An Essential Role for Interleukin 10 in the Function of Regulatory T Cells That Inhibit Intestinal Inflammation

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Summary

A T helper cell type 1–mediated colitis develops in severe combined immunodeficient mice after transfer of $CD45RB^{high}$ $CD4^+$ T cells and can be prevented by cotransfer of the $CD45RB^{low}$ subset. The immune-suppressive activities of the $CD45RB^{low}$ T cell population can be reversed in vivo by administration of an anti-transforming growth factor β antibody. Here we show that interleukin (IL)-10 is an essential mediator of the regulatory functions of the $CD45RB^{low}$ population. This population isolated from IL-10–deficient ($IL\text{-}10^{-/-}$) mice was unable to protect from colitis and when transferred alone to immune-deficient recipients induced colitis. Treatment with an anti–murine IL-10 receptor monoclonal antibody abrogated inhibition of colitis mediated by wild-type (WT) $CD45RB^{low}$ $CD4^+$ cells, suggesting that IL-10 was necessary for the effector function of the regulatory T cell population. Inhibition of colitis by WT regulatory T cells was not dependent on IL-10 production by progeny of the $CD45RB^{high}$ $CD4^+$ cells, as $CD45RB^{low}$ $CD4^+$ cells from WT mice were able to inhibit colitis induced by $IL\text{-}10^{-/-}$ $CD45RB^{high}$ $CD4^+$ cells. These findings provide the first clear evidence that IL-10 plays a nonredundant role in the functioning of regulatory T cells that control inflammatory responses towards intestinal antigens.

Key words: interleukin 10 • inflammatory bowel disease • $CD4^+$ T lymphocyte • regulatory T lymphocyte

The inflammatory bowel diseases encompassing Crohn's disease and ulcerative colitis are complex chronic diseases whose etiology and pathogenesis are poorly understood. Recently, several murine models of colitis have been developed which have highlighted the important role that abnormalities of the immune system, particularly those affecting T cells, may play in disease pathogenesis (for reviews, see references 1 and 2). Studies using T cell–restored immunodeficient mice have provided evidence that $CD4^+$ T cells play a key role in the induction and regulation of intestinal inflammation, as transfer of $CD45RB^{high}$ $CD4^+$ T cells from normal donors into C.B-17 severe combined immunodeficient (SCID)[1] mice led to the development of a severe inflammatory response in the colon (3, 4). Colitis was the result of the development of a Th1 response, as polarized Th1 cells were present in intestinal lesions and disease could be prevented by treatment with an anti–IFN-γ or anti-TNF mAb (5). More recently, $CD45RB^{high}$ $CD4^+$ T cells isolated from signal transducer and activator of transcription (Stat)-4–deficient mice, which are unresponsive to IL-12, were shown to be impaired in their ability to transfer colitis to immune-deficient recipients (6, 7).

Cotransfer of the reciprocal $CD45RB^{low}$ $CD4^+$ T cell subset together with normally pathogenic $CD45RB^{high}$ cells prevented the development of colitis, indicating that the $CD45RB^{low}$ $CD4^+$ subset from normal mice contains a population of regulatory T cells capable of controlling inflammatory responses in the intestine (4). Analysis of the mechanism of immune suppression revealed a role for TGF-β but not IL-4 or IL-10, as anti–TGF-β mAb but not anti–IL-4 mAb or anti–IL-10 mAb was able to abrogate protection from colitis transferred by $CD45RB^{low}$ $CD4^+$ cells. Indeed, IL-4 appeared to play no demonstrable role in either the development or effector function of the regulatory T cell population, as $CD45RB^{low}$ $CD4^+$ cells from IL-4–deficient mice were equally as potent as wild-

[1] *Abbreviations used in this paper:* LP, lamina propria; RAG, recombination activating gene; SCID, severe combined immunodeficient; Tr-1, T regulatory 1; WT, wild-type.

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type (WT) cells in inhibiting colitis (8). Despite the finding that administration of neutralizing anti–IL-10 mAb failed to inhibit the regulatory activity of $CD45RB^{low}$ $CD4^+$ cells, there is evidence that IL-10 plays an important role in mucosal immune regulation, as mice with a targeted disruption of the IL-10 gene developed enterocolitis (9, 10). In addition, administration of murine rIL-10 prevented colitis in SCID mice restored with $CD45RB^{high}$ $CD4^+$ T cells (5). Furthermore, $CD45RB^{high}$ $CD4^+$ cells isolated from transgenic mice that expressed IL-10 under the IL-2 promoter failed to transfer colitis but rather, were able to inhibit colitis induced by WT $CD45RB^{high}$ $CD4^+$ T cells (11). Taken together, these studies provide evidence that IL-10 is able to regulate pathogenic immune responses in the intestine; however, whether this suppression involves the development of regulatory T cells is not known.

In this study, we have analyzed the role of IL-10 in the development and effector function of regulatory T cells that control the immune response to intestinal antigens, and present evidence that IL-10 is essential for the normal functioning of these cells.

Materials and Methods

Mice. Specific pathogen-free BALB/c and C.B-17 SCID mice were obtained from Simonsen Laboratories or Harlan Olac and maintained in the Animal Care Facility of the DNAX Research Institute or in the Biomedical Services Unit (BMSU) at the John Radcliffe Hospital. 129/SvEv WT, 129/SvEv recombination-activating gene (RAG)-2–deficient ($RAG\text{-}2^{-/-}$) and 129/SvEv IL-10–deficient ($IL\text{-}10^{-/-}$) mice were bred in isolators and maintained in microisolator cages with filtered air. Mice were used at 8–12 wk of age.

Antibodies. The following mAbs were used for cell purification: 2-43, anti–mouse-CD8 (TIB210; American Type Culture Collection); M1/70, anti–mouse Mac-1 (TIB128; American Type Culture Collection); RA3-6B2, anti–mouse B220 (12); FITC-conjugated anti–mouse CD45RB (clone 16A; PharMingen); and Cy-Chrome–conjugated anti–mouse CD4 (clone RM4-5; PharMingen). FITC-conjugated anti–mouse IFN-γ (clone AN-16; gift of Dr. A. O'Garra, DNAX Research Institute) and PE-conjugated anti–mouse TNF-α (clone XT-22; PharMingen) were used for intracellular staining. The following mAbs were used in vivo: 1B1.2 (rat IgG1), a blocking mAb reactive with mouse IL-10 receptor (IL-10R) (13), JES5-2A5 (rat IgG1), an anti–mouse IL-10 mAb (14), and GL113 (rat IgG1), an isotype control mAb reactive with β-galactosidase. Unless otherwise indicated, antibodies were purified from supernatants of hybridomas by ion exchange chromatography and shown to contain <3 EU endotoxin/mg of protein.

Cell Purification and Flow Cytofluorography. $CD4^+$ T cell subsets were isolated from spleens as described previously (4). In brief, single cell suspensions were depleted of $B220^+$, $MAC\text{-}1^+$, and $CD8^+$ cells by negative selection using sheep anti–rat coated Dynabeads (Dynal Ltd.). The resulting $CD4^+$-enriched cells were stained with Cy-Chrome–conjugated anti-CD4 and FITC-conjugated anti-CD45RB mAb and sorted into the $CD45RB^{high}$ $CD4^+$ and $CD45RB^{low}$ $CD4^+$ fractions by two-color sorting on a FACS Vantage™ (Becton Dickinson). All populations were >98% pure on reanalysis.

T Cell Reconstitution and Antibody Treatment. $RAG\text{-}2^{-/-}$ mice or C.B-17 SCID mice were injected intraperitoneally with sorted $CD4^+$ T cell subpopulations in PBS. mAbs were injected intraperitoneally in PBS the day after T cell reconstitution and weekly for the duration of the experiments.

Microscopic Examination. Colons were removed from mice 8–12 wk after T cell reconstitution and fixed in buffered 10% formalin. 6 μm paraffin-embedded sections were cut and stained with hematoxylin and eosin. Tissues were graded semiquantitatively from 0 to 5 in a blinded fashion. A grade of 0 was given when there were no changes observed. 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 associated with erosions, with minimal to mild epithelial hyperplasia and minimal to mild mucin depletion from goblet cells; grade 3, mild to moderate inflammatory cell infiltrates that were sometimes transmural, often associated with ulceration, with moderate epithelial hyperplasia and mucin depletion; grade 4, marked inflammatory cell infiltrates that were often transmural and associated with ulceration, with marked epithelial hyperplasia and mucin depletion; and grade 5, marked transmural inflammation with severe ulceration and loss of intestinal glands.

Cell Preparation and Cytokine Analysis. Lamina propria (LP) lymphocytes were purified as described (5). Colons were cut into 0.5–1-cm pieces and incubated in Ca- and Mg-free PBS containing 10% heat-inactivated FCS (GIBCO BRL) and 5 mM EDTA to release intraepithelial lymphocytes. The remaining tissue was further digested with collagenase/dispase (100 U/ml; Sigma Chemical Co.), and the LP cells were then layered on a Percoll gradient (Amersham Pharmacia Biotech). The lymphocyte-enriched population was recovered after centrifugation (600 *g*, 20 min) at the 40/75% interface. For cytokine detection, freshly isolated LP lymphocytes were cultured for 12 h in RPMI 1640 (PAA Laboratories GmbH) 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-bottomed plates coated with anti-CD3ε 145-2C11 (10 μg/ml, CRL1975; American Type Culture Collection). Brefeldin A (10 μg/ml; Sigma Chemical Co.) was added for the final 2 h of incubation, and surface and cytoplasmic stainings were performed as described previously (15). Labeled cells were analyzed on a FACSort™ using CELLQuest™ software (Becton Dickinson).

Statistical Analysis. Colitis scores were compared using the Mann-Whitney test, and differences were considered statistically significant with $P < 0.05$.

Results

$IL\text{-}10^{-/-}$ Mice Lack Regulatory T Cells Capable of Controlling Inflammatory Responses in the Intestine. To determine whether the $CD45RB^{low}$ $CD4^+$ population from $IL\text{-}10^{-/-}$ mice contained regulatory T cells, this population was compared with $CD45RB^{low}$ $CD4^+$ cells from WT mice for the ability to inhibit colitis induced in $RAG\text{-}2^{-/-}$ mice by transfer of WT $CD45RB^{high}$ $CD4^+$ cells. Expression of CD45RB was similar on $CD4^+$ T cells from 10–12-wk-old $IL\text{-}10^{-/-}$ and WT mice (data not shown). WT $CD45RB^{high}$ $CD4^+$ cells (4×10^5) were transferred to syngeneic $RAG\text{-}2^{-/-}$ mice either alone or in combination with 2×10^5 $CD45RB^{low}$ $CD4^+$ cells from WT or $IL\text{-}10^{-/-}$

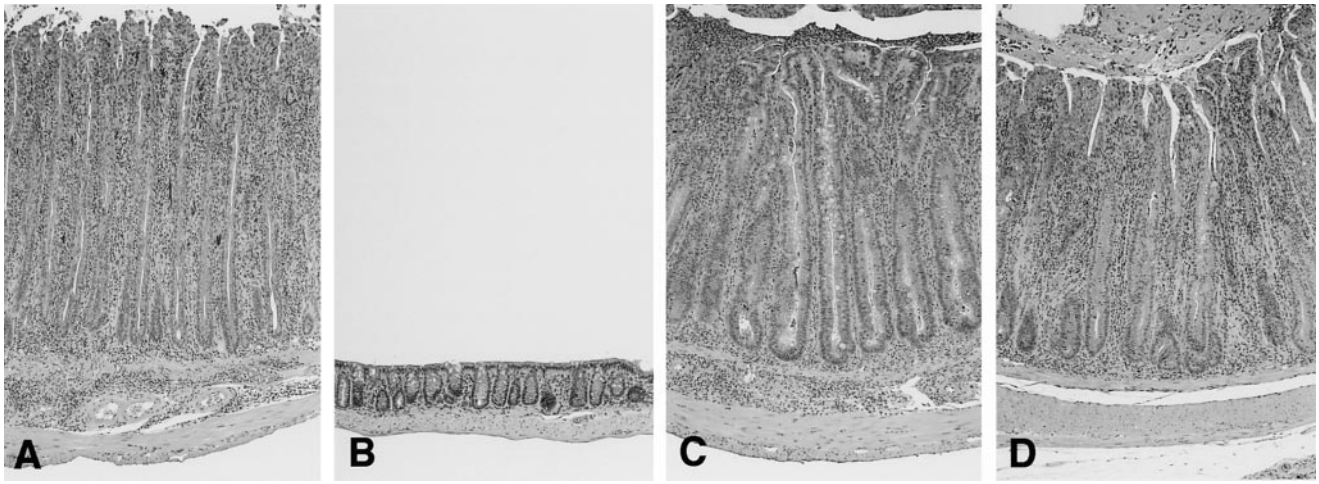

Figure 1. Representative photomicrographs of the descending colon of RAG-$2^{-/-}$ mice after transfer of subpopulations of $CD4^+$ T cells from WT or IL-$10^{-/-}$ mice. (A) Severe colitis in a mouse injected with $CD45RB^{high}$ $CD4^+$ T cells from WT mice. (B) Normal appearance of the colon in a mouse restored with WT $CD45RB^{high}$ and WT $CD45RB^{low}$ $CD4^+$ T cells, indicating that the WT $CD45RB^{low}$ population is able to inhibit disease induced by WT $CD45RB^{high}$ $CD4^+$ T cells. (C) Severe colitis in a mouse cotransferred with WT $CD45RB^{high}$ $CD4^+$ T cells and IL-$10^{-/-}$ $CD45RB^{low}$ $CD4^+$ cells, indicating that the IL-$10^{-/-}$ $CD45RB^{low}$ subpopulation is unable to protect from the disease. (D) Severe colitis in a mouse receiving only IL-$10^{-/-}$ $CD45RB^{low}$ cells, indicating that this population is able to induce disease. Hematoxylin and eosin; original magnifications: ×50.

mice. As controls, the $CD45RB^{low}$ $CD4^+$ populations from IL-$10^{-/-}$ or WT mice were transferred alone and some mice were not reconstituted. 10–12 wk after T cell reconstitution, mice were killed and the development of colonic inflammation was assessed. As described previously (3, 4), transfer of $CD45RB^{high}$ $CD4^+$ cells into RAG-$2^{-/-}$ mice resulted in the development of severe colitis (Fig. 1 A) in the majority of mice (63.4%; Table I, top). This colitis was characterized by an extensive inflammatory cell infiltrate, marked epithelial cell hyperplasia, and depletion of mucin-secreting cells. Cotransfer of the $CD45RB^{low}$ $CD4^+$ population significantly inhibited ($P < 0.005$) the development of colitis, as the majority of mice in this group (83.8%; Table I, top) had minimal changes in the intestine (Fig. 1 B). In contrast, cotransfer of $CD45RB^{low}$ $CD4^+$ cells from IL-$10^{-/-}$ mice failed to protect mice from colitis, as the majority of mice in this group (64.2%; Table I, top) developed intestinal inflammation (Fig. 1 C) identical to that seen in mice restored with $CD45RB^{high}$ $CD4^+$ cells alone. Not only did the $CD45RB^{low}$ population from IL-$10^{-/-}$ mice fail to protect from colitis, this population actually induced disease with similar characteristics (Fig. 1 D) and incidence (59.0%; Table I, top) to that induced by WT $CD45RB^{high}$ $CD4^+$ cells. As described previously (4), immune-deficient mice either unreconstituted or restored with WT $CD45RB^{low}$ $CD4^+$ cells exhibited no pathological changes in the intestine (data not shown). These data indicate that production of IL-10 by $CD45RB^{low}$ $CD4^+$ cells is essential for cells within this population to mediate their immune-suppressive function.

Treatment with Anti–IL-10R mAb Abrogated Inhibition of Colitis Transferred by $CD45RB^{low}$ $CD4^+$Cells. The finding that $CD45RB^{low}$ $CD4^+$ cells from IL-$10^{-/-}$ mice lacked T cells capable of regulating inflammatory responses in the intestine appears at odds with previous studies from this laboratory which showed that treatment with a neutralizing anti–IL-10 mAb failed to abrogate protection from colitis transferred by $CD45RB^{low}$ $CD4^+$ cells (8). The simplest explanation for these data is that the anti–IL-10 mAb used (JES5-2A5) in these studies failed to sufficiently neutralize IL-10. Recently, an mAb reactive with the murine IL-10R has been generated (13) and shown to efficiently neutralize the effects of IL-10. To test whether treatment with anti–IL-10R mAb was able to affect the function of the $CD45RB^{low}$ population, mice restored with a mixture of $CD45RB^{high}$ and $CD45RB^{low}$ $CD4^+$ cells were treated weekly with anti–IL-10, anti–IL-10R, or isotype control mAb. As can be seen in Table I (bottom), treatment with anti–IL-10R abrogated protection from colitis induced by the $CD45RB^{low}$ $CD4^+$ population, as all of the mice in these groups, treated with anti–IL-10R alone or in combination with anti–IL-10, developed colitis. Antibody treatment alone did not induce immune pathology in the absence of T cells, as unreconstituted recipients treated with anti–IL-10R did not develop colitis (data not shown). As reported previously, anti–IL-10 treatment had no effect on the immune-suppressive activities of the $CD45RB^{low}$ population, as mice in this group, like mice treated with isotype control mAb, failed to develop colitis.

Colitis induced by the $CD45RB^{high}$ $CD4^+$ population was characterized by the accumulation of IFN-γ– and TNF-α–secreting Th1 $CD4^+$ T cells in the lesions (Fig. 2 A). A similar expansion of Th1 cells was also present in the colon of mice that developed colitis after transfer of WT $CD45RB^{high}$ $CD4^+$ cells in the presence of WT $CD45RB^{low}$ $CD4^+$ cells plus anti–IL-10R or after transfer of IL-$10^{-/-}$ $CD45RB^{low}$ cells alone. Development of disease correlated with a significant increase in the total num-

Table I. *The Function of Regulatory T Cells That Control Inflammatory Responses in the Colon Is Dependent on IL-10*

Phenotype of $CD4^+$ T cells injected	mAb treatment	No or minimal colitis (0–1)	Mild colitis (2)	Severe colitis (3–5)
RAG-$2^{-/-}$ recipients*				
4×10^5 $CD45RB^{high}$	–	8/41 (19.5%)	7/41	26/41 (63.4%)
4×10^5 $CD45RB^{high}$ + 2×10^5 WT $CD45RB^{low}$	–	26/31 (83.8%)	3/31	2/31 (6.4%)‡
4×10^5 $CD45RB^{high}$ + 2×10^5 IL-$10^{-/-}$ $CD45RB^{low}$	–	6/28 (21.4%)	4/28	18/28 (64.2%)
2×10^5 IL-$10^{-/-}$ $CD45RB^{low}$	–	5/22 (22.7%)	4/22	13/22 (59.0%)
C.B-17 SCID recipients§				
4×10^5 $CD45RB^{high}$	–	0/9	3/9	6/9
4×10^5 $CD45RB^{high}$ + 2×10^5 $CD45RB^{low}$	Isotype control	6/7	1/7	0/7
4×10^5 $CD45RB^{high}$ + 2×10^5 $CD45RB^{low}$	Anti–IL-10	5/5	0/5	0/5
4×10^5 $CD45RB^{high}$ + 2×10^5 $CD45RB^{low}$	Anti–IL-10R	0/8	0/8	8/8
4×10^5 $CD45RB^{high}$ + 2×10^5 $CD45RB^{low}$	Anti–IL-10 + Anti–IL-10R	0/5	2/5	3/5

RAG-$2^{-/-}$ or C.B-17 SCID mice were reconstituted with sorted $CD4^+$ T cell subsets and treated for 8 wk with antibodies as indicated (2 mg the day after T cell reconstitution and 1 mg/wk thereafter for anti–IL-10 mAb and isotype control; 1 mg the day after T cell reconstitution and 0.5 mg/wk thereafter for anti–IL-10R mAb). 8–12 wk after reconstitution, mice were killed and colonic pathology was graded on a scale of 0–5 as described in Materials and Methods.

*Data from four to six independent experiments.

‡Statistically different ($P < 0.005$, Mann-Whitney test) compared with mice restored with 4×10^5 $CD45RB^{high}$.

§Data from two experiments.

ber of $CD4^+$ T cells expressing IFN-γ and TNF-α after polyclonal stimulation (8–20-fold expansion of $CD4^+$ IFN-γ^+ cells, and 6–34-fold expansion of $CD4^+$ TNF-α^+ cells; Fig. 2 A) compared with noncolitic mice restored with a mixture of $CD45RB^{high}$ and $CD45RB^{low}$ cells. The increase in total Th1 cells characteristic of colitis was due to the marked increase of $CD4^+$ cells in the intestine (Table II), as analysis of cytokine production at the single cell level revealed that the few $CD4^+$ T cells present in nondiseased colons were capable of producing similar levels of IFN-γ and TNF-α as those isolated from colitic mice (Fig. 2 B). The finding that in cotransfers of $CD45RB^{high}$ and $CD45RB^{low}$ $CD4^+$ cells, neutralization of the function of IL-10 (by treatment with anti–IL-10R or the transfer of IL-$10^{-/-}$ $CD45RB^{low}$ cells) led to the development of colitis with identical immunological and histological features to that induced by transfer of pathogenic $CD45RB^{high}$ cells indicates that IL-10 is a key mediator of the regulatory activity of the $CD45RB^{low}$ $CD4^+$ T cell population.

The Function of Regulatory T Cells Is Not Dependent on the Differentiation of the Progeny of $CD45RB^{high}$ $CD4^+$ Cells into IL-10–secreting Cells. One way regulatory T cells could mediate their immune-suppressive properties is to induce the differentiation of naive T cells into cells with a similar function, rather than into pathogenic cells. This phenomenon has been termed infectious tolerance (16, 17). Given that production of IL-10 by $CD45RB^{low}$ $CD4^+$ cells was essential for their regulatory function and that culture of T cells in the presence of IL-10 can lead to the generation of regulatory T cells (18), it is possible that immune suppression by these cells requires IL-10 production by the normally pathogenic $CD45RB^{high}$ $CD4^+$ population.

To test this, $CD45RB^{high}$ $CD4^+$ cells from IL-$10^{-/-}$ mice were transferred alone or in combination with WT $CD45RB^{low}$ $CD4^+$ cells to RAG-$2^{-/-}$ mice. As described previously (19), mice restored with IL-$10^{-/-}$ $CD45RB^{high}$ $CD4^+$ cells developed a colitis with similar incidence and severity (68.0%; Table III) to that transferred by this population isolated from WT mice. However, protection from colitis was equally effective when WT $CD45RB^{low}$ $CD4^+$ cells were cotransferred with IL-$10^{-/-}$ $CD45RB^{high}$ $CD4^+$ cells, as the majority of mice (81.2%; Table III) had no colitis or minimal changes in the intestine. These results indicate that inhibition of inflammatory responses in the in-

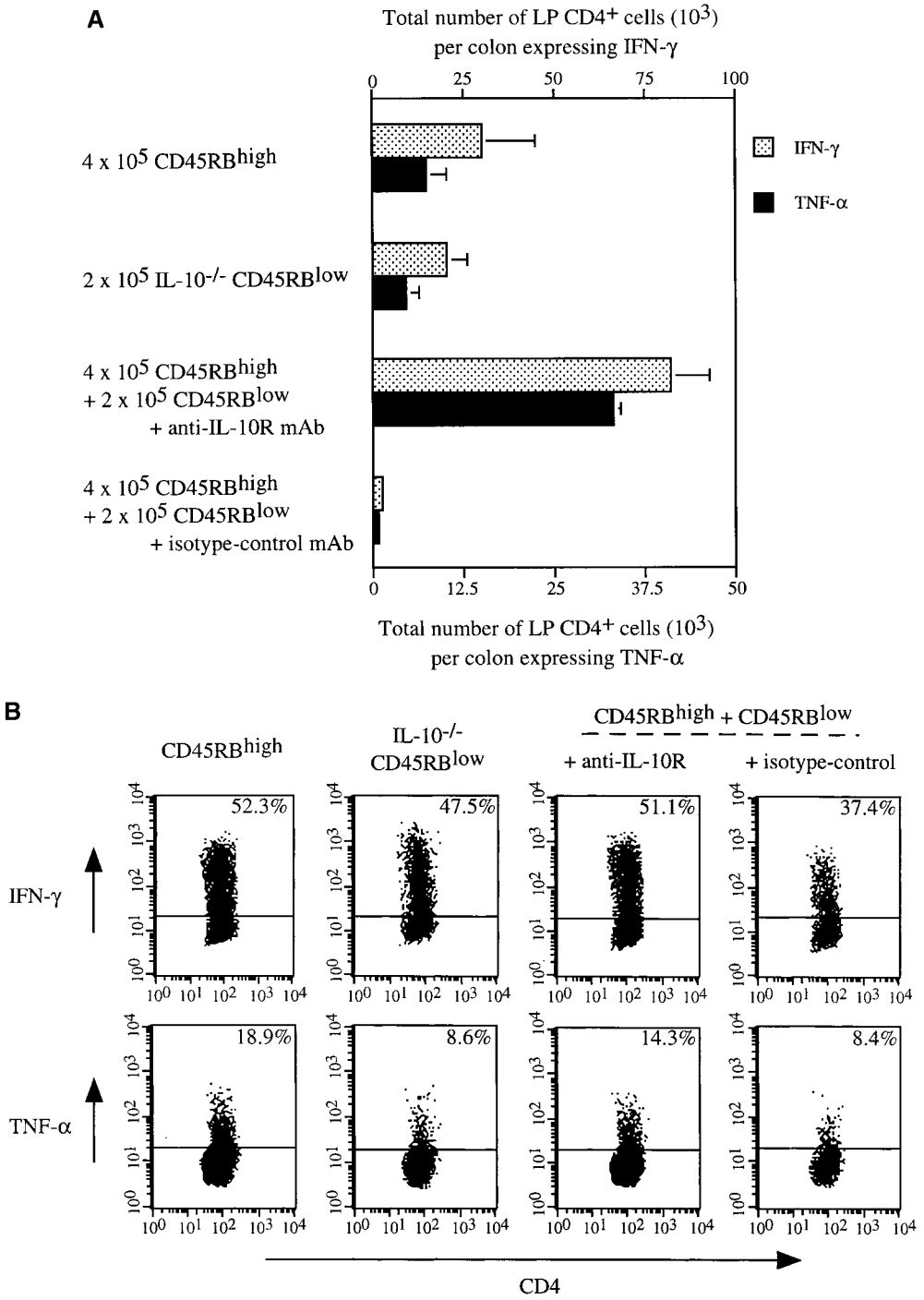

Figure 2. IFN-γ and TNF-α production by LP CD4+ T cells from colons. RAG-2$^{-/-}$ or C.B-17 SCID recipients were reconstituted with cell subsets as indicated. 8–12 wk after cell reconstitution, LP cells were isolated and stimulated for 12 h with anti-CD3ε antibody. Levels of cytokine expression were determined by cytofluorography. (A) Absolute number of CD4+ cytokine-positive cells per colon. Numbers were determined by multiplying the frequency of cytokine-secreting CD4+ T cells by the total number of CD4+ cells. Data represent the mean ± SEM of two to five animals per group. (B) Frequency of cytokine-secreting CD4+ T cells. Data are gated on CD4+ T cells and are representative examples for each group. Horizontal lines represent staining with isotype control mAb.

testine mediated by CD45RB^{low} CD4+ cells is not dependent on the differentiation of the progeny of CD45RB^{high} CD4+ cells into IL-10–secreting cells.

Discussion

Data presented herein provide direct evidence that IL-10 plays an obligate role in the function of regulatory T cells that control inflammatory responses in the intestine. In contrast to CD45RB^{low} CD4+ T cells from WT mice, which inhibit colitis induced in immune-deficient mice after transfer with CD45RB^{high} CD4+ T cells, the CD45RB^{low} CD4+ population from IL-10$^{-/-}$ mice failed to mediate this function. In addition, they induced severe colitis when transferred alone to immune-deficient recipients. Previous studies from this laboratory showed that TGF-β was essential for inhibition of colitis by CD45RB^{low} cells (8). These results, together with the find-

Table II. *Development of Disease Correlates with a Significant Increase in the Total Number of Leukocytes and CD4$^+$ Cells Recovered from the LP*

Phenotype of CD4$^+$ T cells used for reconstitution	mAb treatment	Total no. of LP leukocytes	Total no. of CD4$^+$ cells*
		×10^3 ± SEM	*×10^3 ± SEM*
4×10^5 WT CD45RB^{high}	–	136.0 ± 34.9	92.7 ± 10.2
2×10^5 IL-10$^{-/-}$ CD45RB^{low}	–	64.4 ± 29.7	43.9 ± 22.8
4×10^5 WT CD45RB^{high} + 2×10^5 WT CD45RB^{low}	anti–IL-10R	176.3 ± 104.5	133.3 ± 88.6
4×10^5 WT CD45RB^{high} + 2×10^5 CD45RB^{low}	Isotype control	7.9 ± 0.9	5.4 ± 0.5

RAG-2$^{-/-}$ or C.B-17 SCID recipients were reconstituted with cell subsets as indicated. 8–12 wk after cell reconstitution, cells from LP were isolated. Data represent the number ± SEM of two to five animals per group from two independent experiments.
*Total number of CD4$^+$ cells were determined by multiplying the number of LP leukocytes by the frequency of CD4$^+$ T cells. CD4$^+$ T cell frequencies were similar between groups ranging between 60.2 and 81.6%.

ings reported here, provide the first clear evidence that IL-10 and TGF-β play nonredundant roles in the functioning of regulatory T cells which control inflammatory responses towards intestinal antigens, as the neutralization or absence of one of these cytokines is sufficient to abrogate protection. Furthermore, IL-10 produced by regulatory T cells themselves is crucial for the normal functioning of these cells.

Colitis in the SCID model involves the development of Th1 cells responding primarily to intestinal flora, as transfer of CD45RB^{high} CD4$^+$ T cells to germ-free SCID mice failed to induce disease (20). The fact that the regulatory cells express the phenotype of antigen-experienced cells (CD45RB^{low}) would suggest that their generation in normal mice is antigen driven; however, whether these antigens are of bacterial or self origin is not known. Recent studies of the immune response elicited by *Helicobacter hepaticus* infection, a bacterium that colonizes the cecum, showed that normal mice mounted an IL-10–dependent response, whereas IL-10$^{-/-}$ mice developed a pathogenic Th1 response towards the bacterium (21). These studies support the hypothesis that in immunocompetent hosts, enteric antigens induce IL-10–secreting T cells that are immune suppressive and prevent inflammatory responses towards intestinal antigens. Mucosal T cell unresponsiveness to enteric antigens has similarly been shown in humans to be mediated by antigen-specific CD4$^+$ T cells and production of IL-10 and TGF-β (22).

Injection of rIL-10 inhibited the development of colitis in CD45RB^{high} CD4$^+$ T cell–restored SCID mice (5) and in IL-10$^{-/-}$ mice treated from weaning (10). However, the inhibitory effects of IL-10 treatment were only transitory, as colitis developed after the treatment was stopped, whereas the transfer of CD45RB^{low} CD4$^+$ T cells provided long-lasting protection. This may reflect the capacity of regulatory T cells to provide a constant source of IL-10 upon stimulation by endogenous antigen (bacteria or self). Alternatively, they may produce or induce other regulatory molecules, such as TGF-β, which are not induced by exogenous administration of IL-10.

The finding that CD45RB^{low} CD4$^+$ cells incapable of synthesizing IL-10 failed to inhibit colitis induced by transfer of CD45RB^{high} cells to SCID mice illustrates that the regulatory T cell population itself is the critical source of IL-10 in this model, despite the fact that both the CD45RB^{high} CD4$^+$ T cells and host cells in the SCID mice were capable of making IL-10. It is not clear whether the lack of regulatory T cells in IL-10$^{-/-}$ mice is a result of ab-

Table III. *Prevention of Colitis Is Independent of IL-10 Production by CD45RB^{high} CD4$^+$ T Cells*

Phenotype of CD4$^+$ T cells injected	No or minimal colitis (0–1)	Mild colitis (2)	Severe colitis (3–5)
4×10^5 IL-10$^{-/-}$ CD45RB^{high}	3/25 (12.0%)	5/25	17/25 (68.0%)
4×10^5 IL-10$^{-/-}$ CD45RB^{high} + 2×10^5 CD45RB^{low}	26/32 (81.2%)	2/32	4/32 (12.5%)*

RAG-2$^{-/-}$ mice were reconstituted with sorted CD4$^+$ T cell subsets. 10–12 wk after reconstitution, mice were killed and colonic pathology was graded. Data are pooled from four to six independent experiments.
*Statistically different ($P < 0.05$, Mann-Whitney test).

normalities in the development or effector function of this population. However, the finding that treatment with anti–IL-10R was able to neutralize the immune-suppressive function of differentiated regulatory cells in cotransfers of $CD45RB^{high}$ and $CD45RB^{low}$ cells from normal mice supports the idea that IL-10 is required for the effector function. The reasons for the striking difference in effectiveness between the anti–IL-10 mAb JES5-2A5 and the anti–IL-10R antibody 1B1.2 are not clear, as both are effective neutralizing antibodies in vitro. However, the relative ineffectiveness of JES5-2A5 has been observed in other experimental situations, such as *Leishmania*-infected mice (Mauze, S., and R.L. Coffman, unpublished observations).

Precisely how IL-10 mediates its immune-regulatory function is not known. Inhibition of colitis mediated by IL-10–secreting $CD45RB^{low}$ $CD4^+$ cells was characterized by substantial reductions in total number of Th1 cells recovered from the intestine. This difference was attributable to the reduced number of $CD4^+$ cells present in the intestine (8–24-fold; Table II), as analysis of cytokine production at the single cell level revealed a similar percentage of $CD4^+$ T cells capable of producing IFN-γ and TNF-α in diseased and nondiseased colons. This suggests that the major activity of IL-10–secreting regulatory T cells is to inhibit the accumulation of pathogenic Th1 cells in the intestine. Whether this is due to reduced expansion, or migration, of these cells is not known. IL-10 has been shown to mediate a range of antiinflammatory activities, including the inhibition of antigen-induced proliferation and cytokine secretion by T cells. Inhibition is thought to be mediated mainly by effects on APCs, particularly down-regulation of molecules involved in T cell costimulation (23). It seems likely that IL-10–secreting regulatory T cells act to inhibit Th1 cell activation, and that IL-10 produced locally in the intestine acts on macrophages to prevent their activation and elaboration of proinflammatory molecules and chemokines, thus inhibiting T cell recruitment into the intestine. Consistent with this, mice in which macrophages and neutrophils are unable to respond to IL-10 as a result of a cell type–specific deletion of Stat-3 developed enterocolitis (24), suggesting that IL-10–mediated macrophage and neutrophil deactivation contributes to the immune-suppressive properties of IL-10 in the intestine.

Recently, regulatory cells with activities similar to those within the $CD45RB^{low}$ $CD4^+$ population were cloned in vitro by culturing with IL-10 (18). These cells, termed T regulatory 1 (Tr-1) cells, are characterized by their ability to produce IL-10 but not IL-4 and to inhibit T cell activation in vitro and in vivo. Although immune suppression in vitro was shown to be dependent on IL-10 and TGF-β, the mechanism of action of Tr-1 cells in vivo has not been established. It is likely that regulatory T cells contained within the $CD45RB^{low}$ $CD4^+$ subset represent the in vivo counterpart of in vitro–derived Tr-1 cells. This is based on the fact that the function of the former population is dependent on IL-10 but not IL-4 synthesis, a defining feature of Tr-1 cells in vitro. In addition, a subset of $CD45RB^{low}$ $CD4^+$ T cells, identified by expression of CD38, was shown to be immune suppressive in vitro and to produce IL-10 but not IL-4 after stimulation with anti-CD3 and IL-2 (25). However, further comparison between the in vivo–derived regulatory cells described here and in vitro–derived Tr-1 cells awaits identification of cell surface markers specific for Tr-1 cells and elucidation of their mechanism of action in vivo.

The finding that IL-10 leads to the differentiation of Tr-1 cells in vitro raised the possibility that part of the mechanism by which IL-10–secreting regulatory T cells contained within the $CD45RB^{low}$ $CD4^+$ population inhibit colitis is to induce the differentiation of the progeny of $CD45RB^{high}$ $CD4^+$ cells into IL-10–secreting cells. In some systems, regulatory T cells have been shown to induce the differentiation of naive cells into cells with similar regulatory function, a phenomenon termed infectious tolerance (16, 17). However, differentiation of Tr-1 cells among the progeny of the $CD45RB^{high}$ population did not appear to be a crucial part of the immune-suppressive activities of the $CD45RB^{low}$ population, as these cells were able to inhibit colitis induced by $IL\text{-}10^{-/-}$ $CD45RB^{high}$ $CD4^+$ cells which could not differentiate into IL-10–secreting cells. However, this result does not rule out the possibility that IL-10 secretion by the $CD45RB^{low}$ population leads to the differentiation of the progeny of $CD45RB^{high}$ cells into regulatory T cells secreting other cytokines, for example TGF-β, and experiments are currently underway to test this hypothesis.

In summary, these studies identify IL-10 as an essential mediator of the function of regulatory T cells contained within the $CD45RB^{low}$ $CD4^+$ T cell population. Previous studies identified TGF-β as a critical component of the immune-regulatory function of these cells, and the finding that IL-10 has an identical effect is the first demonstration that both of these immune-suppressive cytokines play mandatory roles. How the functions of these two cytokines are linked is not known. The finding that $IL\text{-}10^{-/-}$ mice have immune pathology restricted to the intestine (9) whereas $TGF\text{-}\beta1^{-/-}$ develop multiple organ disease (26) suggests that IL-10 is not required for the production of TGF-β1. However, TGF-β has been shown to induce IL-10 secretion by APCs (27), making it a possibility that TGF-β alters antigen presentation in favor of the generation of IL-10–secreting regulatory T cells. Alternatively, IL-10 and TGF-β may act entirely separately, and further experiments are required to distinguish between these possibilities.

There is now good evidence that regulatory T cells can be induced after oral exposure to antigen and that their function is dependent on TGF-β (28, 29). In addition, regulatory T cells that inhibit the development of autoimmune disease have been shown to exist naturally in mice and rats (for a review, see reference 30). However, information regarding the role of IL-10 and TGF-β in the function of these cells is incomplete, as in most cases only one or the other has been examined. Thus, TGF-β–dependent mechanisms were shown to be involved in the regulation of autoimmune nephritis (31) and thyroiditis (32), whereas inhibition of diabetes by NK1.1 T cells was dependent on

IL-4 and IL-10 (33). It remains to be established whether regulatory T cells that control inflammatory responses in the gut are the same as those shown to regulate organ-specific autoimmune disease. However, more information on the relative roles of IL-10 and TGF-β in the function of these different populations of cells will facilitate their comparison. Differentiation of IL-10–secreting TGF-β–dependent regulatory T cells is one of the host's natural mechanisms for avoiding immune pathology. Further understanding of the antigen specificity, development, and mechanism of action of these cells is crucial for the design of effective immune interventions that seek to capitalize on this potent immune-regulatory mechanism.

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