

A Critical Role for Transforming Growth Factor- β but Not Interleukin 4 in the Suppression of T Helper Type 1-mediated Colitis by CD45RB^{low} CD4⁺ T Cells

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Summary

A T helper type 1 (Th1)-mediated colitis with similarities to inflammatory bowel disease in humans developed in severe combined immunodeficiency mice reconstituted with CD45RB^{high} CD4⁺ splenic T cells and could be prevented by cotransfer of CD45RB^{low} CD4⁺ T cells. Inhibition of this Th1 response by the CD45RB^{low} T cell population could be reversed in vivo by an anti-transforming growth factor (TGF) β antibody. Interleukin (IL) 4 was not required for either the differentiation or function of protective cells as CD45RB^{low} CD4⁺ cells from IL-4-deficient mice were fully effective. These results identify a subpopulation of peripheral CD4⁺ cells and TGF- β as critical components of the natural immune regulatory mechanism, which prevents the development of pathogenic Th1 responses in the gut, and suggests that this immunoregulatory population is distinct from Th2 cells.

Mucosal immune responses are highly regulated to allow activation of effector mechanisms that eliminate pathogenic microorganisms while specifically preventing cell-mediated inflammatory responses against dietary antigens and enteric flora. It has been suggested that a breakdown in this mucosal immune regulation is important in the development of idiopathic inflammatory bowel disease (IBD) in humans (1). Dissection of the regulatory pathways that act normally to prevent the development of pathogenic immune responses in the intestine may therefore be key to the understanding of the pathogenesis of human IBD.

We and others have described a model of colitis in mice that has proven useful for dissecting the mechanisms of this form of immune regulation. Colitis was induced in immunodeficient C.B-17 *scid* mice by transfer of the CD45RB^{high} subset of CD4⁺ T cells from normal C.B-17 (2) or congenic BALB/c (3, 4) donors. Progressive colon inflammation was caused by a dysregulated Th1 response as CD4⁺ T cells purified from the lamina propria of diseased mice produced very high levels of IFN- γ , and TNF and disease was prevented by systemic treatment with antibodies to IFN- γ or TNF or with recombinant IL-10 (4). Significantly, transfer of the reciprocal CD45RB^{low} population did not result in disease, and this population, when transferred together with CD45RB^{high}CD4⁺ T cells, completely inhibited disease development (3–5). These data identify a subpopulation of CD4⁺ T cells, present in normal mice, that are critical for the regulation of pathogenic Th1 responses in the gut.

It seems likely that the inhibition of colitis transferred by CD45RB^{low} cells is mediated via the production of immunosuppressive cytokines. Th2 cells are known to inhibit Th1 effector cell development and function, principally by the production of IL-4 and IL-10 (6–8). More recently, TGF- β has been shown to be a mediator of the induced suppression associated with oral tolerance to foreign antigens (9, 10). Such TGF- β -producing regulatory cells are often found together with Th2-like cells; however, it is not known whether these cells constitute part of the Th2 subset or represent a distinct subset of cells. Here, we use neutralizing anti-TGF- β mAbs and IL-4-deficient mice to examine the role of these cytokines in the suppression of Th1-mediated colitis by CD45RB^{low}CD4⁺ T cells and to examine the relationship of regulatory cells to Th2 cells.

Materials and Methods

Mice. Specific pathogen-free BALB/c and C.B-17 *scid* mice were obtained from Simonsen Laboratories (Gilroy, CA); 129/SvEv mice were obtained from Taconic Farms, Inc. (Germantown, NY). Mice were maintained in the Animal Care Facility of the DNAX Research Institute. 129SvEv RAG-2-deficient mice and 129/J IL4T and 129 SvEv IL-4T (IL-4-deficient) mice were bred and maintained in the Animal Care Facility of the DNAX Research Institute. C.B-17 *scid* mice and RAG-2-deficient mice were housed in microisolator cages with sterile filtered air. Female mice were used at 8–12 wk of age.

Antibodies. The following mAbs were used for cell purification: AMS-32.1, anti-I-A^d (PharMingen, San Diego, CA); 2-43,

anti-mouse CD8 (TIB210; American Type Culture Collection, Rockville, MD); M1/70, anti-mouse Mac-1 (TIB128; American Type Culture Collection); RA3-6B2, anti-mouse B220 (11); FITC-conjugated 16A, anti-mouse CD45RB (PharMingen); PE-conjugated anti-mouse CD4 (Becton Dickinson & Co., Mountain View, CA); and PE-conjugated isotype control mAb (PharMingen). The following mAbs were used in vivo: 1D11.16 (mouse IgG1), anti-TGF- β 1, 2, and 3 (12), and 1410KG7 (mouse IgG1) isotype control mAb (12). For in vivo use, mAbs were purified by column chromatography from tissue culture supernatants. The resulting antibodies were >98% pure and contained <3 EU endotoxin per milligram of protein.

Cell Purification and Flow Cytofluorography. CD4⁺ T cell subsets were purified from the spleens of mice as previously described (3). Briefly, cells were depleted of B220⁺, MAC-1⁺, I-A^{d+}, and CD8⁺ cells by negative selection using sheep anti-rat-coated Dynabeads (Robbins Scientific Corp., Sunnyvale, CA). The remaining cells were labeled with FITC-conjugated anti-CD45RB (25 μ g/ml) and PE-conjugated anti-CD4 and fractionated into CD4⁺CD45RB^{high} and CD4⁺CD45RB^{low} fractions by two-color sorting on a FACSTAR[®] PLUS (Becton Dickinson & Co.). All populations were >98% pure on reanalysis.

Reconstitution of C.B-17 scid Mice with T Cell Subpopulations. C.B-17 scid mice were injected intraperitoneally with 100 μ l of PBS containing sorted CD4⁺ T cell subpopulations or unseparated CD4⁺ T cells. Anticytokine or control mAbs were injected intraperitoneally in PBS.

Microscopic Examination of Colons. Colons were removed from mice 8–18 wk after T cell reconstitution and fixed in PBS containing 10% formaldehyde. 6- μ m paraffin-embedded sections were cut and stained with hematoxylin and eosin. Microscopic sections were graded semiquantitatively as 0 (no change) to 5 (most severe) in a blinded fashion by the same pathologist (M.W. Leach).

Nucleic Acid Preparation and Semiquantitative PCR of Cytokine mRNA. Quantitation of cytokine mRNA in the colon was performed as previously described (3). Briefly, RNA was prepared from 40–200-mg pieces of distal colon by a modification of the guanidium isothiocyanate method. 2–3 μ g of RNA from test samples was reverse transcribed using Superscriptase II RNase H⁻reverse transcriptase (GIBCO BRL, Gaithersburg, MD). IFN- γ , TNF- α , and hypoxanthine phosphoribosyl transferase (HPRT) cDNAs were amplified by PCR and detected by dot blot hybridization and phosphoimaging. Relative quantitation of samples was performed using quantitation of the housekeeping enzyme HPRT as an internal standard. HPRT levels in the samples were measured first, and samples with equivalent amounts of HPRT were then analyzed for cytokine cDNA. For each cytokine, the standard Th cDNA was run and specific cytokine levels were determined (IFN- γ 156 – 1.2 pg, TNF- α 10 – 0.019 ng). Units of specific cytokine were determined for each sample by comparison with a standard curve constructed with cDNA from pooled RNA of an activated Th1 and Th2 clone. 1 U of cytokine was defined as the amount in 1 ng Th standard cDNA, and values were expressed as units of cytokine/10⁵ U HPRT.

Results

Suppression of Colitis by CD45RB^{low} CD4⁺ T Cells Is Dependent on TGF- β . In previous studies, mice restored with CD45RB^{high} and CD45RB^{low}CD4⁺ T cells remained protected from colitis when treated with anti-IL-4, anti-IL-10,

or both mAbs (5), suggesting that suppression of colitis was mediated by other immunosuppressive cytokines. To test the role of TGF- β , mice reconstituted with CD45RB^{high} and CD45RB^{low}CD4⁺ cells were treated with a mouse mAb that neutralized TGF- β 1, 2, and 3 (12). In the initial experiments (Table 1, protocol 1), mice were treated with 2 mg anti-TGF- β at the time of reconstitution followed by 1 mg weekly for 6 wk and then killed for histological examination at 7 wk after transfer. Neutralization of TGF- β partially, but significantly, diminished the ability of CD45RB^{low} T cells to protect from colitis. The average colitis score in these mice was 2, and 6 of 11 mice developed severe colitis compared with an average colitis score of 0.3 and zero incidence of severe colitis in isotype control-treated mice (Table 1, protocol 1). Anti-TGF- β -treated mice that developed severe colitis also developed a wasting disease, indistinguishable from mice restored with CD45RB^{high}CD4⁺ T cells alone (Table 1). Anti-TGF- β treatment of unreconstituted scid mice (Table 1) or scid mice reconstituted only with CD45RB^{low}CD4⁺ T cells alone (data not shown) did not induce colitis or wasting, demonstrating that the antibody did not cause disease in the absence of the CD45RB^{high} population.

To address the possibility that insufficient antibody was being administered to inhibit TGF- β activity completely, the weekly dose of anti-TGF- β was doubled to 2 mg/wk in subsequent experiments (Table 1, protocol 2). At this dose, 100% (7/7) of mice given a mixture of CD45RB^{high} and CD45RB^{low}CD4⁺ T cells developed severe colitis and wasting disease, a frequency indistinguishable from mice given CD45RB^{high}CD4⁺ T cells alone. The colitis in the two groups of mice was histologically identical (Fig. 1, A and B), being characterized by a prominent lymphocytic infiltrate, extensive epithelial cell hyperplasia, and goblet cell depletion. Mice given the mixture of CD45RB^{high} and CD45RB^{low} cells and treated with isotype control mAb were completely protected from these pathological changes (Fig. 1 C).

Colitis Induced in Recipients of CD45RB^{high} and CD45RB^{low} CD4⁺ T Cells Treated with Anti-TGF- β Is Identical to Colitis Induced by CD45RB^{high} Cells Alone. Colitis induced by the CD45RB^{high}CD4⁺ population has been shown to be a Th1 response mediated by IFN- γ and TNF (4). To determine whether the disease that developed in anti-TGF- β -treated mice restored with both CD45RB^{high} and CD45RB^{low} cells had a similar pathogenesis, colons were isolated from these mice 7 wk after T cell reconstitution and tested by reverse transcriptase-PCR analysis for the expression of IFN- γ and TNF- α mRNA (Fig. 2). Mice restored with CD45RB^{high} cells and mice restored with CD45RB^{high} and CD45RB^{low} cells and treated with anti-TGF- β had 7–8- and 6–10-fold higher levels, respectively, of TNF- α and IFN- γ mRNA than mice protected from colitis by cotransfer of both T cell subsets. The extensive similarities in kinetics, histology, and cytokine mRNA expression between the diseases caused by CD45RB^{high} T cells without the regulatory population, and CD45RB^{high} T cells plus the regulatory population and anti-TGF- β demonstrate that TGF- β is

Table 1. Protection from Colitis Transferred by CD45RB^{low}CD4⁺ T Cells Is Dependent on TGF- β

Phenotype of CD4 ⁺ T cell inoculum	Protocol 1			Protocol 2		
	Colitis score*	Number of mice with severe colitis [‡]	Weight 6w/0w % [§]	Colitis score*	Number of mice with severe colitis [‡]	Weight 6w/0w % [§]
4 × 10 ⁵ CD45RB ^{high}	‡3.3 ± 0.3	8/10	†89.7 ± 2	‡2.9 ± 0.1	9/10	‡89.9 ± 2
4 × 10 ⁵ CD45RB ^{high} + 2 × 10 ⁵ CD45RB ^{low} + anti-TGF- β mAb	‡2.0 ± 0.4	6/11	‡85.7 ± 4	‡3.1 ± 0.1	7/7	‡87.9 ± 1
4 × 10 ⁵ CD45RB ^{high} + 2 × 10 ⁵ CD45RB ^{low} + isotype control mAb	0.3 ± 0.2	0/11	110.8 ± 3	**0 ± 0	0/7	104.2 ± 4
Unreconstituted SCID + anti-TGF- β mAb	0 ± 0	0/5	108.3 ± 3	ND	ND	ND

C.B-17 *scid* mice were injected intraperitoneally with sorted CD4⁺ T cell subsets; some mice were treated with anti-TGF- β or isotype control mAb intraperitoneally as indicated. Protocol 1 contains pooled data from two independent experiments in which antibody treated mice were given 2 mg mAb at the time of T cell reconstitution and 1 mg weekly up to 6 wk. Protocol 2 contains data from one experiment in which antibody-treated mice were given 2 mg mAb weekly up to 6 wk.

*7 wk after reconstitution, mice were killed and colon pathology was graded. Data represent the mean colitis score plus SE.

‡Severe colitis means grade 3 and above.

§Weight at 6 wk as a percentage of starting weight. Data represent the mean with SE for the group.

‖Statistically significantly different ($P < 0.05$, Wilcoxin rank sum test) to mice restored with 4 × 10⁵ CD45RB^{high} + 2 × 10⁵ CD45RB^{low} + isotype control mAb.

†Weight loss of mice in group with severe colitis.

**Mice in this group were not treated with isotype control mAb.

a key mediator of the regulatory activity of CD45RB^{low} T cells, rather than a direct inducer of an unrelated colon inflammation.

Differentiation and Effector Function of the Regulatory CD45RB^{low} Population Is IL-4 Independent. Inhibition of

colitis by CD45RB^{low} CD4⁺ T cells was not blocked by administration of anti-IL-4 mAbs (5), suggesting that IL-4 production was not necessary for the TGF- β -dependent regulatory mechanism. However, these experiments did not establish whether IL-4 was necessary for the develop-

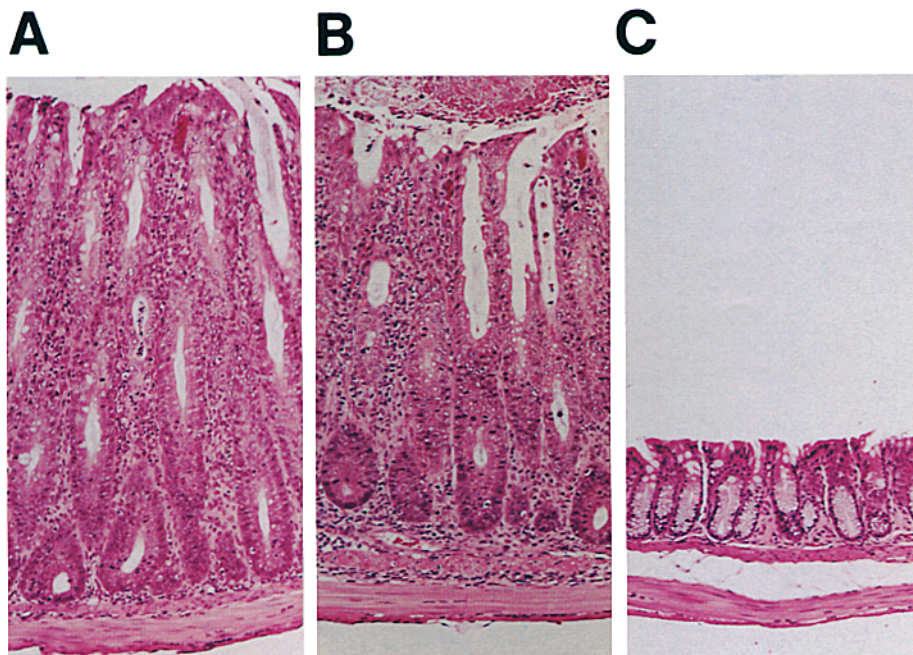


Figure 1. Treatment with anti-TGF- β mAb abrogates protection from colitis transferred by CD45RB^{low}CD4⁺ T cells. Severe colitis in C.B-17 *scid* mice restored with (A) 4 × 10⁵ CD45RB^{high}-CD4⁺ cells or (B) 4 × 10⁵ CD45RB^{high} plus 2 × 10⁵ CD45RB^{low}CD4⁺ cells, treated weekly with 2 mg anti-TGF- β mAb as described in Table 1. (C) Protection from colitis in mice restored with 4 × 10⁵ CD45RB^{high} plus 2 × 10⁵ CD45RB^{low}CD4⁺ cells and treated weekly with isotype control mAb. Colon sections were stained with hematoxylin and eosin and photographed. ×90.

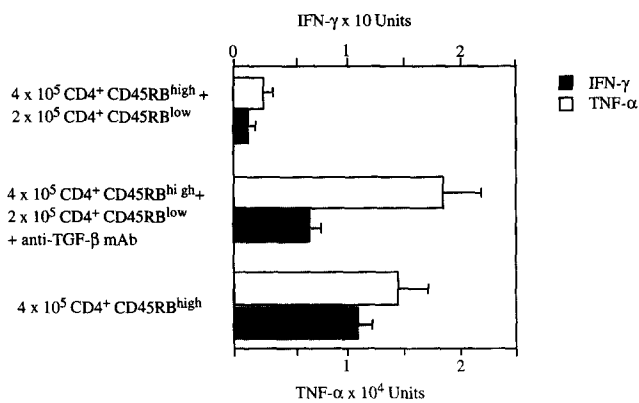


Figure 2. Elevations in Th1 cytokine mRNA in the colon of mice with severe colitis. 7 wk after T cell reconstitution *scid* mice were killed, and the levels of cytokine mRNA in the colons were determined by semiquantitative reverse transcriptase-PCR. Values are expressed as units of cytokine per 10^5 U HPRT. Data represent the mean plus SEM of three animals per group. A repeat experiment gave a similar result.

ment of the regulatory population of cells themselves. The relationship, if any, between Th2 cells and TGF- β -producing regulatory cells is not yet clear, although several lines of evidence suggest a common origin. T cell clones from orally tolerized mice include many that secrete TGF- β and the Th2 cytokines IL-4 and IL-10 (10), and some human Th2-like clones change to TGF- β -producing cells when stimulated with altered peptide ligands (13). IL-4 is the only known inducer of Th2 differentiation in mice (14, 15), and a requirement for IL-4 in the development of regulatory CD45RB^{low} cells would provide additional support for a relationship between the two populations. To address this, CD45RB^{low} cells from IL-4-deficient and wild-type (WT) mice were compared for their ability to inhibit colitis induced by WT CD45RB^{high} cells. As IL-4-deficient mice were available on the 129 but not the BALB/c background, RAG-2-deficient mice on the 129 background were used as immunodeficient recipients. The great majority of RAG-2-deficient mice restored with CD45RB^{high} CD4⁺ T cells from syngeneic donors developed colitis very similar to that observed in C.B-17 *scid* mice (Table 2). CD45RB^{low}CD4⁺ T cells were present in both IL-4-deficient and WT mice (data not shown), and the populations from both mice were equally able to inhibit colitis and wasting disease. The average grade of colitis in the group reconstituted with IL-4-deficient cells was 0.8, and only 1 of 21 mice developed severe colitis (Table 2). In one experiment, neutralizing doses of the anti-IL-4 mAb 11B11 were administered weekly to mice reconstituted with CD45RB^{high} cells from WT donors and CD45RB^{low} cells from IL-4-deficient mice to block any possible IL-4 production from sources other than the donor CD45RB^{low} population. These mice were still significantly protected from severe colitis (data not shown), demonstrating unambiguously that the regulatory population of CD45RB^{low} cells is present and mediates its immunoregulatory function in IL-4-deficient mice.

Table 2. Protection from Colitis Transferred by CD45RB^{low} CD4⁺ T Cells is IL-4 Independent

Phenotype of CD4 ⁺ T cell inoculum	Colitis score*	Number of mice with severe colitis [‡]	Weight 9W/0W% [§]
4×10^5 CD45RB ^{high}	3.1 ± 0.35	13/17	84.3 ± 2.5
4×10^5 CD45RB ^{high} + 2×10^5 CD45RB ^{low}	0.8 ± 0.3	4/19	104.7 ± 5.3
4×10^5 CD45RB ^{high} + 2×10^5 CD45RB ^{low} IL-4 deficient	0.3 ± 0.2	1/21	107.7 ± 2.7
2×10^5 CD45RB ^{low} IL-4 deficient	0 ± 0	0/5	ND

CD4⁺ T cell subsets were isolated from the spleen of 129SvEv or IL-4-deficient 129/J or 129SvEv mice and injected intraperitoneally into 129SvEv RAG-2-deficient mice. Data represent three independent experiments; in two of these, 129/J IL-4-deficient mice were used as a source of CD45RB^{low} cells, and in a third experiment, 129SvEv IL-4-deficient mice were used. Results using these two strains were similar therefore data were pooled.

*20 wk after reconstitution, mice were killed and colon pathology was graded. Data represent the mean colitis score plus SE.

[‡]Severe colitis means grade 3 and above.

[§]Weight at 9 wk as a percentage of starting weight. Data represent the mean with SE for the group.

^{||}Statistically significantly different ($P < 0.05$, Wilcoxin rank sum test) to mice restored with 4×10^5 CD45RB^{high} + 2×10^5 CD45RB^{low} cells from WT mice.

Discussion

The experiments presented here identify a subpopulation of CD4⁺ T cells that is present in normal mice and is essential for the prevention of Th1-mediated inflammation in the intestine. Inhibition of colitis by CD45RB^{low} cells was completely ablated by an anti-TGF- β mAb, providing direct evidence that TGF- β is an important mediator of the natural immune regulatory mechanism involved in control of intestinal immune responses. Immunoregulatory subpopulations of CD4⁺ T cells that have the ability to inhibit the development of spontaneous organ-specific autoimmune diseases, including thyroiditis, gastritis, oophritis, and diabetes, have been identified in normal mice and rats; however, their mechanism of action has not been determined (16–18). It is possible that TGF- β plays a similar role in this active mechanism of peripheral tolerance, as has been established here for the regulation of mucosal immune responses.

The suppression of Th1 responses has been shown, in some instances, to be a property of Th2 cells, in part by production of IL-4 (5, 19, 20). In this model of colitis, however, the protection could not be blocked by an anti-IL-4 mAb (5), and CD45RB^{low} cells isolated from IL-4-

deficient mice were able to protect from colitis as well as the same population isolated from WT mice. Taken together, these data indicate that the TGF- β -dependent regulatory population both develops and functions normally in the absence of IL-4. Recently, similar forms of colitis have been shown to develop in TCR- α chain-deficient mice (21), IL-2-deficient mice (22), and IL-10-deficient mice (23), among others, providing support for the idea that intestinal immune responses are regulated normally by a subpopulation of regulatory T cells (24). Whether these other models are deficient in the TGF- β -dependent regulation described here remains to be established.

There is now a large body of data correlating the induction of TGF- β with a subsequent state of antigen-specific systemic hyporesponsiveness induced by oral administration of antigen (25). CD4⁺ T cell clones (10) and CD8⁺ populations (9) derived from mice fed myelin basic protein were able to inhibit the induction of active experimental allergic encephalomyelitis, a Th1-mediated autoimmune disease, by a mechanism that involved TGF- β . Many of the regulatory CD4⁺ T cell clones also secreted IL-4 and IL-10; however, the roles of these cytokines in the regulatory activity of the clones were not tested. The present findings identify TGF- β as a key inhibitor of pathogenic Th1 responses in a very different disease model and show the importance of this mode of regulation in normal mucosal unresponsiveness *in vivo*.

TGF- β 1-deficient mice developed a severe multiple organ inflammatory disease that was lethal by 3–5 wk of age, with the most severe lesions being present in the heart and lungs (26, 27). The cellular infiltration in affected organs was associated with increases in the inflammatory cytokines

IFN- γ and TNF- α (26), suggesting that TGF- β 1 is important for the prevention of dysregulated inflammatory responses in many organs. Interestingly, inflammation in the intestine of the TGF- β 1-deficient mice was usually mild, although gastric inflammation was severe on some genetic backgrounds. These observations appear to be at odds with the findings from the *scid* transfer experiments, in which an absence of CD45RB^{low}, TGF- β -dependent regulatory cells led to the development of severe inflammation restricted primarily to the colon. Mild inflammation was seen in the lung and stomach of some *scid* mice restored with CD45RB^{high}CD4⁺ T cells (3). This difference may be related to (a) a strain dependency in the susceptibility of mice to develop colitis, or (b) the presence of TGF- β 2 and TGF- β 3 in TGF- β 1-deficient mice, isoforms that would be inhibited in reconstituted *scid* mice treated with the mAb that neutralizes all three isoforms of TGF- β . TGF- β 1, 2, and 3 have all been shown to be present in murine small and large intestine (28), and it may be that TGF- β 2 and TGF- β 3 play a protective role in the intestine of TGF- β 1-deficient mice.

In summary, the finding that suppression of colitis by CD45RB^{low} cells was dependent on TGF- β but independent of IL-4 has provided valuable information on the mechanism of action of these natural regulatory cells. A thorough understanding of the development, effector function, and antigen specificity of these cells will provide a backbone of information with which to devise immune interventions favoring the development of TGF- β -dependent regulatory cells rather than pathogenic Th1 responses. Such immune therapy may be useful for treatment of a number of autoimmune diseases, including IBD.

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