag-experienced CD45RB^{low} pool is less well characterized. In general, transfer of CD4$^+$CD45RB^{low} T cells does not induce colitis (11–13, 16, 32), although exceptions to this have been observed (14). In the latter case, it remains to be determined whether the colitis reflects the activity of naive cells, recent thymic emigrants (RTE), or Th1 effector/memory cells. The answer to this has important implications for our understanding of how T$_R$ cells regulate

T cell responses in vivo, as the presence of primed colitogenic Th1 cells in the CD45RB^{low} population would suggest that T_R cells do not control T cell responses at the level of activation. In this study, we have further investigated the ontogeny of colitogenic T cells within the CD4$^+$CD45RB^{low} population. Our data suggest that these cells are not RTE, but represent Ag-experienced Th1 cells that are driven by resident bacteria. The pathogenic potential of these cells can be controlled by CD4$^+$ T_R cells and IL-10.

Materials and Methods

Mice

Specific pathogen-free BALB/c, BALB/c IL-10-deficient (IL-10$^{-/-}$), C.B-17 SCID mice, 129/SvEv, 129/SvEv recombination-activating gene (RAG)-2-deficient (RAG-2$^{-/-}$), and 129/SvEv IL-10$^{-/-}$ mice were bred under specific pathogen-free (SPF) conditions and maintained in microisolator cages with filtered air at the Biomedical Services Unit at John Radcliffe Hospital (Oxford, U.K.). All colonies used in these experiments were free of *Helicobacter hepaticus*, as assessed by PCR (33). GF BALB/c mice were obtained from the Center de Développement des Techniques Avanées pour L'Experimentation Animale (Orléans, France). Mice were used at 8–12 wk of age, unless specified. For thymectomy, 6-wk-old BALB/c mice were anesthetized with Hypnorm (Janssen Pharmaceuticals, Piscataway, NJ) and Hypnoval (Roche, Nutley, NJ) before removal of both lobes (34). All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986.

Antibodies

The following Abs were used for cell purification: YTS169 (35), anti-mouse CD8; TIB120, anti-mouse MHC class II (American Type Culture Collection (ATCC), Manassas, VA); M1/70, anti-mouse Mac-1 (TIB128; ATCC); RA3-6B2, anti-mouse B220 (36); FITC-conjugated anti-mouse CD45RB (clone 16A; BD PharMingen, Oxford, U.K.); CyChrome-conjugated anti-mouse CD4 (clone RM4-5; BD PharMingen); biotinylated anti-mouse CD25 (clone 7D4; BD PharMingen); and PE-conjugated streptavidin (BD PharMingen). PerCP-conjugated anti-mouse CD4 (clone RM4-5; BD PharMingen) and PE-conjugated anti-mouse TCR β-chain (clone H57-597; BD PharMingen) were used for analysis of T cell populations in reconstituted SCID mice. FITC-conjugated anti-mouse IFN-γ (clone XMG1-2; BD PharMingen) and PE-conjugated anti-mouse TNF-α (clone XT-22; BD PharMingen) were used for intracellular cytokine staining. For in vivo use, anti-mouse IL-10R mAb (clone 1B1.2) (37), and GL113, an isotype control mAb (rat IgG1), were purified from hybridoma supernatant by affinity chromatography and shown to contain <0.1 EU endotoxin per mg of protein.

Purification of T cell subsets

CD4$^+$ T cell subsets were isolated from spleen or mesenteric lymph nodes (MLN), as described previously (12). Briefly, single cell suspensions were depleted of CD8$^+$, MHC class II$^+$, Mac-1$^+$, and B220$^+$ cells by negative selection using sheep anti-rat-coated Dynabeads (Dynal Biotech, Wirral, U.K.). The resulting CD4$^+$-enriched population was stained with Cy-Chrome-conjugated anti-CD4, FITC-conjugated anti-CD45RB, and for some experiments biotinylated anti-CD25 mAb and streptavidin-PE. Subpopulations of CD4$^+$ cells were isolated by cell sorting on a FACSVantage (BD Biosciences, Oxford, U.K.). All populations were >98.5% pure on reanalysis. In some experiments, mice were thymectomized 6–8 wk before isolation of CD4$^+$ T cells.

T cell reconstitution and Ab treatment

C.B-17 *scid* mice or RAG-2$^{-/-}$ mice were injected i.p. with sorted CD4$^+$ T cell subpopulations in PBS. mAbs were also injected i.p. in PBS the day after T cell reconstitution, and hence once per week for the duration of the experiment.

Histological examination

Colons were removed from mice 6–8 wk after T cell reconstitution and fixed in buffered 10% Formalin. Paraffin-embedded sections (6 μm) were cut and stained with H&E. Inflammation was scored in a blinded fashion, on a scale of 0–4, representing no changes to severe inflammation. Changes typically associated with other grades are as follows: grade 1, minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; grade 2, mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and asso-

ciated with erosions, with mild to moderate epithelial hyperplasia and mild to moderate mucin depletion from goblet cells; grade 3, moderate inflammatory cell infiltrates that were sometimes transmural, with moderate to severe epithelial hyperplasia and mucin depletion; grade 4, marked inflammatory cell infiltrates that were often transmural and associated with crypt abscesses and occasional ulceration, with marked epithelial hyperplasia, mucin depletion, and loss of intestinal glands.

Cell preparation and cytokine analysis

Lamina propria (LP) lymphocytes were purified, as described (3). Briefly, colons were cut into 0.5- to 1.0-cm pieces and incubated in Ca- and Mg-free PBS containing 10% heat-inactivated FCS (Invitrogen, Paisley, U.K.) and 5.0 mM EDTA to remove the epithelial layer. The remaining tissue was further digested with collagenase/dispase (100 U/ml; Sigma-Aldrich, Poole, U.K.), and the LP cells were then layered on a Percoll gradient (Amersham Pharmacia Biotech, Amersham, U.K.). The lymphocyte-enriched population was recovered after centrifugation (600 × *g*, 20 min) at the 40–75% interface. The resulting population was labeled with fluorochrome-conjugated Abs recognizing CD4 and TCR β-chain, and the proportion of CD4$^+$ T cells was determined by flow cytometric analysis on a FACSort using CellQuest software (BD Biosciences). For detection of cytokines, freshly isolated LP lymphocytes were cultured for 12 h in RPMI 1640 (Invitrogen) containing 10% FCS, 2 mM L-glutamine, 0.05 mM 2-ME, and 100 U/ml each of penicillin and streptomycin in 24-well flat-bottom plates coated with anti-mouse CD3ε (10 μg/ml) (clone 145-2C11, CRL1975; ATCC). Brefeldin A (10 μg/ml; Sigma-Aldrich) was added for the final 2 h of incubation, and surface and cytoplasmic staining was performed, as described previously (38). Labeled cells were analyzed on a FACSort using CellQuest software.

Statistical analysis

Where indicated, colitis scores were compared using a Mann-Whitney *U* test, and differences were considered statistically significant when $p < 0.05$.

Results

IL-10 controls pathogenic T cells contained within the CD4$^+$CD45RB^{low} population

The essential role that IL-10 plays in intestinal homeostasis is illustrated by the fact that IL-10$^{-/-}$ mice develop an IBD-like syndrome (39, 40) and mount a pathogenic Th1 response toward the murine intestinal pathogen *H. hepaticus* (41). Previously, we found that CD4$^+$CD45RB^{low} T cells from IL-10$^{-/-}$ mice, but not wild-type (wt) mice, were able to induce colitis after transfer into immune deficient recipients (29). This demonstrates that pathogenic T cells are present in the Ag-experienced pool of IL-10$^{-/-}$ mice; however, it is not clear whether this occurs as a consequence of the lack of IL-10 through ontogeny, or whether colitogenic T cells are present in the Ag-experienced pool of normal mice, but remain silent as a result of active control by IL-10.

To investigate this, CD4$^+$CD45RB^{low} cells were isolated from the spleen or MLN of wt mice and injected into SCID mice together with anti-IL-10R mAb or an isotype-matched control mAb. As expected, colons from mice restored with CD4$^+$CD45RB^{low} cells isolated from either spleen or MLN and treated with isotype control mAb exhibited no detectable pathological changes (Figs. 1 and 2*A*). In contrast, 80–90% of CD4$^+$CD45RB^{low} cell-restored mice treated with anti-IL-10R mAb showed characteristic features of intestinal inflammation, including marked epithelial cell hyperplasia, leukocytic infiltration of the mucosa, and depletion of goblet cells (Fig. 2*B*). Disease induced by transfer of CD4$^+$CD45RB^{low} cells together with anti-IL-10R treatment had similar characteristics to that induced by transfer of CD4$^+$CD45RB^{high} cells (Fig. 2*C*), including the presence of high numbers of IFN-γ- and TNF-α-expressing CD4$^+$ Th1 cells in the large intestine (Table I) (29). Importantly, induction of colitis with anti-IL-10R mAb was strictly dependent on the presence of CD4$^+$ T cells, as unreconstituted SCID mice treated with anti-IL-10R failed to show any signs of inflammation (data not
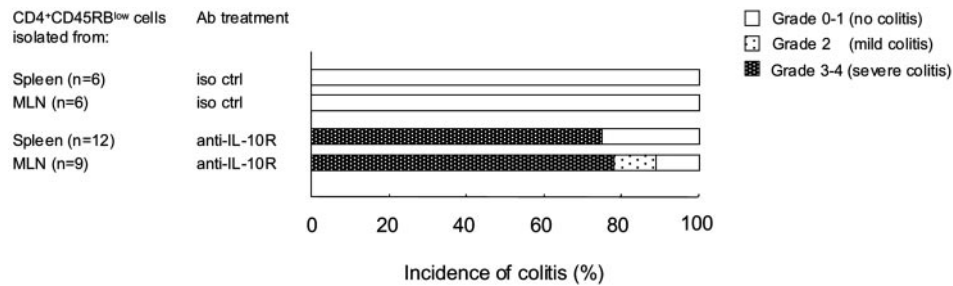
FIGURE 1. Induction of colitis in mice restored with wt CD4$^+$CD45RB^{low} T cells and treated with anti-IL-10R mAb. C.B-17 SCID mice were reconstituted with 2×10^5 CD4$^+$CD45RB^{low} cells isolated from spleen or MLN and treated with Abs, as indicated (1 mg the day after T cell reconstitution and 0.5 mg/wk thereafter). Eight to twelve weeks after reconstitution, mice were killed and colonic pathology was assessed. Data from four independent experiments. Whether SCID mice received CD4$^+$CD45RB^{low} cells isolated from spleen or MLN, administration of anti-IL-10R mAb resulted in significant disease: $p < 0.001$.

shown). Taken together, these data indicate that potentially pathogenic cells are contained within the CD4$^+$CD45RB^{low} subset, but that their activity is controlled by IL-10.

Pathogenic cells within the CD45RB^{low} subset are not RTE and are driven into the Ag-experienced pool by commensal bacteria

The CD4$^+$CD45RB^{low} T cell population is heterogeneous, containing T$_R$ cells (11, 12), Ag-experienced/memory cells (42), and, in the rat, RTE (43). The latter were found to be responsible for the diabetogenic potential associated with CD4$^+$CD45RC^{low}CD25$^-$ cells (20). To determine whether the pathogenicity revealed by anti-IL-10R mAb treatment was the result of the expansion of unprimed RTE, the CD45RB^{low} population was isolated from mice thymectomized 5–8 wk previously. Because postthymic maturation of RTE is thought to occur within 1–2 wk, these mice can be considered to be devoid of RTE (44, 45). As shown in Fig. 3, CD4$^+$CD45RB^{low} cells isolated from these mice, although devoid of pathogenicity themselves, were able to induce colitis in the presence of anti-IL-10R mAb. These results suggest that the colitogenic T cells within the CD4$^+$CD45RB^{low} population are mature peripheral T cells and do not represent the activity of RTE.

Development of colitis after transfer of CD4$^+$CD45RB^{high} cells is dependent on the resident flora (16, 17). However, this population isolated from GF mice is still able to induce colitis (unpublished observations), suggesting naive T cells differentiate into colitogenic Th1 cells upon exposure to resident bacteria in the immune deficient recipient. To investigate whether the presence of colitogenic T cells within the CD4$^+$CD45RB^{low} population requires prior exposure to enteric bacteria, this population was isolated from the spleens of GF mice and tested for its ability to induce colitis when transferred to immune deficient recipients treated with anti-IL-10R mAb. As shown in Fig. 3, the incidence and severity of colitis were significantly reduced when CD4$^+$CD45RB^{low} cells were taken from GF mice, suggesting that the pathogenic T cells in the CD4$^+$CD45RB^{low} population represent a primed population of T cells driven by resident bacteria.

Pathogenic cells are enriched within the CD4$^+$CD45RB^{low}CD25$^-$ subset

To determine whether the colitogenic T cells within the Ag-experienced pool reside together with T$_R$ cells within the naturally activated CD25$^+$ subset or are present within the reciprocal CD25$^-$ subset, CD4$^+$CD45RB^{low} cells were fractionated into CD25$^+$ and CD25$^-$ subsets and transferred to SCID mice. CD4$^+$CD45RB^{low}CD25$^-$ cells were able to expand in immune deficient recipients and migrate to the LP. However, this population, isolated from either spleen or MLN, did not induce inflammation unless the recipients were treated with anti-IL-10R mAb (Table II). Consistent with this, CD4$^+$CD45RB^{low}CD25$^-$ cells from IL-10$^{-/-}$ mice also accumulated in the colon and were as capable of inducing colitis as wt CD4$^+$CD45RB^{low}CD25$^-$ cells transferred to recipients treated with anti-IL-10R (Table II).
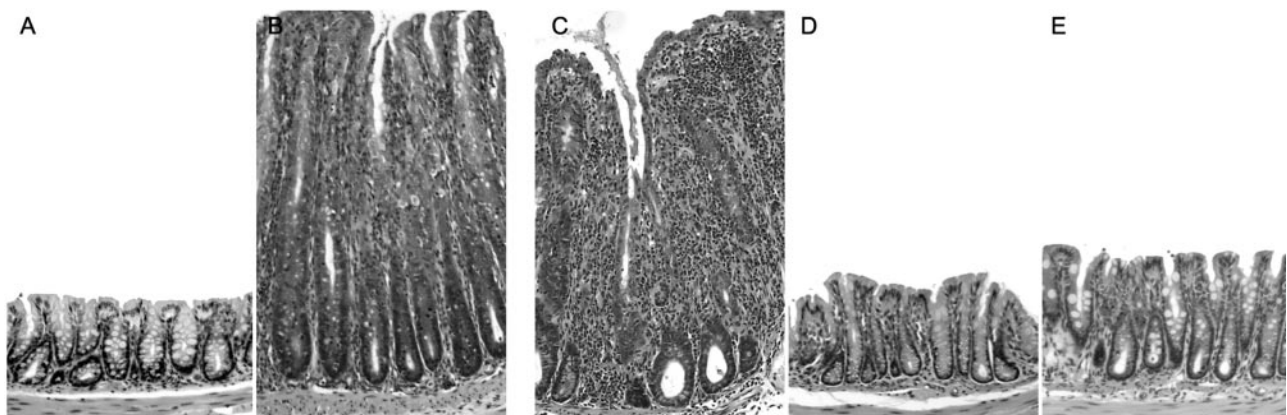

FIGURE 2. Representative photomicrographs of the colons of C.B-17 SCID mice after transfer of CD4$^+$ T cells. The colons in mice restored with wt CD4$^+$CD45RB^{low} cells and treated with isotype control mAb (*A*) were histologically normal; however, mice receiving CD4$^+$CD45RB^{low} cells in combination with anti-IL-10R mAb developed severe colitis (*B*). Injection of 4×10^5 CD4$^+$CD45RB^{high} cells also resulted in the development of severe colitis (*C*); this was prevented by cotransfer of wt CD4$^+$CD25$^+$ T cells (not shown). Inhibition of colitis mediated by CD4$^+$CD25$^+$ cells was not abrogated by administration of anti-IL-10R mAb (*D*). In addition, IL-10$^{-/-}$ CD4$^+$CD25$^+$ cells also retained the ability to inhibit the development of colitis induced by transfer of CD4$^+$CD45RB^{high} cells (*E*). Original magnification: $\times 250$.

Table I. *Expansion of Th1 cells in the LP of mice with colitis induced by anti-IL-10R treatment of SCID recipients restored with wt CD4$^+$ CD45RB^{low} T cells[a]*

Cell Inoculum	Total No. of LP Leukocytes × 10^3	% of CD4$^+$ T Cells	Total No. of CD4$^+$ T Cells × 10^3	% of CD4$^+$ Cells Expressing IFN-γ/TNF-α
wt CD4$^+$CD45RB^{low}				
+ isotype control	34.9 ± 17.1	22.4 ± 11.9	9.2 ± 8.8	7.0 ± 1.1/3.4 ± 1.7
+ anti-IL-10R	1199.4 ± 819.0	78.5 ± 6.7	920.6 ± 657.2	29.4 ± 6.5/9.3 ± 3.5

[a] CB-17 SCID mice were reconstituted with 2 × 10^5 MLN CD4$^+$CD45RB^{low} cells and treated either with anti-IL-10R mAb or an isotype control. Eight to 12 wk after cell reconstitution, mononuclear cells from LP were isolated and stained for CD4, TCR β-chain. IFN-γ, and TNF-α. Data represent pooled results ± SEM of two to three animals per group from four independent experiments.

In contrast, wt CD4$^+$CD45RB^{low}CD25$^+$ T cells isolated from the spleen or MLN expanded poorly in SCID recipients. In line with a previous report (25), ~10-fold fewer cells were found to accumulate in the colon than after transfer of wt CD4$^+$CD45RB^{low} CD25$^-$ cells (8.0 × 10^3 ± 0.4 × 10^3 compared with 1.1 × 10^5 ± 0.7 × 10^5 LP CD4$^+$), and in the absence of anti-IL-10R mAb, these populations did not induce colitis (Table II). Strikingly, treatment with anti-IL-10R led to development of colitis only after transfer of CD25$^+$ cells isolated from MLN and not from spleen (Table II). A similar frequency of mice developed colitis (6 of 17; Table II) after transfer of CD4$^+$CD45RB^{low}CD25$^+$ cells from the MLN of IL-10$^{-/-}$ mice, consistent with the observations of others (25). Taken together, these data indicate that Ag-experienced, potentially pathogenic T cells are enriched within the CD4$^+$CD45RB^{low} CD25$^-$ subset present in both the spleen and MLN, and that their ability to cause disease is regulated by the presence of IL-10. In addition, in the absence of IL-10, pathogenic T cells can also be revealed within the CD25$^+$ population taken from MLN, but not from the spleen. The difference between MLN and spleen may reflect a higher frequency of chronically stimulated bacteria-reactive T cells in the former, and is consistent with the finding that the presence of colitogenic T cells in the CD45RB^{low} pool is dependent on exposure to bacteria in the donor.

Treatment with anti-IL-10R induces colitis in normal mice

It is possible that development of colitis in immune deficient mice restored with CD4$^+$CD45RB^{low} cells and treated with anti-IL-10R is a consequence of expansion of pathogenic cells in a lymphopenic environment, and that colitis in IL-10$^{-/-}$ mice is attributable to a lack of IL-10 throughout ontogeny. However, treatment of normal BALB/c mice with anti-IL-10R led to the induction of severe colitis in 8 of 12 treated mice (Fig. 4; mean colitis score, 3.8 ± 0.0; $n = 8$), demonstrating that IL-10 is actively required in adult mice to maintain intestinal homeostasis.

IL-10 is required for the control of colitis induced by Ag-experienced, but not naive T cells

Transfer of wt CD4$^+$CD25$^+$ T$_R$ cells prevents the development of colitis induced by cells contained within the IL-10$^{-/-}$ CD45RB^{low} CD25$^-$ population (2 × 10^5 IL-10$^{-/-}$ CD45RB^{low}CD25$^-$ cells, 6 of 7 colitic; 2 × 10^5 IL-10$^{-/-}$ CD45RB^{low}CD25$^-$ + 1 × 10^5 CD25$^+$ cells, 1 of 7 colitic), indicating that in addition to regulating naive T cells, CD25$^+$ T$_R$ cells also suppress Ag-experienced colitogenic T cells. This regulation is IL-10 dependent, as anti-IL-10R treatment induced colitis in recipients of unseparated CD45RB^{low} cells (Fig. 1), which are a mixture of approximately one-third CD25$^+$ and two-third CD25$^-$ cells. The requirement for IL-10 in the suppression of colitis induced by transfer of naive CD4$^+$CD45RB^{high} cells has only been examined using unseparated CD45RB^{low} cells as a source of T$_R$ cells (29). Given that anti-IL-10R treatment reveals pathogenic T cells within the CD45RB^{low} pool, it was important to determine whether the regulation of naive T cells by CD4$^+$CD25$^+$ T$_R$ cells also requires IL-10.

To this end, CD4$^+$CD45RB^{high} cells were transferred together with splenic CD4$^+$CD25$^+$ cells into recipient mice treated with
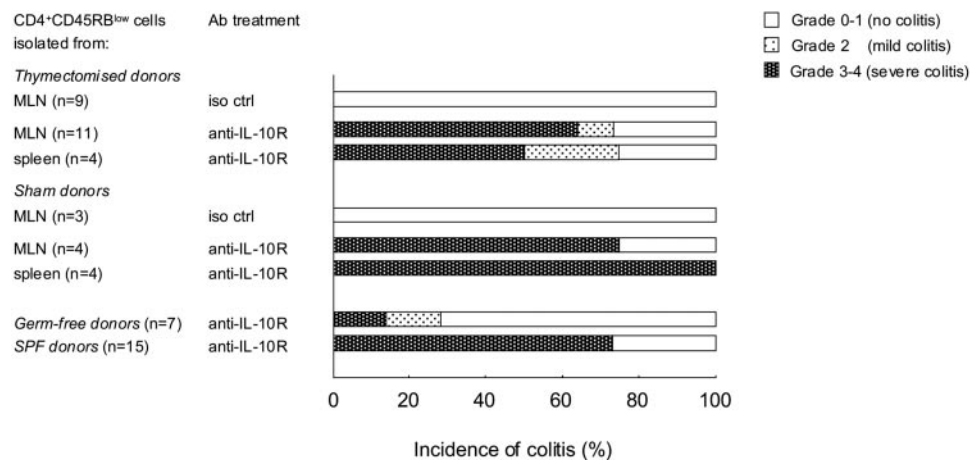
FIGURE 3. Pathogenic T cells in the CD4$^+$CD45RB^{low} pool are not RTE, but are driven by exposure to bacteria in the donor. C.B-17 SCID mice were reconstituted with 2 × 10^5 splenic or MLN CD4$^+$CD45RB^{low} cells from donor mice, as indicated, and treated with Abs, as indicated in Fig. 1. For some experiments, donor mice were thymectomized at least 8 wk before sacrifice; the absence of thymic tissue was confirmed on sacrifice. For other experiments, donor mice were taken from colonies housed under either GF or SPF conditions. Eight weeks after reconstitution, SCID mice were killed and colonic pathology was assessed. Data from four independent experiments. In the presence of anti-IL-10R mAb, CD4$^+$CD45RB^{low} cells from SPF mice induce significantly more severe colitis compared with the equivalent population from GF mice ($p < 0.01$).

Table II. *In the absence of IL-10, pathogenic T cells are revealed in both CD25$^+$ and CD25$^-$ subsets of CD4$^+$CD45RB^{low} T cells*

Cell Inoculum		Incidence of Colitis[c]	Colitis Score[d]
wt CD25^{-a}	MLN	0/8	
	Spleen	0/3	
	MLN + anti-IL-10R	6/7	3.2 ± 0.2[e]
	Spleen + anti-IL-10R	3/3	2.5 ± 0.5
IL-10$^{-/-}$ CD25^{-b}	MLN	13/18	3.7 ± 0.1
wt CD25^{+a}	MLN	0/11	
	Spleen	0/5	
	MLN + anti-IL-10R	5/15	2.6 ± 0.2[f]
	Spleen + anti-IL-10R	0/7	
IL-10$^{-/-}$ CD25^{+b}	MLN	6/17	2.8 ± 0.3

[a,b] CB-17 SCID mice[a] or 129Sv/ev RAG-2$^{-/-}$ mice[b] received 2 × 10^5 CD4$^+$ T cells and anti-IL-10R or isotype control mAb, as indicated.

[c] Colitis score >2.

[d] Data represent mean colitis score ± SEM of affected mice, from two to three independent experiments for each experimental group.

[e] Administration of anti-IL-10R mAb induced significant colitis in mice reconstituted with CD4$^+$ CD45RB^{low} CD25$^-$ cells isolated from either MLN ($p = 0.001$) or spleen ($p < 0.025$).

[f] In the presence of anti-IL-10R mAb, CD4$^+$ CD45RB^{low} CD25$^-$ cells from the MLN induce a significantly more severe colitis when compared to the equivalent population isolated from spleen ($p < 0.05$).

isotype control or anti-IL-10R mAb. Splenic CD4$^+$CD25$^+$ cells were used because in contrast to the equivalent population taken from the MLN, anti-IL-10R treatment did not reveal the presence of pathogenic cells. Cotransfer of CD4$^+$CD25$^+$ cells significantly inhibited the development of colitis induced by CD4$^+$CD45RB^{high} cells, even in the presence of anti-IL-10R (Figs. 2D and 5). However, IL-10 does appear to play some role in CD4$^+$CD25$^+$ T$_R$ cell-mediated regulation of naive T cells, as administration of anti-IL-10R led to a small, but significant increase in the incidence of colitis compared with isotype control-treated mice ($p < 0.02$). Importantly, CD4$^+$CD25$^+$ cells themselves do not have to synthesize IL-10, as this population isolated from IL-10$^{-/-}$ mice was able to inhibit colitis induced by wt CD4$^+$CD45RB^{high} cells (Figs. 2E and 5).

Discussion

It is now well established that T$_R$ cells can control a number of T cell-mediated immune diseases (26, 46, 47). Whether T$_R$ cells act to prevent the priming of potentially pathogenic T cells or actively control these cells as part of the Ag-experienced/memory T cell pool is not known. In this study, we extend previous findings to show that colitogenic T cells reside alongside T$_R$ cells in the Ag-experienced T cell pool in normal mice. These cells are not RTE and are driven into the Ag-experienced T cell pool by exposure to commensal bacteria in the donor. The pathogenic potential of these cells is controlled by both CD4$^+$CD45RB^{low}CD25$^+$ and CD4$^+$CD45RB^{low}CD25$^-$ T$_R$ populations and is highly dependent on IL-10. Surprisingly, control of colitogenic T cells within the naive CD4$^+$CD45RB^{high} T cell population by CD4$^+$CD25$^+$ T$_R$ cells is less dependent on IL-10, suggesting that T$_R$ populations may use distinct mechanisms to inhibit intestinal inflammation depending on the nature of the immune response that drives the inflammation.

The CD4$^+$CD45RB^{low} T cell pool is heterogeneous, containing Ag-experienced cells (42, 48), T$_R$ cells (49, 50), and RTE. In rats, RTE reside within the CD4$^+$CD45RC^{low} population (43), and removal of these cells prevents transfer of diabetes by CD4$^+$CD45RC^{low}CD25$^-$ cells, suggesting RTE can contain pathogenic autoreactive cells (20). It has been reported that transfer of CD4$^+$CD45RB^{low} cells into immune deficient recipients can induce colitis (14); however, it was not established whether the colitogenic T cells derive from naive RTE or Ag-experienced cells. In this study, we show that the colitogenic cells within CD4$^+$CD45RB^{low} T cell pool are enriched within the CD25$^-$ fraction and are present in both spleen and MLN. These cells are not RTE, and their activity is significantly reduced in the CD45RB^{low} population isolated from GF mice, suggesting that commensal bacteria in the host drive these pathogenic T cells into the Ag-experienced pool. The most straightforward interpretation of these data is that bacteria-reactive Th1 cells are primed in donor mice, but fail to induce pathology as a result of their control by IL-10.

It is notable that in the presence of the anti-IL-10R mAb, CD4$^+$CD45RB^{low}CD25$^+$ cells taken from the MLN, but not the spleen, were also able to induce colitis. Whether this is a result of differential homing properties or reflects an increased frequency of recently activated bacteria-reactive T cells in the MLN as compared with the spleen is not known. It does, however, emphasize the limitations of using expression of CD25 as a marker unique to CD4$^+$ T$_R$ cells, and illustrates that the make-up of cells within the CD25$^+$ population varies with anatomical location.
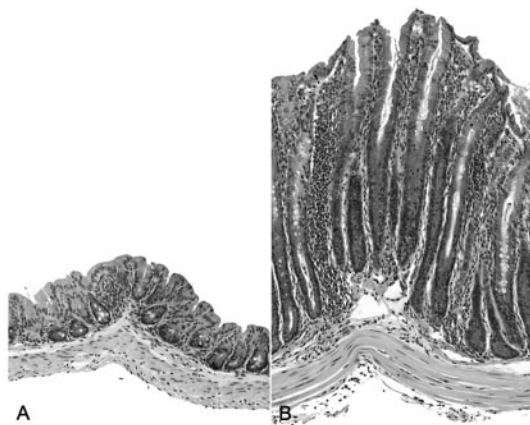

FIGURE 4. Representative photomicrographs of the colons of wt BALB/c mice after administration of anti-IL-10R mAb. Wt BALB/c mice were treated with anti-IL-10R mAb (*B*) or isotype control Abs (*A*), 0.5 mg/wk from 5 wk of age for 8 wk. Mice were sacrificed 7 days after the final injection of Ab. Eight of twelve mice treated with anti-IL-10R developed severe colitis. Original magnification: ×250.
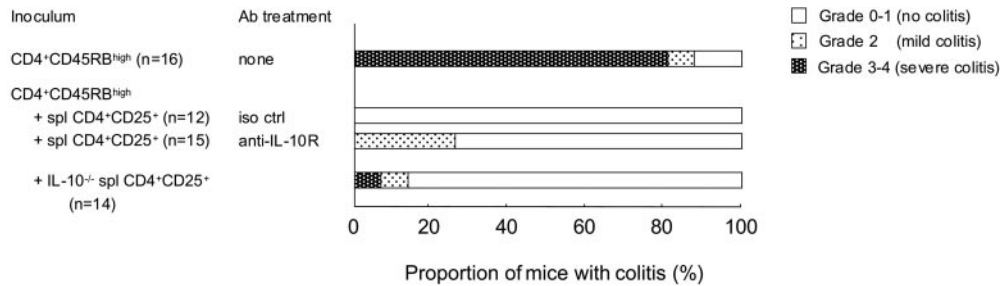
FIGURE 5. Control of colitogenic T cells within the naive T cell pool by CD4$^+$CD25$^+$ T cells is IL-10 independent. C.B-17 *scid* mice received 4 × 10^5 CD4$^+$CD45RB^{high} cells alone or in combination with 1 × 10^5 CD4$^+$CD25$^+$ T cells from spleen of wt BALB/c or IL-10$^{-/-}$ mice. mAb treatments were as described in Fig. 1. Eight weeks posttransfer, mice were sacrificed and tissues were taken for histological examination. Data from three independent experiments. In the presence of anti-IL-10R mAb, wt CD4$^+$CD25$^+$ cells confer significant inhibition of colitis ($p < 0.001$).

The finding that anti-IL-10R mAb treatment reveals colitogenic Th1 cells that enrich within the CD4$^+$CD45RB^{low}CD25$^-$ population emphasizes the importance of IL-10 in the control of Ag-experienced T cell responses. This is not an artifact of T cell transfer to immune deficient recipients, as treatment of adult BALB/c mice with anti-IL-10R mAb was sufficient to induce colitis with similar kinetics and severity to that induced by T cell transfer. Whether IL-10 is produced by T cells or by host-derived cells such as myeloid cells is not clear. However, the observation that IL-10$^{-/-}$ CD4$^+$CD45RB^{low}CD25$^-$ cells are able to induce pathology when transferred to IL-10$^{+/+}$ recipients would suggest that in wt mice, T cells present within the CD25$^-$ population regulate the pathogenic cells that coexist within this population via the production of IL-10. CD4$^+$CD25$^-$ cells have also been shown to inhibit colitis induced by transfer of naive T cells, albeit with reduced potency when compared with CD4$^+$CD25$^+$ T$_R$ cells (18), and to inhibit both diabetes (20) and experimental autoimmune encephalomyelitis (51). The data presented in this study suggest that IL-10 is an important molecule for suppression mediated by these cells; indeed, among CD4$^+$ T cells, IL-10 production enriches within the CD4$^+$CD45RB^{low}CD25$^-$ population (52) (our own unpublished results). Consistent with this, it was recently reported that infection of wt mice with *H. hepaticus* results in the generation of *H. hepaticus*-specific T$_R$ cells. The regulatory activity was found to enrich within the CD4$^+$CD45RB^{low}CD25$^-$ population and to be mediated via IL-10 (53). It is possible that the IL-10-dependent T$_R$ cells within the CD45RB^{low}CD25$^-$ population represent the in vivo counterpart of the in vitro derived T regulatory type 1 cells shown to inhibit both colitis (54) and experimental autoimmune encephalomyelitis (55).

An area of controversy within the T$_R$ cell field is how CD4$^+$CD25$^+$ T$_R$ cells function to inhibit T cell-mediated immune pathology (26, 46). Our data concerning control of intestinal inflammation and the role of IL-10 suggest that the situation is complex and that multiple mechanisms of suppression may operate in vivo. Thus, while control of Ag-experienced colitogenic T cells was found to be highly dependent on IL-10, suppression of naive T cell responses was less dependent, as treatment with anti-IL-10R induced only a marginal reduction in the ability of CD4$^+$CD25$^+$ cells to prevent colitis. Furthermore, CD4$^+$CD25$^+$ cells isolated from IL-10$^{-/-}$ mice retained the ability to inhibit colitis induced by CD4$^+$CD45RB^{high} cells, indicating that IL-10 secretion by CD4$^+$CD25$^+$ T$_R$ cells themselves is not required. Depletion of CD25$^+$ cells from IL-10$^{-/-}$ CD4$^+$ T cells before transfer to RAG-2$^{-/-}$ mice led to an enhanced incidence of colitis (30), providing support for the presence of CD4$^+$CD25$^+$ T cell-mediated IL-10-independent mechanisms of intestinal regulation. TGF-β has been shown to be required for CD4$^+$CD25$^+$ T$_R$ cell-mediated suppression of colitis induced by CD4$^+$CD45RB^{high} cells (18), suggesting that this cytokine may be sufficient to prevent colitis induced by the activation of primarily naive cells, whereas IL-10 is required to control previously activated/differentiated Th1 cells. CD4$^+$CD25$^+$ cells have been shown to express membrane-bound TGF-β (52, 56), suggesting T$_R$ themselves may be a source of this cytokine. A recent report has shown that activated/memory cells become refractory to TGF-β signaling via down-regulation of TGF-β receptor type II expression (57). This could be reversed by IL-10, indicating that IL-10 may play an important role in potentiating the effects of TGF-β on differentiated effector T cells.

It should be noted that in some studies, IL-10 synthesis by CD4$^+$CD25$^+$ cells has been found to be required for prevention of CD4$^+$CD45RB^{high} cell-induced immune pathology in immune deficient recipients (25, 58). This suggests that the state of differentiation of the pathogenic T cells is not the only factor that influences mechanisms of regulation. As intestinal inflammation was not assessed in these studies, it is difficult to make direct comparisons between these studies and our own; however, differences may be attributable to the use of immune deficient recipients of different genetic background or to the environmental factors that drive the inflammatory response. The nature of the endogenous microbiota in immune deficient recipients may be an important and variable factor when comparing results between different laboratories. Recently, we have found that control of both T cell-dependent and T cell-independent intestinal inflammation induced by the mouse pathogen *H. hepaticus* requires IL-10 secretion by CD4$^+$CD25$^+$ cells (59). Thus, in the presence of a potent proinflammatory stimulus, such as *H. hepaticus*, IL-10 is required in addition to TGF-β to control naive T cells. Although TGF-β may act directly on T cells (60–62), IL-10 may serve to inhibit proinflammatory cytokine production, particularly IL-12, by macrophages and DC (63–65). In support of this, IL-10 was shown to enhance TGF-β-mediated suppression of trinitrobenzene sulfonic acid-induced colitis via a mechanism that involved suppression of IL-12 and Th1 responses, which in turn favored expansion of TGF-β-producing Th3 cells (66).

The finding that naive T cells can in some circumstances be controlled by CD4$^+$CD25$^+$ T$_R$ cells via an IL-10-independent mechanism raises the question of why anti-IL-10R mAb treatment induces colitis in normal BALB/c mice. It would appear that the IL-10-independent control mechanism does not prevent priming of potentially colitogenic T cells in these animals, but is sufficient to prevent the development of colitis in SCID recipients following transfer of naive T cells. One possible explanation is that the IL-10-independent regulation is somewhat leaky such that in a normal mouse, some T cells become primed and are then able to induce pathology when IL-10 activity is removed. If this is so, then the

IL-10-dependent mechanism would represent a second layer of regulation able to suppress primed T cell responses. However, when colitis is induced by transfer of naive cells, the IL-10-independent mechanism is sufficient to limit the proliferative expansion of potentially colitogenic cells to the extent that the mice fail to develop disease. Another and not mutually exclusive possibility is that in a normal mouse, only T cells with higher affinity for enteric Ag will be primed, such that those remaining in the naive T cell pool will be expressing lower affinity TCRs. Thus, transfer of the lower affinity cells that remain in the naive T cell pool is able to cause colitis in the absence of T_R and yet remains controllable by T_R even in the absence of IL-10. Clearly, additional experiments are required to identify the factors that control the requirement for IL-10 in T_R cell-mediated control of pathologic T cell responses. The complexity is revealed by a recent report in which it was found that control of the immune response to *Leishmania major* by $CD4^+CD25^+T_R$ cells involved both IL-10-dependent and IL-10-independent mechanisms (67).

Therapeutic use of T_R cells will require that they suppress Ag-experienced T cell responses and reverse established disease. The finding that control of Ag-experienced colitogenic T cells by T_R cells is dependent on IL-10 suggests that production or induction of IL-10 may be a key factor in the therapeutic potential of these cells.

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