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Characterization of Foxp3+CD4+CD25+ and IL-10-Secreting CD4+CD25+ T Cells during Cure of Colitis

Holm H. Uhlig,† Janine Coombes, Christian Mottet, Ana Izcue, Claire Thompson, Andrea Fanger, Andrea Tannapfel, Jason D. Fontenot, Fred Ramsdell, and Fiona Powrie

CD4+CD25+ regulatory T cells can prevent and resolve intestinal inflammation in the murine T cell transfer model of colitis. Using Foxp3 as a marker of regulatory T cell activity, we now provide a comprehensive analysis of the in vivo distribution of Foxp3+CD4+CD25+ cells in wild-type mice, and during cure of experimental colitis. In both cases, Foxp3+CD4+CD25+ cells were found to accumulate in the colon and secondary lymphoid organs. Importantly, Foxp3+ cells were present at increased density in colon samples from patients with ulcerative colitis or Crohn’s disease, suggesting similarities in the behavior of murine and human regulatory cells under inflammatory conditions. Cure of murine colitis was dependent on the presence of IL-10, and IL-10-producing CD4+CD25+ T cells were enriched within the colon during cure of colitis and also under steady state conditions. Our data indicate that although CD4+CD25+ T cells expressing Foxp3 are present within both lymphoid organs and the colon, subsets of IL-10-producing CD4+CD25+ T cells are present mainly within the intestinal lamina propria suggesting compartmentalization of the regulatory T cell response at effector sites. The Journal of Immunology, 2006, 177: 5852–5860.

Increasing evidence suggests that functionally specialized subsets of CD4+ T cells play a key role in the regulation of immune responses (1, 2). In particular, naturally occurring CD4−CD25+ regulatory T cells (Treg) have been shown to prevent both T cell-mediated and innate immune pathology in a number of disease models (3–5). CD4+CD25+ Treg cells with similar properties to those described in mice are also present in humans and impaired function of these cells has been observed in patients with autoimmune disease (6–11). The transcription factor Foxp3 is differentially expressed by CD4+CD25+ Treg cells and plays a key role in their development and function (12–14). Accordingly, mice lacking functional Foxp3 develop a multiorgan-inflammatory disease (12, 15, 16). Similarly, loss of function mutations in FOXP3 have been shown to be responsible for the human autoimmune and inflammatory disease, immune polyendocrine X-linked enteropathy, providing evidence that Treg cells also contribute to immune homeostasis in humans (17, 18).

The T cell transfer model of colitis provides a good system with which the mechanisms of Treg function can be dissected. In this model, transfer of naive CD4+CD45RBhigh T cells into immunodeficient mice leads to a Th1-mediated colitis, while cotransfer of CD4+CD25+ Treg cells can completely prevent disease (4). In addition, recent studies in models of type 1 diabetes and inflammatory bowel disease (IBD) have shown that CD4+CD25+ Treg not only prevent development of disease but can also actually reverse established inflammation (19–22). Thus, using the T cell transfer model of colitis, we found that CD4+CD25+ T cells can reverse an established T cell-mediated inflammatory response in the intestinal mucosa by reducing the pathogenic T cell infiltrate, ultimately leading to restoration of normal intestinal architecture. It is likely that the mechanisms by which Treg can prevent or cure colitis differ. In prevention of colitis, Treg must control activation of a predominantly naive population of cells, while in cure of colitis, they must act on Ag-experienced cells and an aggressive inflammatory response.

The ability of CD4+CD25+ Treg cells to resolve established inflammation in model systems raises the possibility that these cells may be useful as therapeutic agents for chronic inflammatory diseases in humans. With this in mind, it will be important to establish whether the properties of Treg cells determined through study of prevention of colitis also apply to cure of colitis. Attempts to design effective therapeutic strategies will also be aided by knowledge of the location and behavior of Treg in the human IBDs.

We have previously shown that, during cure of experimental colitis, CD4+CD25+ T cells proliferate and accumulate in the mesenteric lymph nodes (MLN) and also in the colonic lamina propria (LP). At both sites, the progeny of CD4+CD25+ T cells are in direct contact with CD11c+ dendritic cells, as well as effector T cells (20). These findings suggest that regulation of an active immune response by CD4+CD25+ T cells occurs in the draining lymph node, as well as at the site of inflammation. However, these studies have been limited by the lack of specific markers for naturally arising Treg cells. Although useful, CD25 expression does not uniquely identify Treg cells, as the CD4+CD25− pool can contain activated effector cells and not all Treg cells express CD25. Identification of Foxp3 as a more specific marker for Treg cells provides an opportunity to track the fate of Treg cells in vivo.
Cure of colitis by CD4+CD25+ T cells has also been shown to be functionally dependent on IL-10, however, it is not known whether T<sub>R</sub> cells themselves are the important source of IL-10 and where it is produced (19). In this study, we have used anti-Foxp3 Abs together with analysis of IL-10 secretion to further investigate the functional and phenotypic characteristics of CD4+CD25+ T cells in physiological conditions and during resolution of intestinal inflammation. Our results show that during cure of intestinal inflammation the majority of Foxp3<sup>+</sup> cells and IL-10-secretory cells derive from CD4<sup>+</sup>CD25<sup>+</sup> precursors. However, whereas Foxp3<sup>+</sup> cells are present in similar frequencies in both the secondary lymphoid organs and LP of colitic animals, IL-10-producing CD4<sup>+</sup>CD25<sup>+</sup> T cells selectively enrich within the colonic LP. In addition, we have extended these findings to analysis of human IBD and show an accumulation of Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells in the inflamed intestine of patients with IBD. This suggests that chronic intestinal inflammation in humans is not simply a consequence of a lack of T<sub>R</sub> cells at the inflammatory site.

**Materials and Methods**

**Mouse and human tissue samples**

BALB/c, C57BL/6, congenic C57BL/6.SJL, CD45, C57BL6 recombinase-activating gene 1-deficient (rag1<sup>−/−</sup>), CB17 SCID, IL-10-knockout (IL-10<sup>−/−</sup>), and Foxp3<sup>−/−</sup> mice (12) were bred under specific pathogen-free conditions. All mice used were >6 wk old.

Colonic tissue sections from patients with Crohn’s disease (CD) and ulcerative colitis (UC) as well as inflamed and noninflamed human colon, appendix, and tonsil tissue were obtained from the Department of Pathology and from the Department of Ear Nose and Throat diseases of the University of Leipzig. All tissues were obtained for therapeutic and diagnostic reasons. The studies were approved by the local ethical review committee (HU242/04).

**Cell purification and flow cytometry**

CD4<sup>+</sup> T cell subsets were isolated from spleens as described (20). Essentially, erythrocyte-depleted spleen cell suspensions were enriched for CD4<sup>+</sup> cells by negative selection following incubation with anti-CD8 (clone YTS169 (25), anti-B220 (clone RA3-6B2 (26), anti-CD11b (TIB128; American Type Culture Collection (ATCC)) and anti-MHC-II Abs (TIB120, ATCC). Ag-positive cells were depleted using sheep anti-rat-IgG Dynal beads (Dynal Biotech).

For MACS sorting of CD4<sup>+</sup>CD25<sup>+</sup> T cells, CD4<sup>+</sup>-enriched cells were incubated with biotinylated anti-CD25 (clone 7D4), followed by streptavidin MACS beads, and sorted on a MoFlo (DakoCytomation). Ag-negative cells were depleted using sheep anti-rat-IgG Dynal beads (Dynal Biotech).

For FACS sorting of CD4<sup>+</sup>CD45RB<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells, CD4<sup>+</sup>-enriched cells were stained with anti-CD45RB (clone 16A), anti-CD25 (clone 7D4), and anti-CD4 (clone H129.19; all BD Biosciences), and sorted on a MoFlo (DakoCytomation). The purity of MACS- and FACS-sorted cells was >90 and >99%, respectively. Because similar results were obtained using MACS or FACS sorting, data were pooled. Unless otherwise stated, all Abs were obtained from BD Biosciences. FACS analysis of Foxp3 expression was performed using the Foxp3 staining kit from eBioscience.

**T cell transfer experiments**

SCID and rag1<sup>−/−</sup> mice were injected i.p. with 4 × 10<sup>7</sup> syngeneic CD4<sup>+</sup>CD45RB<sup>+</sup>-T cells. Mice developed colitis 3.5–4.5 wk posttransfer.

**Histology**

Tissue sections were stained with H&E and colitis severity was graded semiquantitatively from 0 to 4, as outlined in Ref. 27.

**Intracellular cytokine staining**

Cell suspensions were prepared from spleen, MLN, and the LP. For preparation of LP cell suspensions, colons were cut into 0.5-cm pieces and incubated in RPMI 1640 containing 10% heat-inactivated FCS and 5 mM EDTA for 15 min to remove epithelial cells. This step was performed in a shaking incubator at 37°C, and was repeated a further two times, with all supernatant being discarded. The remaining tissue was digested twice using RPMI 1640 containing 10% FCS, 15 mM HEPES, and 0.3 mg/ml Type II Collagenase/Dispase (Warthogton Biological) in h at a 1 in 7 h shaking incubator. LP cells were collected and layered on a Percoll gradient (Amersham Biosciences) and centrifuged at 600 × g for 20 min. The lymphocyte-enriched population was recovered from the 40 to 75% interface. Cell suspensions were stimulated with 100 ng/ml PMA and 1 μg/ml ionomycin for 4 h in the presence of 20 μg/ml brefeldin A. After this in vitro stimulation, cells were stained for CD4 (clone H129.19) and CD45.1 (clone A20) followed by fixation in 2% paraformaldehyde and permeabilization in 0.5% saponin. This was followed by staining for intracellular IL-10 (clone J501, BD Biosciences), IFN-γ (clone XMG1.2, BD Biosciences), and Foxp3 using anti-Foxp3 followed by donkey anti-rabbit-POD (Jackson ImmunoResearch Laboratories) and mouse anti-Foxp3 (clones 105/221/D3 and 236/A/E7; Abcam). Sections were subsequently incubated with appropriate donkey POD-labeled Abs (Jackson ImmunoResearch Laboratories) followed by tyramide amplification.

**Quantitative real-time PCR analysis**

Cells were FACs sorted to >99% purity and RNA extracted using the RNeasy minikit (Qiagen), including a DNase digestion step. cDNA was transcribed with Superscript II (Invitrogen Life Technologies) and used as a template for real-time quantitative PCR. A multiplex reaction for CD3 and Foxp3 was performed using the chromo4 (MJ Research) machine and reporter signals were measured using a SybrGreen standard of CD4<sup>+</sup> T cell cDNA. Foxp3 expression was then normalized to the internal standard, CD3. The following primers and TaqMan probe sequences were used: CD3<sub>α</sub> probe VIC-ACATAGGACCACTATCCTGGCTTTATCTTG-TAMRA- TAMRA; CD3<sub>β</sub> 5′-TTCAGAATGTGGAATACGTCAAGT; CD3<sub>γ</sub> 5′-CACCAGAGCGAGGGAGGAAGGATG; Foxp3 probe FAM-ATCTTCAACGTCTGGCACAATGGACTT-TAMRA; Foxp3<sub>α</sub> 5′-CCCAAGGAACAGACGACCACTT; Foxp3<sub>β</sub> 5′-TTCCTCAACCAGGCACTTGG.
for IL-10 as indicated above. Finally, the cells were permeabilized in eBio-science buffer and stained for Foxp3 according to the manufacturer’s instructions. Cells were analyzed using a FACScalibur or FACSsort (BD Biosciences).

**Statistics**

Two-tailed Mann-Whitney \( U \) test was performed using GraphPad Prism 3.00 (GraphPad). Values of \( p < 0.05 \) were regarded as significant. Data are presented as mean ± SD.

**Results**

**In situ distribution of Foxp3\(^{+}\) CD4\(^{+}\) CD25\(^{+}\) T cells in wild-type mice**

Numerous studies have demonstrated that T\(_{R}\) activity enriches within the CD4\(^{+}\) CD25\(^{+}\) subset, and this is consistent with its high level of expression of Foxp3. However, most studies have analyzed expression of Foxp3 mRNA at the population level. The level of expression of Foxp3. However, most studies have analyzed expression of Foxp3 mRNA at the population level. The frequency of bona fide Foxp3 protein-expressing T\(_{R}\) cells within the CD4\(^{+}\) CD25\(^{+}\) T cell pool and their distribution in vivo remain to be established. To investigate this, expression of CD25 was analyzed in combination with Foxp3 (Fig. 1). In tissue sections from spleen, MLN, and colon of wild-type mice, a minority of DAPI-stained cell nuclei also stained in a dotted pattern with the polyclonal rabbit anti-Foxp3 serum (Fig. 1a). Control experiments using normal rabbit serum did not lead to any nuclear staining, whereas the rabbit anti-histone H2B Ab stained all cell nuclei (data not shown). The specificity of the staining was further confirmed by the absence of nuclear staining in secondary lymphoid organs of Foxp3\(^{-/-}\) mice (Fig. 1, b and c).

Foxp3 staining was found within a subpopulation of CD4\(^{+}\) cells homogenously distributed in the T cell areas of secondary lymphoid organs. Furthermore, Foxp3 expression was largely associated with the expression of CD25 (Fig. 1, d–j). Thus, 48 ± 7 and 49 ± 18% of CD25\(^{+}\) cells were Foxp3\(^{+}\) in the spleen or MLN, respectively. In contrast, only 0.7 ± 0.2 and 0.6 ± 0.4% of CD25\(^{-}\) cells were Foxp3\(^{+}\) (Fig. 1j). This enrichment of Foxp3\(^{+}\) cells within the CD25\(^{+}\) pool is in accordance with the mRNA expression of these cells (Fig. 1k). The frequency of Foxp3\(^{+}\) cells among CD25\(^{+}\) cells in the colon, at 20 ± 18%, was lower than in the spleen and MLN. Whether this represents a real reduction in the frequency of T\(_{R}\) cells or is secondary to an increased frequency of activated CD4\(^{+}\) CD25\(^{+}\) T cells is not known.

Costaining of CD25, Foxp3 and MHC-II indicated that ~60–90% of CD25\(^{+}\)Foxp3\(^{+}\) cells were in contact with MHC-II\(^{+}\) cells (Fig. 1, f–i), including in the intestine where they were preferentially contained within organized leukocytic clusters (Fig. 1, h and i).

The presence of Foxp3 cells in colonic lymphoid structures argues for immunosuppressive activity of T\(_{R}\) cells not only in the spleen and MLN, but also in the colon.

**FIGURE 1.** Expression of Foxp3 by CD4\(^{+}\) CD25\(^{+}\) T cells in the lymphoid organs and colon of wild-type BALB/c mice. a, Wild-type MLN stained for Foxp3 and DAPI. b, Wild-type spleen and c) Foxp3\(^{-/-}\) spleen stained for CD4 and Foxp3. d, Overview and (e) high power magnification of wild-type MLN stained for CD4, CD25, and Foxp3 and analyzed using laser-scanning microscopy. f–i, Wild-type spleen (f), MLN (g), and colon (h) stained for Foxp3, CD25\(^{+}\), and MHC-II and analyzed using conventional fluorescence microscopy. j and i show the same section of colon at different magnifications. j, Percentage of Foxp3\(^{+}\) among CD25\(^{+}\) cells (filled symbols) and Foxp3\(^{+}\) among CD25\(^{-}\) cells (open symbols) in the T cell area of spleen, MLN, and colon of BALB/c mice. The quantification is based on the approximate number of cells per area of lymphoid organ as indicated by the DAPI nuclear staining. Statistical significance was tested using the Mann-Whitney \( U \) test. Similar expression patterns of Foxp3 were found in spleen, MLN, and colon of C57BL/6 mice. k, Expression of Foxp3 mRNA by splenic CD4\(^{+}\) CD25\(^{+}\) CD45RB\(^{hi}\) and CD4\(^{+}\) CD25\(^{+}\) CD45RB\(^{hi}\) T cells. Foxp3 mRNA expression was normalized to CD3\(^{+}\) expression. Data from RNA expression analysis of three independent FACS sorts were pooled.
In situ distribution of Foxp3+CD4+CD25+ T cells during cure of colitis

We next investigated the distribution of Foxp3+ cells following cure of colitis by transfer of CD4+CD25+ T cells. Colitis was induced in immune-deficient (C57BL/6J Rag−/−) mice by transfer of wild-type (CD45.2+) CD4+CD45RBhigh T cells. After development of the first signs of colitis, indicated by weight loss and diarrhea, a group of mice received a second transfer of congenic CD45.1+CD4+CD25+ T cells. As previously described (20) this second transfer led to increased weight and resolution of colitis (Fig. 2). At the time of transfer, ~90% of CD25+ T cells expressed Foxp3 (data not shown).

Two weeks after transfer of CD4+CD25+ T cells, when colitis was still present, ~50% of the CD45.1+ progeny of the CD4+CD25+ T cells expressed Foxp3 irrespective of whether spleen, MLN, or colon were analyzed (Fig. 2a and data not shown). Our data suggest that Foxp3+CD4+CD25+ regulatory cells migrate not only to lymphoid organs but also into the inflamed LP. Following resolution of colitis, 5–10 wk after the secondary transfer of CD4+CD25+ T cells, Foxp3 expression was still primarily restricted to the CD25+ progeny and still detectable within ~50% of these cells (Fig. 2b). Less than 2% of the CD4+CD45RBhigh progeny expressed Foxp3 at this time (n = 4, data not shown), compared with <1% of donor CD45RBhigh T cells directly ex vivo (n = 3, data not shown). Furthermore, in similar experiments performed in mice on the BALB/c background, we again saw no increase in the percentage of Foxp3+ cells among the CD45RBhigh progeny. This suggests that cure of colitis in this model situation is not associated with the induction of Foxp3 in the progeny of CD4+CD45RBhigh T cells.

Presence of Foxp3+IL-10+ cells in the LP of the human colon

The T cell transfer model of colitis reflects several features of the human IBDs, UC and CD. However, the human diseases present distinct histological morphology and are of heterogeneous pathogenicity. Consequently, we investigated the distribution of Foxp3+ cells in human tissue. Within lymphoid organs, as well as in the appendix, Foxp3 expression was confined to the nuclei of CD4+ and CD3+ cells (Fig. 3, a and e) and present predominantly in the T cell areas. In tissue samples from noninflamed human colons, and in patients with intestinal inflammation, nuclear Foxp3-positive cells of lymphocyte morphology were present with the highest density in lymphoid follicles, although there were also scattered cells within the LP (Fig. 3b). The total number of Foxp3+ cells was greater in inflamed tissue than in normal controls (Fig. 3c).

This in part reflected the increase in CD3+ cells and lymphoid follicles in the inflamed colon (Fig. 3, c and d). There was no increase in the actual density of Foxp3+ cells in the T cell areas in inflamed vs uninflamed samples. By contrast there was a higher density of Foxp3+ cells in the inflamed LP compared with controls. Together, the data suggest that inflammation is driving the accumulation of regulatory cells, particularly in effector sites.

To analyze whether the accumulation of Foxp3+ cells in inflamed IBD LP is a specific feature of CD or UC or is due to a general accumulation of Foxp3+ cells in inflamed intestinal tissue, we compared the density of Foxp3+ cells in colon sections of patients with IBD with those with diverticulitis, pseudomembranous colitis, or CMV-induced colitis (CMV) (Fig. 3, b–d). In all cases we found an increased density of Foxp3+ cells indicating that the accumulation of Foxp3+ cells is driven by inflammation in the intestine and is not a specific feature of IBD. The presence of Foxp3+ cells in patients with IBD argues against a simple lack of Foxp3+ TR as a cause of the intestinal inflammation, but for ineffective activity of these cells, or nonresponsiveness to their activity, under conditions of chronic intestinal inflammation.

To investigate whether there is an association of Foxp3 positive cells with IL-10 secretion within the human intestine, we stained human appendix for IL-10 and Foxp3. IL-10-producing cells were found in high density within the germinal center as well as in the subepithelial dome area (Fig. 3e). We show the presence of IL-10-positive CD3+Foxp3+ TR cells as well CD3+Foxp3+ cells that are IL-10 negative but are in close contact with IL-10-positive CD3+Foxp3+ cells (Fig. 3e). This indicates that some Foxp3+ cells secrete IL-10 within the intestine but that these cells are frequently in close contact with IL-10-producing non-T cells (CD3+Foxp3− cells).

Cure of colitis is dependent on IL-10

We have described the presence of Foxp3+CD4+CD25+ T cells in both the secondary lymphoid organs and colonic LP during cure of experimental colitis, suggesting that regulation occurs at both sites. We next decided to further investigate the mechanism by which CD4+CD25+ T cells cure colitis. Again, we transferred CD4+CD25+ cells into animals with established T cell-induced colitis. However, if the transfer of CD4+CD25+ cells was combined
with injection of a neutralizing anti-IL-10R mAb, mice continued to lose weight and remained colitic (Fig. 4). Many different cell types have been shown to be able to produce IL-10. To investigate whether the IL-10 needs to be secreted by the CD4<sup>+</sup>CD25<sup>+</sup> T cell progeny a third group of colitic mice received CD4<sup>+</sup>CD25<sup>+</sup> T cells from IL-10<sup>-/-</sup> mice. Interestingly, although CD4<sup>+</sup>CD25<sup>+</sup>IL-10<sup>-/-</sup> T cells were less effective at controlling intestinal inflammation than wild-type CD4<sup>+</sup>CD25<sup>+</sup> cells they did lead to some amelioration of disease (Fig. 4). The data demonstrates that the presence of IL-10 is required for treatment of colitis, and also suggests that functionally relevant IL-10 may derive from both TR and non-TR sources.
IL-10-producing CD4⁺CD25⁺ T cells enrich within the colon

To further investigate the source of IL-10 and the frequency of IL-10-producing T cells within the CD4⁺CD25⁺ T cell progeny during cure of colitis, we used the conogenic system described above. T cells were isolated from colitic and cured mice and activated using PMA/ionomycin. This permitted analysis of the potential of the progeny of the CD4⁺CD45RB⁺ high cells, and the progeny of the CD4⁺CD25⁺ cells, to produce IL-10 and IFN-γ (Fig. 5). A significant proportion of T cells derived from the CD4⁺CD45RB⁺ high progeny produced IFN-γ, irrespective of whether they were isolated from spleen, MLN, or colonic LP (Fig. 5). Indeed the frequency of IFN-γ⁺ cells among the CD4⁺CD45RB⁺ high progeny in the LP was similar in colitic mice and mice with Tα1-mediated resolution of colitis (Fig. 5b). Within this population there was no significant induction of IL-10 production, indicating that cure of colitis does not involve immune deviation of the colitogenic T cell population into one producing immune suppressive cytokines (Fig. 5b). A significantly higher proportion of the progeny of CD4⁺CD25⁺ cells produced IL-10 than the progeny of CD4⁺CD45RB⁺ high cells (p < 0.05). Strikingly, IL-10-producing CD4⁺CD25⁺ progeny were selectively enriched within the colonic LP (Fig. 5b).

To test whether the enrichment of IL-10-producing cells within the colonic LP was a general feature of CD4⁺CD25⁺ T cells, or due to the specific inflammatory conditions present during the cure of colitis, we analyzed IL-10 production by CD4⁺CD25⁺ T cells from the colons of wt mice. Consistent with the results obtained in the T cell transfer model, we were able to identify IL-10-producing CD4⁺CD25⁺ cells in wild-type mice that selectively enriched within the colonic LP (data not shown). We were also able to show that in wt mice the IL-10 secreting T cells isolated from the colonic LP expressed Foxp3 (Fig. 6).

Discussion

In previous studies, we and others have shown that transfer of CD4⁺CD25⁺ T<sub>R</sub> cells to colitic mice is sufficient to resolve the inflammatory response leading to restoration of normal intestinal architecture (19, 20). Here, we have analyzed the anatomical localization and mechanism by which small numbers of T<sub>R</sub> cells are able to overcome sustained activation of the innate and adaptive immune response. Using expression of Foxp3 protein as a marker of bona fide naturally arising T<sub>R</sub> cells, we show that cure of colitis involves the accumulation of Foxp3⁺ T<sub>R</sub> cells in the secondary lymphoid tissue as well as in the colonic LP. Such cells are also present in the intestine of normal mice and are increased in number in the intestine of patients with IBD. We also show that cure of colitis by CD4⁺CD25⁺ T<sub>R</sub> cells involves an IL-10-dependent mechanism, and that CD4⁺CD25⁺ T<sub>R</sub> cells are able to secrete IL-10. Strikingly, IL-10-producing CD4⁺CD25⁺ T cells were most prominent in the colonic LP of both colitic mice and unmanipulated wild-type mice suggesting further functional specialization of T<sub>R</sub> cells in effector sites.

Although naturally occurring T<sub>R</sub> cells enrich within the CD4⁺CD25⁺ subset, CD25 itself is a marker of activation and therefore unsuitable as an indicator of T cells with regulatory potential. However, the transcription factor, Foxp3, is highly expressed among the CD4⁺CD25⁺ T cell pool, and is in fact required for regulatory T cell development and function (12–14). Foxp3 is therefore a useful and specific marker for naturally occurring T<sub>R</sub>. Elegant studies by Fontenot et al. (23) using mice with a GFP-Foxp3 fusion protein-reporter knockin allele have shown that approximately half of the CD4⁺CD25⁺ population isolated from lymph nodes express Foxp3. However, detailed analysis of Foxp3 expression in situ, and in pathogenic situations, was not performed in that study. Our results using in situ analysis with a Foxp3 polyclonal Ab showed that Foxp3⁺ cells were located in the T cell areas in close contact with APCs. Foxp3-expressing CD4⁺CD25⁺ T cells were also found in the colon under normal physiological conditions suggesting an in situ role for T<sub>R</sub> cells in intestinal homeostasis.

Transfer of naïve CD45RB⁺ high cells into immunodeficient mice leads to the development of a severe colitis, but it is now well-established that cotransfer of CD4⁺CD25⁺ T<sub>R</sub> can prevent disease. More recently, we showed that CD4⁺CD25⁺ T<sub>R</sub> can also bring an existing inflammatory response under control. It can be envisaged that the focus of T<sub>R</sub> activity may differ in these two situations, with the need for T<sub>R</sub> activity in the periphery being greater during cure of colitis when T cells have already become activated and migrated into peripheral effector sites. Consistent with this, during and after cure of colitis the progeny of the CD4⁺CD25⁺ cells were found both in the secondary lymphoid organs and the LP and follicles of the inflamed colon (20). CD4⁺CD25⁺ T<sub>R</sub> have also been shown to localize to colonic follicles when cotransferred with CD4⁺ T cells reactive to bacterially expressed OVA (28). Using Foxp3 as a more accurate indicator of regulatory activity, we now show that Foxp3⁺ cells are also found in both the lymphoid tissue and intestine of colitic mice. By contrast with normal physiological conditions, where Foxp3⁺ cells account for 20% of CD4⁺CD25⁺ cells in the colon, during cure of experimental colitis, Foxp3⁺ cells accounted for some 50% of the CD25⁺ progeny. This could indicate that the accumulation of Foxp3⁺ CD4⁺CD25⁺ T cells may be in part driven by intestinal inflammation, or may simply reflect an even distribution of the transferred splenic CD4⁺CD25⁺ T cells in all of the organs analyzed.

We have also investigated the role of IL-10 in the cure of colitis by CD4⁺CD25⁺ T cells. Our previous studies showed that mice typically started to recover, by around two weeks after T<sub>R</sub> cell transfer. At this time point, effector T cells, as well as other inflammatory cells, greatly outnumber the CD4⁺CD25⁺ progeny. This indicates that CD4⁺CD25⁺ T<sub>R</sub> suppressor mechanisms are locally very potent. This potency may be a consequence of the
secretion of high concentrations of immunosuppressive cytokines, or instruction of effector cells to produce such cytokines. Indeed, previous studies have demonstrated the necessity of IL-10 for the cure of T cell-induced colitis, and of CD4+CD25+ T cell-derived IL-10 for inhibition of colonic inflammation and dysplasia (19, 29). Our own results confirm IL-10 is required for the resolution of established colitis, and furthermore demonstrate that a proportion of the CD4+CD25+ cell progeny in the colons of treated mice produced IL-10. Similarly, recent studies have demonstrated the presence of IL-10-producing CD4+CD25+ T cells in prediabetic and Leishmania major lesions, and in the CNS in EAE (30–32).

However, we also show that IL-10−/− CD4+CD25+ T cells are, although less potent, able to cure colitis. It would appear that although T_R are a major source of the IL-10 required for cure of colitis, other cell types, possibly under the instruction of T_R, can make an important contribution. Indeed, experiments investigating the protective role of IL-10 in schistosomiasis have shown that both innate immune cells and T cells contribute to IL-10 production (33). Many different cell types, including epithelial cells, macrophages, DC, and other T cell subsets, have been shown to produce IL-10. For example, naive T cells can differentiate into IL-10 producers with regulatory function both in vitro and in vivo (2). Importantly, such cells are also able to cure colitis in the T cell transfer model (21). However, we do not consider the naive T cell population to be an essential source of IL-10 in our model. This is based on the finding that colitis induced by IL-10−/− CD45RBhigh T cells was efficiently cured by CD4+CD25+ T cell progeny

Foxp3+ T_R cells can also arise from naive progenitors in vivo following chronic exposure to low doses of Ag (34) or in vitro when activated in the presence of TGF-β (24, 35–37). However, we found no evidence that resolution of intestinal inflammation, at least in the T cell transfer model, was associated with significant immune deviation of CD4+CD45RBhigh progeny into either Foxp3+ or IL-10-producing T_R cells. Cure of colitis was also not

**FIGURE 5.** Accumulation of the IL-10-producing progeny of CD4+CD25+ T cells within the colon. rag-1−/− mice were injected with 4 × 10⁵ wild-type CD45.2+ CD4+CD45RBhigh T cells. After development of colitis, mice received a second transfer of 1 × 10⁶ congenic CD45.1+ CD4+CD25+ T cells. After 4 wk, the ability of CD4+CD45RBhigh (n = 6) and CD4+CD25+ (n = 6) progeny to produce IL-10 and IFN-γ was analyzed. Lymphocytes were prepared from spleen, MLN, and the colonic LP and stimulated with PMA/ionomycin and brefeldin A. The results of two independent experiments are pooled. a, Representative FACS plot showing preferential IL-10 production by LP CD4+CD25+ T cell progeny (CD45.1+) and preferential IFN-γ production by the CD4+CD45RBhigh T cell progeny (CD45.1+). b, Production of IL-10 and IFN-γ by CD4+CD25+ T cell progeny and comparison of IL-10 and IFN-γ production by CD4+CD45RBhigh T cell progeny in the presence or absence of CD4+ CD25+ T cells. Data are shown for spleen, MLN, and LP. Significance was tested using the Mann-Whitney U test; n.s., not significant.
dependent on a reduction in the proportion of IFN-γ-secreting cells among the CD4⁺CD45RBhigh progeny, although, as reported previously, the total number of CD4⁺ T cells present in the colon was greatly reduced (20).

We have previously demonstrated that IL-10 is not required for the prevention of colitis by CD4⁺CD25⁺ T_R. However, when the whole CD4⁺CD45RBhigh fraction is transferred instead of CD4⁺CD25⁺ T_R, prevention of colitis becomes dependent on IL-10. In this situation, T_R-derived IL-10 appears to be essential for the control of colitogenic cells contained within the Ag-experienced CD45RBhigh pool, but not for control of naive T cells (27). The finding that IL-10 is essential for cure of colitis, where a large population of Ag-experienced cells are present, is consistent with this.

We have shown that the IL-10-producing subset of CD25⁺ selectively enriches within the colon of cured mice. The population of T_R used in our model was isolated from the spleen and as such expressed only low levels of IL-10 upon transfer. It is therefore possible that the gut environment conditioned the T_R to further differentiate into IL-10-producing cells. Alternatively, the accumulation of IL-10⁺ T_R in the colon could be attributed to the preferential expansion and homing of a minor subset of IL-10 producers contained within the transferred population. The inability of CD4⁺CD25⁺ cells isolated from peripheral lymphoid organs to secrete IL-10 in vitro has led to the notion that naturally arising Foxp3⁺ T_R cells function via cytokine independent mechanisms (38). However, our finding that IL-10⁺Foxp3⁺ cells are enriched within tissue sites illustrates the problems with extrapolating observations in vitro to predicted function in vivo. The identification of IL-10-producing CD4⁺CD25⁺ T cells in the normal colon suggests a constitutive role for those cells in the prevention of intestinal inflammatory responses. Our data is supported by the earlier observation by Cong et al. (39) that bacterially reactive IL-10-secreting CD4⁺ T cells are present in the colons of normal mice. However, that study did not establish whether such bacterially reactive cells belonged to the naturally occurring Foxp3⁺ T_R pool, or acquired regulatory activity in the periphery. We now show that IL-10-secreting CD4⁺CD25⁺ T_R in the normal colon are contained within the Foxp3⁺ subset. It is however likely that under certain conditions, other IL-10-producing T cells that do not express Foxp3 may be induced (40).

Human IBDs consist of two dominant disease subtypes. CD is largely Th1 in nature (41), while IL-5-, and IL-13-producing T or NKT cells are found in UC (42). Although there is evidence for T cell-mediated immune dysregulation in human IBD, there is limited evidence that disease results from a primary lack or dysfunction of T_R cells. However, clear evidence that a lack of functionally sufficient T_R cells can lead to chronic intestinal inflammation in humans does come from the study of patients with immune polyendocrine X-linked enteropathy syndrome (17, 18). CD4⁺CD25⁺ Foxp3 mRNA expressing T cells have recently been isolated from human intestinal tissue samples, and CD4⁺CD25⁺ T cells from the colons of IBD patients have been shown to be suppressive in vitro (43, 44). Our data show that Foxp3-positive cells are present within the noninflamed LP, and are also present in increased numbers in patients with acute UC and CD. The increase in Foxp3⁺ cell density was seen largely in the LP, rather than in the T cell areas of isolated lymphoid follicles. This can be explained by considering the T cell areas of the isolated lymphoid follicles to be sites of induction of immune responses, analogous to those in the lymph node. Therefore, during an active inflammatory response, T_R may be expected to be preferentially recruited to the site of inflammation. A recent study also found an increase in CD25⁺Foxp3⁺ cells in the colon of UC and CD patients, although this increase was modest compared with that seen in diverticulitis (45). In our study, a similar increase in Foxp3⁺ cells is seen in UC, CD, and diverticulitis, but also in CMV colitis and pseudomembranous colitis, strongly suggesting that chronic intestinal inflammation is not simply a consequence of the absence of Foxp3⁺CD4⁺ T_R cells at the site of inflammation. Whether the T_R cells present have, at least in some patients, impaired intrinsic immunosuppressive activity, or whether the inflamed tissue environment is resistant to immunosuppression by T_R cells remains to be investigated. In any case, our data on human Foxp3⁺ cells supports the hypothesis that intestinal immune regulation in mice and humans may share common features. Consequently, further investigation of the mechanisms by which T_R can cure experimental colitis may give us insight into how regulatory T cell function might be enhanced in human IBD.

**FIGURE 6.** IL-10 secretion by Foxp3⁺ cells in the LP of wild-type mice. Lamina propria and spleen cell suspensions were restimulated with PMA/ionomycin as described in Materials and Methods. a, Representative dot plots showing Foxp3 expression against IL-10 or an isotype control in spleen and LP of B6 SJL CD45 congenic mice. Plots are gated on CD4⁺ cells. The numbers indicate the percentage of IL-10⁺ cells in the Foxp3⁺ or Foxp3⁻ populations. b, Percentage of IL-10-secreting cells in the Foxp3⁺ (filled symbols) and Foxp3⁻ (open symbols) populations from spleen (Sp) and LP CD4⁺ lymphocytes (LPL) from B6 SJL CD45 congenic mice. Each symbol represents data from an individual mouse. In all cases, the isotype control stained <1% of total cells. Similar results were obtained in an experiment using BALB/c mice.