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# T Cell-Produced Transforming Growth Factor-β1 Controls T Cell Tolerance and Regulates Th1- and Th17-Cell Differentiation

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## SUMMARY

**TGF-β1 is a regulatory cytokine with a pleiotropic role in immune responses. TGF-β1 is widely expressed in leukocytes and stromal cells. However, the functions of TGF-β1 expressed by specific lineages of cells remain unknown in vivo. Here, we show that mice with a T cell-specific deletion of the *Tgfb1* gene developed lethal immunopathology in multiple organs, and this development was associated with enhanced T cell proliferation, activation, and CD4$^+$ T cell differentiation into T helper 1 (Th1) and Th2 cells. TGF-β1 produced by Foxp3-expressing regulatory T cells was required to inhibit Th1-cell differentiation and inflammatory-bowel disease in a transfer model. In addition, T cell-produced TGF-β1 promoted Th17-cell differentiation and was indispensable for the induction of experimental autoimmune encephalomyelitis. These findings reveal essential roles for T cell-produced TGF-β1 in controlling differentiation of T helper cells and controlling inflammatory diseases.**

## INTRODUCTION

Transforming growth factor-β1 (TGF-β1) is a regulatory cytokine with an essential role in immune responses (Li et al., 2006b). TGF-β1 is produced by virtually all cell types and mediates pleiotropic functions on leukocytes. The utmost importance of TGF-β1 in the immune system is underscored by the finding that TGF-β1-deficient mice developed a multifocal inflammatory disease that led to their early demise at approximately 3–4 weeks of age (Kulkarni et al., 1993; Shull et al., 1992).

Studies with cell-type-specific targeting strategies have revealed that T cells are a key target of TGF-β1 in vivo. Expression of a dominant-negative form of TGF-β receptor II (TGF-βRII) from the *Cd4* promoter (CD4-DNRII) in mice attenuated TGF-β signaling in T cells (Gorelik and Flavell, 2000). These mice developed an inflammatory phenotype associated with T cell activation and differentiation (Gorelik and Flavell, 2000). In another report, expression of DNRII from the *Cd2* promoter leads to a CD8$^+$ T cell lymphoproliferative disorder (Lucas et al., 2000). These transgenic mice are likely to retain some TGF-β signaling in T cells because T cell-specific deletion of TGF-βRII results in an autoimmune phenotype as severe as that of TGF-β1-deficient mice (Li et al., 2006a; Marie et al., 2006). These recent studies have also revealed pleiotropic functions for TGF-β signaling in T cell development and tolerance. Thus, TGF-β promotes CD8$^+$ T cell and natural-killer T cell development and inhibits the proliferation of Foxp3-expressing thymic regulatory T (Treg) cells. In peripheral tissues, TGF-β inhibits T cell proliferation, T cell activation, and effector T-cell differentiation and maintains Treg cells (Li et al., 2006a; Marie et al., 2006). This dual effect of TGF-β on effector T cells and Treg cells is likely to contribute to its regulation of peripheral T cell tolerance.

Recent studies have also uncovered an important function for TGF-β in the differentiation of IL-17-producing helper T cells called Th17 cells (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006a). In vitro antigen stimulation of T cells in the presence of TGF-β induces Foxp3 expression and Treg-cell differentiation (Chen et al., 2003; Fantini et al., 2004). The addition of IL-6 reprograms T cells to differentiate into Th17 cells (Bettelli et al., 2006; Veldhoen et al., 2006a). Although the precise in vivo functions of Th17 cells remain to be fully explored, Th17 cells drive autoimmune diseases including experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (Cua et al., 2003; Langrish et al., 2005; Murphy et al., 2003). In support of an important role for TGF-β in Th17-cell differentiation, CD4-DNRII mice are resistant to the induction of EAE (Veldhoen et al., 2006b).

TGF-β1 is produced by multiple lineages of leukocytes and stromal cells (Li et al., 2006b). However, the essential sources of TGF-β1 and the cellular mechanisms whereby T cells are regulated are unknown. Endocrine expression of active TGF-β1 in TGF-β1-deficient mice failed to ameliorate the lethal phenotype (Longenecker et al., 2002). Local but not systemic administration of neutralizing TGF-β1 antibody inhibits Th17-cell differentiation and the induction of EAE (Veldhoen et al., 2006b). These

studies suggest that TGF-β regulation of T cell tolerance and Th17-cell differentiation might be mediated by autocrine, paracrine, or both sources of TGF-β1.

Early studies showed that activated T cells produce TGF-β1 (Kehrl et al., 1986). TGF-β1 produced by Treg cells has also been suggested to be a mechanism for Treg-cell-mediated immune suppression, although conflicting results have been reported. A fraction of Treg cells express TGF-β1 on their surface, and this has been suggested to mediate their suppressor function in vitro (Green et al., 2003; Nakamura et al., 2001). However, TGF-β1-deficient Treg cells inhibit proliferation of responder T cells in vitro (Piccirillo et al., 2002), indicating that TGF-β1 synthesis by Treg cells is not required for cell-contact-dependent suppression in vitro. The protective activity of Treg cells against inflammatory-bowel disease induced by transfer of $CD4^+CD45Rb^{hi}$ T cells into severe combined immune deficiency (SCID) mice is reversed by the treatment with TGF-β antibody (Powrie et al., 1996). However, in an autoimmune gastritis model, Treg-cell-mediated protection is not alleviated by the same treatment (Piccirillo et al., 2002). Even in the same colitis model, TGF-β1-deficient Treg cells were shown to be protective or nonfunctional depending on experimental systems (Fahlen et al., 2005; Kullberg et al., 2005; Nakamura et al., 2004). One complication of these studies is that TGF-β1-deficient mice develop an early inflammatory disease, and activated T cells may contaminate the isolated $CD4^+CD25^+$ Treg-cell population. Therefore, the function of Treg-cell-produced TGF-β1 remains to be established.

To investigate the cell-type-specific function of TGF-β1, we generated mice in which the *Tgfb1* gene can be inactivated by using the Cre-loxP system (Rajewsky et al., 1996). In this report, we show that T cell-produced TGF-β1 inhibited thymic Treg-cell expansion and peripheral T cell proliferation, activation, and differentiation; in the absence of this inhibition, mice developed a lethal inflammatory disorder. In addition, Treg-cell-produced TGF-β1 was required for the inhibition of Th1-cell differentiation and colitis. To our surprise, T cell-produced TGF-β1 was essential for the differentiation of Th17 cells and the induction of EAE.

## RESULTS

### Generation of Mice with T Cell-Specific Deletion of *Tgfb1* Gene

To investigate the cell-type-specific function of TGF-β1 in vivo, we generated mice with a mutated *Tgfb1* allele by the insertion of two *loxP* sites flanking its promoter region and the first exon (Figure 1A). *Tgfb1* exon 1 encodes the leader peptide that directs secretion of TGF-β1 protein. *loxP* sites were introduced into the *Tgfb1* locus by homologous recombination in mouse embryonic stem (ES) cells (Figure 1B, left). The neomycin resistance gene (*Neo*) was removed from homologous recombinants by transient transfection with a Cre expression plasmid (Figure 1B, right). Clones carrying the *Tgfb1* locus with exon 1 flanked by *loxP* sites (floxed allele, f) were used for generating chimeric mice that produced heterozygous mice after germline transmission. At the time that we generated these mice, there was no known gene in close proximity to *Tgfb1* promoter region. However, a hypothetical gene (LOC232987) was later predicted in this region, the exon 4 of which resided 340 base pair upstream of the transcriptional start site of *Tgfb1* gene (Figure 1A). Therefore, in addition to *Tgfb1* exon 1, LOC232987 exon 4 was also encompassed by the *loxP* sites (Figure 1A).

Mice with two floxed alleles (f/f) developed normally, and did not show any sign of disease. To study the function of T cell-produced TGF-β1, we initially crossed f/f mice with CD4-Cre transgenic mice (Lee et al., 2001), in which Cre is specifically expressed in T cells. Recombination of the *Tgfb1* locus (deleted allele, d) was detected in the thymus of the $CD4\text{-}Cre^+$f/f (4cre-f/f) mice (Figure 1C), which resulted in more than 90% deletion of the *loxP*-flanked region in peripheral $CD4^+$ and $CD8^+$ T cells but not in B cells, dendritic cells, or macrophages (Figure 1C). Activated 4cre-f/f $CD4^+$ T cells did not produce detectable amounts of TGF-β1 protein compared to control f/f $CD4^+$ T cells (Figure 1D), demonstrating efficient ablation of TGF-β1 expression in these cells. The hypothetical protein LOC232987 was expressed in T cells (Figure 1F, left lane). As predicted, its exon 4 was also deleted in 4cre-f/f $CD4^+$ T cells (Figure 1F, middle lane).

To eliminate the influence of lack of LOC232987 exon 4 in T cells, we used a strain of mice with an insertion of the coding region of green-fluoresent protein (GFP) to the first exon of the *Tgfb1* gene (Figure 1E). Because the stop codon of the GFP open-reading frame precedes the start codon of the *Tgfb1* gene, the GFP knockin allele was predicted to generate a *Tgfb1* null allele (n). This was confirmed by the generation of mice homozygous for the GFP knockin allele. These mice developed an early lethal multifocal inflammatory disorder (Figure S1A in the Supplemental Data available online), which was associated with T cell activation (Figure S1B). These phenotypes were similar to those of the originally reported TGF-β1-deficient mice (Kulkarni et al., 1993; Shull et al., 1992). We crossed n/+ mice with 4cre-f/+ mice to generate 4cre-f/n mice. Because the LOC232987 locus is intact in the *Tgfb1* null allele, 4cre-f/n $CD4^+$ T cells expressed LOC232987 (Figure 1F, right lane). Importantly, $CD4^+$ T cells isolated from 4cre-f/n mice (both Foxp3-positive Treg cells and Foxp3-negative non-Treg cells) did not produce detectable amounts of TGF-β1 protein compared to that of control +/n T cells (Figure 1G). These observations reveal that *Tgfb1* gene was specifically abrogated in T cells from 4cre-f/n mice.

### Development of Immunopathology in the Absence of T Cell-Produced TGF-β1

4cre-f/n mice appeared to be healthy until 4 months of age, when they started to display signs of wasting and diarrhea that led to their early lethality starting at 6 months of age. Upon histological examination, we observed heavy mononuclear cell infiltration in mucosal lamina propria and the subglandular area of the colons in all 4cre-f/n

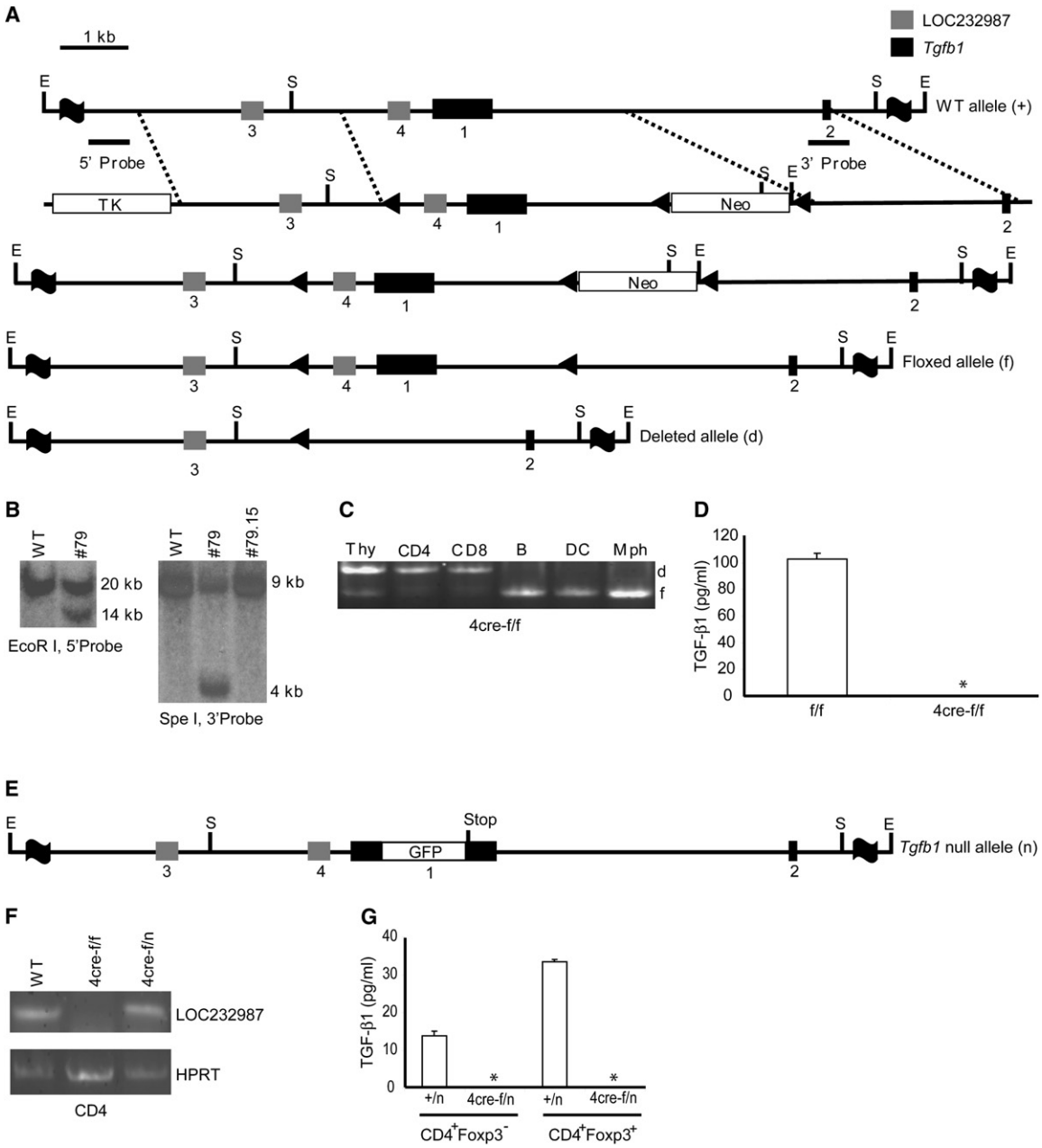


**Figure 1. Generation of Mice with T Cell-Specific Deletion of the *Tgfb1* Gene**

(A) Schematic presentation of the wild-type allele (WT, +), the mutated alleles after homologous recombination, and Cre-mediated deletion (Floxed allele, f; deleted allele, d). The locations of the probes for Southern blotting and restriction-enzyme sites (E, EcoRI; S, SpeI) are indicated. Filled boxes represent exons. loxP sites are depicted as arrowheads.

(B) Southern-blot analysis of an embryonic stem cell clone (#79) positive for recombination (left) and a subclone (#79.15) positive for the selective removal of the *neo* gene (right).

(C) PCR analysis of purified DNA from thymocytes, $CD4^+$ T cells, $CD8^+$ T cells, B cells (B), dendritic cells (DC), and macrophages (Mph) of a 4cre-f/f mouse. The floxed allele (f) and the deleted allele (d) are indicated.

(D) f/f and 4cre-f/f $CD4^+$ T cells were stimulated with CD3 and CD28 antibodies in the presence of IL-2 for 3 days and were restimulated with CD3 antibody for 24 hr. TGF-β1 amounts in culture supernatant were determined by ELISA. The asterisk indicates what is undetectable by assays.

(E) Schematic presentation of a *Tgfb1* null allele (n) with the knockin of GFP open-reading frame to the first exon of *Tgfb1* gene. The stop codon of the GFP gene is depicted.

(F) Wild-type (WT), 4cre-f/f, and 4cre-f/n $CD4^+$ T cells were purified. Expression of LOC232987 and HPRT in these cells was analyzed by RT-PCR.

(G) +/n and 4cre-f/n $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ T cells were stimulated with CD3 and CD28 antibodies in the presence of IL-2 for 24 hr. TGF-β1 amounts in culture supernatant were determined by ELISA. Asterisks indicate what are undetectable by assays.

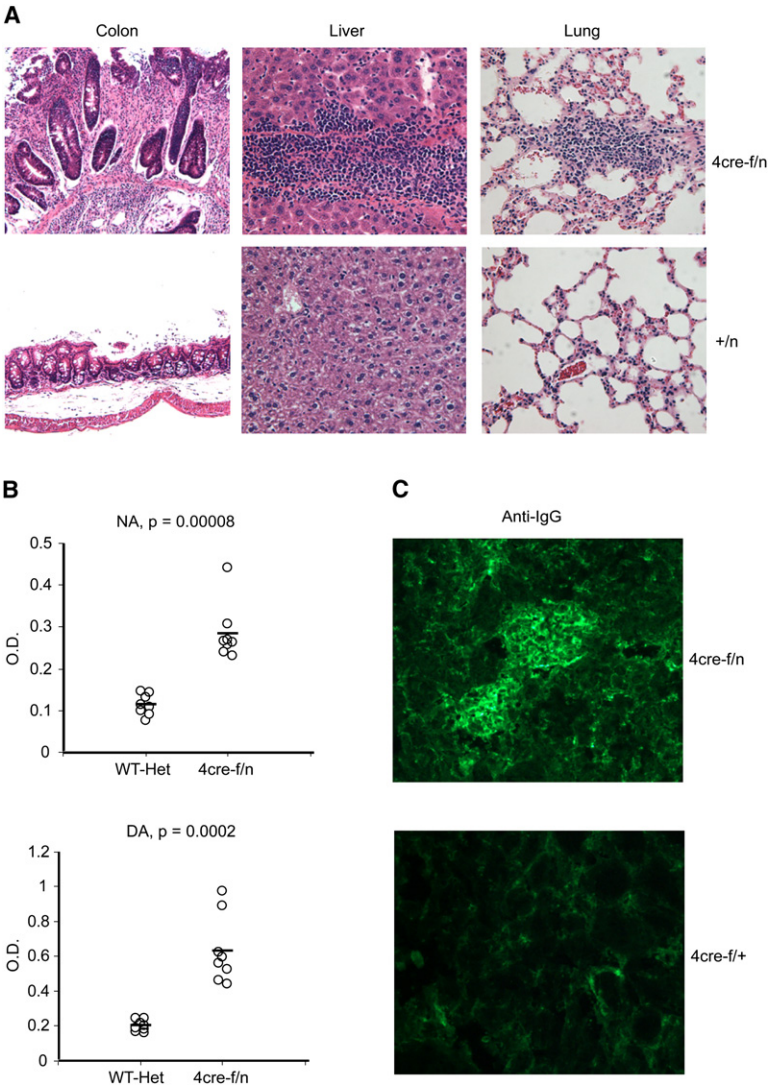


**Figure 2. Development of Immunopathology in the Absence of T Cell-Produced TGF-β1**

(A) Hematoxylin and eosin staining of colon, liver, and lung sections (200×) of 4cre-f/n and +/n mice at 6 months old. These are representative results of eight mice per group analyzed.

(B) Nuclear antibody (NA) and dsDNA antibody (DA) titers in the sera of 4cre-f/n mice compared to those of control +/n, 4cre-f/+, f/n, or f/f (WT-Het) mice aged 4–7 months (n = 8). The p values of antibody titers between the two groups of mice are shown.

(C) Frozen kidney sections of 4cre-f/n or 4cre-f/+ mice were stained with Alexa-Fluor-488-labeled goat anti-mouse IgG antibody. Five mice per group were analyzed. A representative picture is shown (200×).

mice aged 4–12 months (Figure 2A, left panel). Severe colitis was associated with the disruption of crypt architecture, formation of crypt abscesses, and compensatory epithelium hyperplasia (Figure 2A and data not shown). In the livers of these mice, clusters of mononuclear cells were detected in parenchema (Figure 2A, middle panel). Mononuclear cell infiltration was also evident in the lungs of these mice (Figure 2A, right panel). A similar inflammatory disorder was observed in 4cre-f/f mice (data not shown). However, none of these changes was detected in control +/n, 4cre-f/+, f/n, or f/f mice (Figure 2A and data not shown).

TGF-β1 complete knockout mice lose self tolerance and produce autoreactive antibodies (Dang et al., 1995; Yaswen et al., 1996). To investigate whether T cell-produced TGF-β1 is required for maintaining self tolerance, the amounts of autoantibodies in the sera of these mice were measured. Importantly, increased titers of both nuclear and dsDNA antibodies were detected in the sera of 4cre-f/n mice (Figure 2B). Because the presence of autoimmune antibodies frequently leads to formation of immune complexes and their deposition in kidney glomeruli, we tested whether this process occurred in 4cre-f/n mice. Specific IgG deposits were detected in the kidney sections of 4cre-f/n mice but not those of 4cre-f/+ mice (Figure 2C), suggesting pathogenic functions of the autoreactive antibodies. These observations demonstrate that T cell-produced TGF-β1 is essential for the inhibition of immunopathology in multiple organs.

## T Cell Development and Homeostasis in the Absence of T Cell-Produced TGF-β1

The immunopathology developed in 4cre-f/n mice resembled that of CD4-DNR mice, which had attenuated TGF-β signaling in T cells (Gorelik and Flavell, 2000), suggesting that T cell-produced TGF-β1 regulated T cell responses. To investigate the T cell phenotypes, we first studied T cell development in 4cre-f/n mice. Our recent study of mice with T cell-specific deletion of the *Tgfbr2* gene showed that TGF-β positively regulates $CD8^+$ T cell

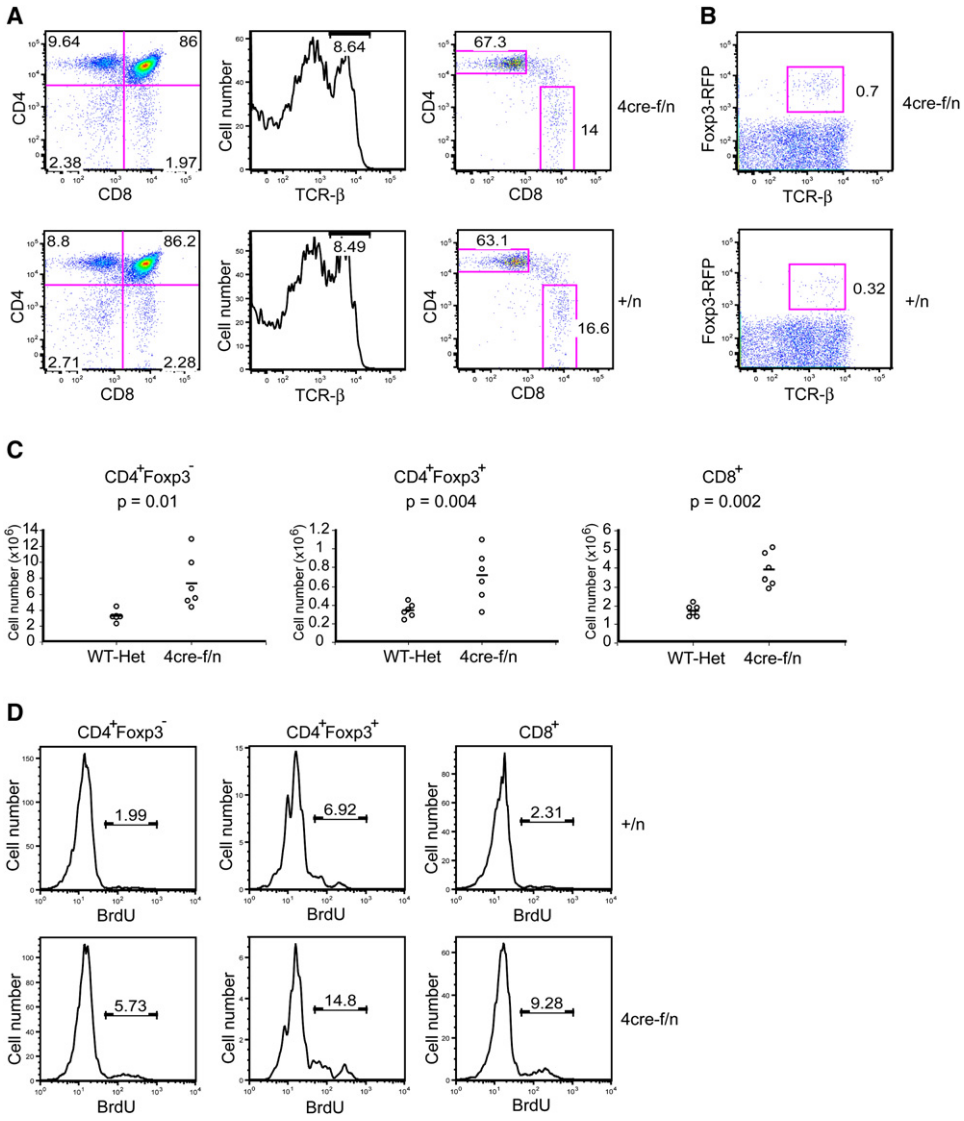


**Figure 3. T Cell Development and Homeostasis in the Absence of T Cell-Produced TGF-β1**

(A) The thymic CD4, CD8, and TCR-β profile of 4cre-f/n and +/n mice at 6 weeks old. These are representative profiles of six mice per group analyzed.

(B) The thymic Foxp3-RFP and TCR-β profile of 4cre-f/n and +/n mice at 6 weeks old. This is a representative picture of eight mice per group analyzed.

(C) Number of $CD4^+Foxp3^-$, $CD4^+Foxp3^+$, or $CD8^+$ T cells in the mesenteric lymph nodes of 4cre-f/n or littermate control +/n, 4cre-f/+, f/n, or f/f (WT-Het) mice (n = 6) at 6–8 weeks old. The p values of cell numbers between the two groups of mice are indicated.

(D) BrdU staining of mesenteric-lymph-node $CD4^+Foxp3^-$, $CD4^+Foxp3^+$, and $CD8^+$ T cells isolated from +/n and 4cre-f/n mice at 8 weeks old. These are representative results of three mice per group analyzed.

maturation (Li et al., 2006a). Analysis of young 4cre-f/n mice aged 4–8 weeks revealed normal thymic cellularity (data not shown). The subset distribution of 4cre-f/n $CD4^+$ and $CD8^+$ T cells is not drastically different from that of control T cells, although a slight decrease of $CD8^+TCR\text{-}\beta^{hi}$ T cells was observed (Figure 3A). Thymic selection also results in the generation of Treg cells whose development and function are specified by the transcription factor Foxp3 (Fontenot and Rudensky, 2005; Sakaguchi, 2004). To investigate the function of T cell-produced TGF-β1 in Treg-cell development, we crossed 4cre-f/n mice with Foxp3-RFP mice that contain knockin alleles of red-fluorescent protein (RFP) in the Foxp3 locus (Wan and Flavell, 2005). Deficiency of T cell-produced TGF-β1 led to a 2-fold increase of thymic Foxp3-positive Treg cells (Figure 3B). We have reported a similar increase of thymic Treg cells in mice with T cell-specific deletion of the *Tgfbr2* gene (Li et al., 2006a). These observations demonstrate that T cell-produced TGF-β1 plays a nonredundant role in limiting the expansion of thymic Treg cells.

Studies of mice with T cell-specific inactivation of TGF-β signaling, or of TGF-β1-deficient mice, showed that TGF-β1 is required for the maintenance of peripheral Treg cells (Li et al., 2006a; Marie et al., 2005; Marie

et al., 2006). We have also found that despite the decrease of peripheral Treg cells, TGF-βRII-deficient Treg cells undergo high rates of proliferation (Li et al., 2006a). These observations suggest that the TGF-β pathway has a dual role in the inhibition of Treg-cell proliferation and in the maintenance of Treg cells. For investigating the function of T cell-produced TGF-β1 in these processes, peripheral Treg cells were examined in 4cre-f/n mice. Deletion of *Tgfb1* gene in T cells did not result in a decrease of the frequencies of Treg cells in the mesenteric lymph nodes, peripheral lymph nodes, and spleens of 4cre-f/n mice (Figure S2A). Substantial numbers of Treg cells were also detected among the lamina propria mononuclear cells that infiltrated the colons of 4cre-f/n mice (Figure S2B). Interestingly, the expression of Foxp3 was lower in colonic Treg cells than mesenteric-lymph-node Treg cells (Figure S2B). Importantly, the number of Treg cells increased 2-fold in the mesenteric lymph nodes of 4cre-f/n mice compared to that of control mice (Figure 3C). To test whether increased Treg cells were due to enhanced proliferation, we pulse-labeled 4cre-f/n and +/n mice with BrdU for 16 hr. A higher percentage of Treg cells from the mesenteric lymph nodes of 4cre-f/n mice incorporated BrdU than that of control +/n mice (Figure 3D). Increased number and enhanced proliferation of $CD4^+Foxp3^-$ T cells and $CD8^+$ T cells were also observed in the mesenteric lymph nodes of 4cre-f/n mice compared to those of control mice (Figures 3C and 3D), and such a finding was associated with the development of severe colitis in these mice (Figure 2A). These findings reveal that T cell-produced TGF-β1 is required to inhibit T cell proliferation but is dispensable for the maintenance of Treg cells in peripheral lymphoid organs. By inference, TGF-β1 sources other than T cells may be able to provide this function.

## T Cell Activation and Differentiation in the Absence of T Cell-Produced TGF-β1

In addition to the maintenance of Treg cells, we and others have reported that TGF-β signaling is required for the inhibition of T cell activation and differentiation (Li et al., 2006a; Marie et al., 2006). To study the function of T cell-produced TGF-β1 in T cell activation, we examined the expression of T cell activation markers CD44 and CD62L in $CD4^+$ and $CD8^+$ T cells isolated from the mesenteric lymph nodes of 4cre-f/n mice. Compared to control +/n T cells, a higher percentage of 4cre-f/n T cells exhibited an activated $CD44^{hi}CD62L^{lo}$ phenotype (Figure 4A). 4cre-f/n $CD4^+$ and $CD8^+$ T cells also expressed greater amounts of surface CD122 (Figure 4B), the shared receptor for IL-2 and IL-15. We and others have recently shown that CD122 expression is controlled by transcription factors T-bet and eomesodermin that also regulates effector Th1 and CTL functions (Intlekofer et al., 2005; Li et al., 2006a). These observations suggested that 4cre-f/n $CD4^+$ and $CD8^+$ T cells may undergo effector T cell differentiation.

To determine T cell differentiation, we activated mesenteric-lymph-node T cells with CD3 and CD28 antibodies for 24 hr and examined the secretion of IFN-γ, IL-4, and IL-17, the signature cytokines of Th1, CTL, Th2, and Th17 cells. 4cre-f/n T cells produced greater amounts of IFN-γ and IL-4 but lesser amounts of IL-17 compared to control T cells (Figure 4C). In order to determine the proportion of T cells capable of cytokine production, we stimulated T cells from mesenteric lymph nodes with PMA and inonomycin for 4 hr and performed intracellular cytokine staining. Compared to T cells from the +/n mouse, which had only a few $CD4^+$ and $CD8^+$ T cells capable of IFN-γ and IL-4 production, a higher percentage of $CD4^+$ and $CD8^+$ T cells from the 4cre-f/n mouse produced these cytokines (Figure 4D, left and middle panels). In contrast, fewer 4cre-f/n $CD4^+$ T cells produced IL-17 than +/n $CD4^+$ T cells (Figure 4D, right panel). In addition, we examined the differentiation of $CD4^+$ T cells that infiltrated the colons of 4cre-f/n mice. Similar to mesenteric-lymph-node T cells, the frequency of colonic IFN-γ-producing $CD4^+$ T cells was higher, whereas the frequency of IL-17-producing $CD4^+$ T cells was lower in 4cre-f/n $CD4^+$ T cells compared to control f/n $CD4^+$ T cells (Figure S3). These observations demonstrate that T cell-produced TGF-β1 promotes Th17-cell differentiation but inhibits Th1, Th2, and CTL differentiation in vivo; the lack of this function leads to severe colitis in 4cre-f/n mice.

## Inhibition of Colitis by $CD4^+Foxp3^+$ Treg-Cell- and Naive T Cell-Produced TGF-β1

We are interested in the prevalent colitis phenotype that developed in 4cre-f/n mice. Because both $CD4^+Foxp3^+$ Treg cells and $CD4^+Foxp3^-$ T cells that were activated in vitro produced TGF-β1 (Figure 1G), we wished to determine their function in vivo. To this end, we crossed 4cre-f/n mice with Foxp3-RFP mice and isolated Treg cells on the basis of the expression of RFP, which faithfully marks Foxp3-expressing Treg cells (Wan and Flavell, 2005). We first tested the function of TGF-β1-deficient Treg cells in vitro. 4cre-f/n Treg cells inhibited naive T cell proliferation as potently as control Treg cells (Figure S4). These observations are consistent with cell-contact-dependent but cytokine-independent mechanisms of Treg-cell suppression of proliferation in vitro.

To determine the activity of 4cre-f/n Treg cells in vivo, we used a transfer model of colitis. In this model, transfer of naive $CD4^+$ cells into lymphopenic hosts leads to the development of colitis, which can be prevented by the cotransfer of Treg cells (Powrie et al., 1994). Previous studies showed that the protection can be abrogated by the treatment with TGF-β antibody (Powrie et al., 1996), and Treg cells fail to inhibit colitis induced by naive CD4-DNR $CD4^+$ T cells, which have attenuated TGF-β signaling (Fahlen et al., 2005). These results suggested that Treg-cell-mediated protection is dependent on the TGF-β pathway. To determine the function of TGF-β1 that originated from naive T cells, we also isolated $CD4^+Foxp3^-CD62L^{hi}CD45Rb^{hi}$ naive T cells from 4cre-f/n mice or control (wild-type, WT) mice by FACS sorting. Purified T cells were transferred alone or in combination into $Rag1^{-/-}$ recipients. These mice were monitored for body-weight change and the development of colitis.

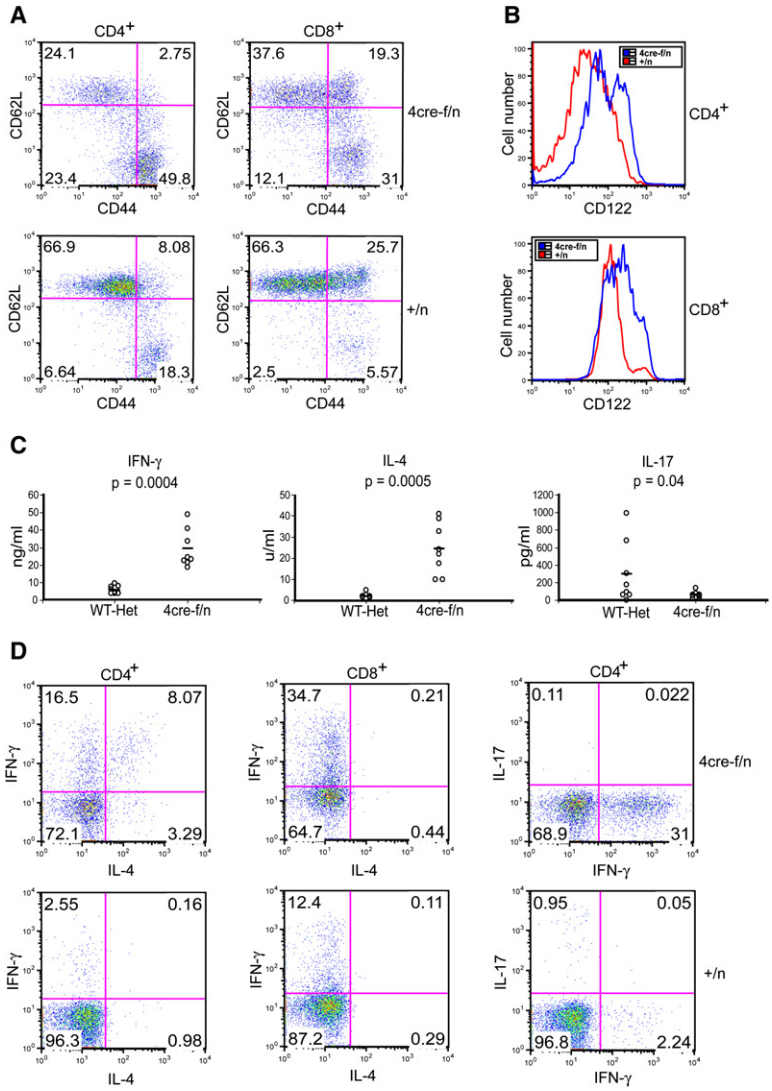


**Figure 4. T Cell Activation and Effector T Cell Differentiation in the Absence of T Cell-Produced TGF-β1**

(A) $CD4^+$ and $CD8^+$ T cells from the mesenteric lymph nodes of 5-month-old 4cre-f/+ and +/n mice were analyzed for the expression of CD44 and CD62L by flow cytometry. Eight mice per group were analyzed. Representative results are presented.

(B) Mesenteric-lymph-node $CD4^+$ and $CD8^+$ T cells of 4cre-f/n and +/n mice at the age of 5 months were analyzed for the expression of CD122. These are representative results of five mice per group analyzed.

(C) Mesenteric-lymph-node cells of 4cre-f/n or littermate control +/n, 4cre-f/+, f/n, or f/f (WT-Het) mice (n = 8) at 4–7 months old were stimulated with CD3 and CD28 antibodies for 24 hr. Cytokine amounts in the supernatants were determined by ELISA. The p values of cytokine amounts between the two groups of mice are indicated.

(D) Mesenteric-lymph-node $CD4^+$ and $CD8^+$ T cells from 5-month-old 4cre-f/n and +/n mice were stimulated with PMA and ionomycin for 4 hr and were analyzed for the expression of IL-4, IFN-γ, and IL-17. These are representative results of eight mice per group analyzed.

Recipients of WT or 4cre-f/n Treg cells alone gained body weight (Figure 5A, upper panel) and remained disease free for up to 12 weeks (data not shown). In contrast, transfer of WT or 4cre-f/n naive T cells resulted in severe bodyweight loss (Figure 5A, middle panel) and the development of colitis in all recipients in 5 weeks (data not shown). These observations suggest that TGF-β1 originated from naive T cells does not play a major role in limiting the development of colitis.

We also performed cotransfer experiments with Treg cells and naive T cells in four groups (Figure 5A, lower panel). Recipients of WT Treg cells and WT naive T cells gained weight and showed no signs of disease in 8 weeks (Figures 5A and 5B). Transfer of 4cre-f/n Treg cells and WT naive T cells resulted in wasting disease and the development of colitis in four out of five mice (Figures 5A and 5B), thus demonstrating an essential role for Treg-cell-produced TGF-β1 in inhibiting colitis. *Rag1*$^{-/-}$ recipients of WT Treg cells and 4cre-f/n naive T cells gained weight and remained disease free (Figures 5A and 5B). However, transfer of 4cre-f/n Treg cells and 4cre-f/n naive T cells led to the most severe wasting disease and colitis in all recipients (Figures 5A and 5B), suggesting that TGF-β1 originating from naive T cells also contributed to the protection of colitis under these conditions. These observations reveal an essential role for TGF-β1 produced by Treg cells and to a lesser extent by nonregulatory T cells in inhibiting the wasting disease and colitis.

Previous studies showed that colitis that developed in the transfer model is dependent on Th1 cells and can be prevented by an antibody that neutralizes IFN-γ (Powrie et al., 1994). To determine the molecular mechanisms by which Treg-cell-produced TGF-β1 inhibits naive T cell-induced colitis, we FACS sorted naive $CD4^+$ T cells from C57BL/6 mice that express the congenic marker CD45.1. Naive T cells were labeled with CFSE and were transferred alone or in combination with WT or 4cre-f/n Treg cells that express the congenic marker CD45.2. The use of different CD45 markers enabled us to analyze the naive T cell population in recipients. Cotransfer of WT Treg cells did not

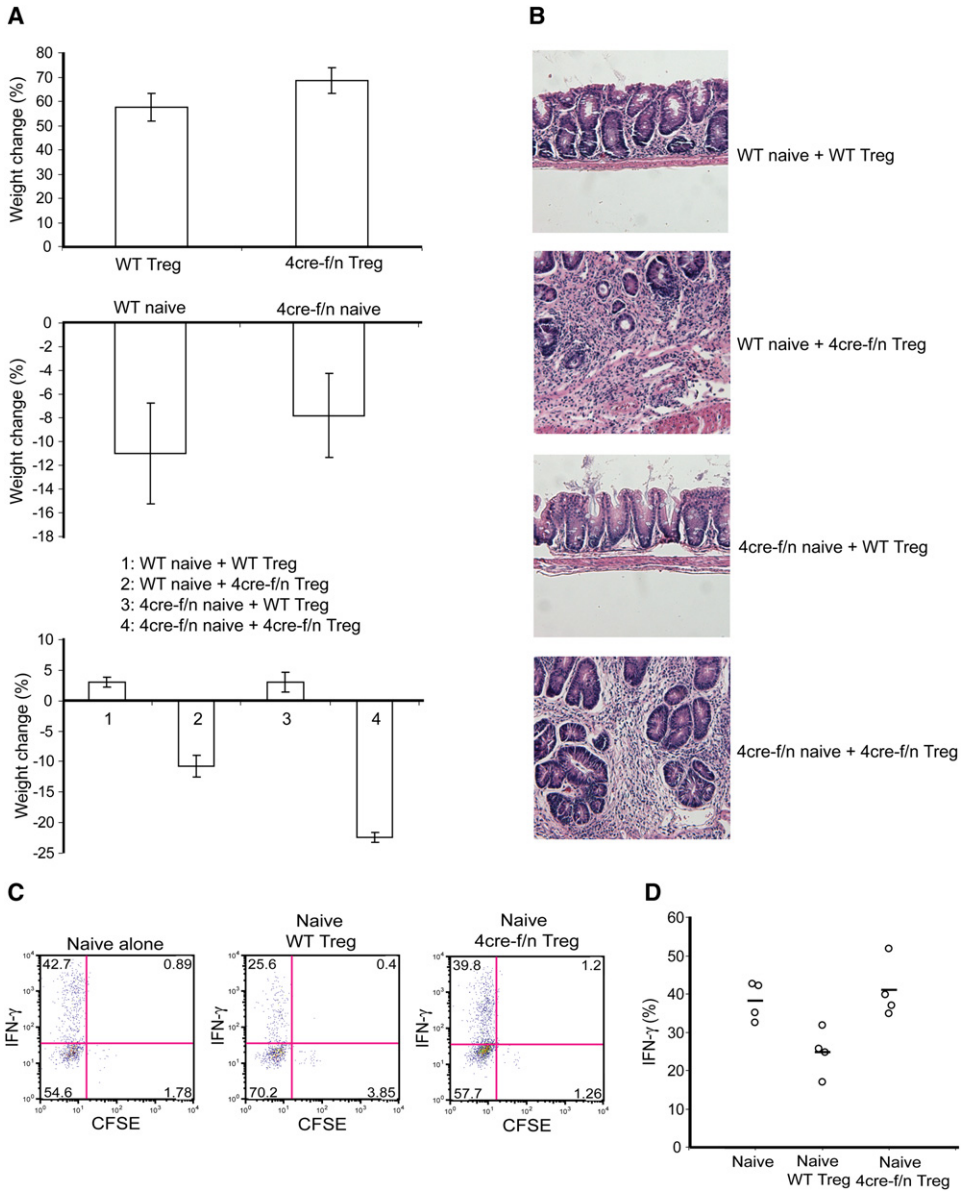


**Figure 5. Failed Inhibition of Colitis and Th1-Cell Differentiation in the Absence of Treg-Cell-Produced TGF-β1**

(A) Purified 4cre-f/n or control (wild-type, WT) Treg cells and naive $CD4^+$ T cells were transferred alone or in combination into *Rag1*$^{-/-}$ mice. The body-weight change of these mice is plotted as mean ± SEM.

(B) Treg cells and naive $CD4^+$ T cells from WT and 4cre-f/n mice were transferred to *Rag1*$^{-/-}$ mice in four groups. Representative colon histology is shown (n = 5).

(C and D) CFSE-labeled $CD45.1^+$ naive T cells were transferred alone or in combination with $CD45.2^+$ WT or 4cre-f/n Treg cells into *Rag1*$^{-/-}$ mice. One week later, $CD4^+$ T cells from the mesenteric lymph nodes were stimulated with PMA and ionomycin for 4 hr and were analyzed for IFN-γ expression and CFSE levels. The percentiles of $CD45.1^+CD4^+$ T cells that produced IFN-γ are depicted in (D). Cotransfer of WT Treg cells but not 4cre-f/n Treg cells significantly inhibited IFN-γ production ($p = 0.01$).

inhibit the proliferation of naive T cells revealed by CFSE dilution (Figure 5C). However, the frequencies of IFN-γ-producing T cells were markedly reduced in the presence of WT Treg cells (Figures 5C and 5D). Importantly, inhibition of IFN-γ production was not observed when naive T cells were transferred with 4cre-f/n Treg cells (Figures 5C and 5D). These observations are consistent with enhanced Th1-cell differentiation in 4cre-f/n mice (Figures 4C and 4D) and also demonstrate an essential role for Treg-cell-produced TGF-β1 in inhibiting Th1-cell differentiation in vivo.

## T Cell-Produced TGF-β1 Regulation of Th17-Cell Differentiation and EAE

TGF-β1 is a pleiotropic cytokine with a pivotal role in regulating the differentiation of effector T cells including the

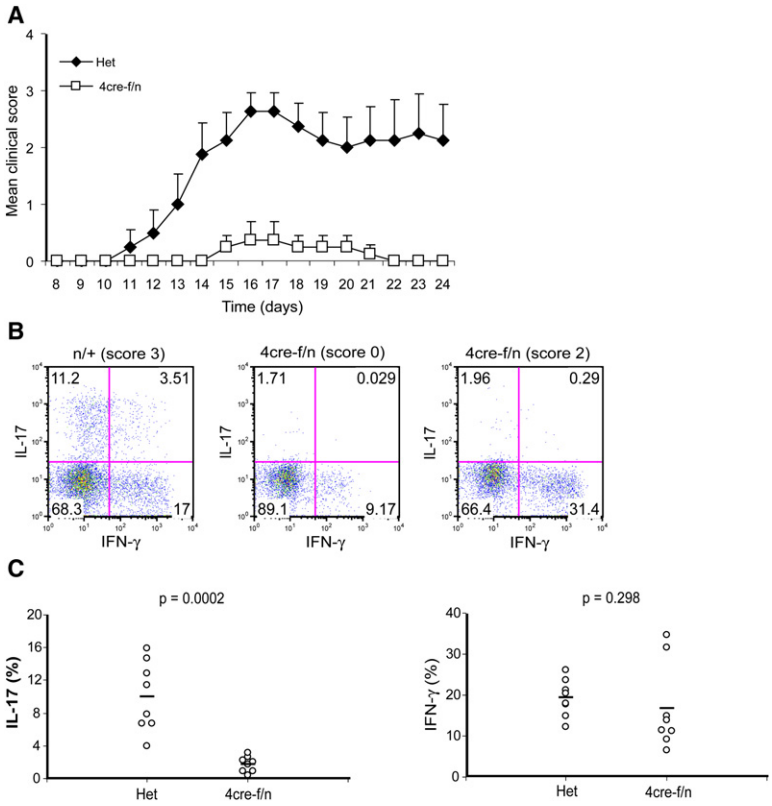


**Figure 6. Inhibited EAE Induction and Th17-Cell Differentiation in the Absence of T Cell-Produced TGF-β1**

(A) EAE disease course in 4cre-f/n and +/n, f/n, or 4cre-f/+ (Het) mice (n = 8). Disease scores are plotted as mean ± SEM. These are representative results of two independent experiments.

(B and C) Cytokine production by $CD4^+$ T cells isolated form the spinal cords day 24 after disease induction. The cells were stimulated with PMA and ionomycin for 4 hr and were analyzed for IFN-γ and IL-17 expression. Six out of eight 4cre-f/n mice did not develop any clinical sign of disease (score 0), whereas the remaining two developed mild disease (score 2). The representative profiles of cytokine staining are shown in (B). The percentiles of IL-17 and IFN-γ producers in all mice are shown in (C). The p values of percentiles of cells producing cytokines between the two groups of mice are indicated.

recently described Th17 cells that secrete the inflammatory cytokine IL-17. Recent studies showed that in the presence of IL-6, TGF-β promotes the differentiation of Th17 cells (Bettelli et al., 2006; Veldhoen et al., 2006a). Importantly, Th17 cells are reduced in TGF-β1 complete knockout mice (Mangan et al., 2006), and CD4-DNR mice with attenuated TGF-β signaling in T cells fail to develop Th17 cells or EAE (Veldhoen et al., 2006b). These observations suggest that the TGF-β pathway is important for the differentiation of Th17 cells in vivo.

Intriguingly, T cell production of IL-17 was greatly reduced in 4cre-f/n mice (Figure 4C, 4D), suggesting that T cell-produced TGF-β1 promoted Th17-cell differentiation. We wished to extend these observations in the EAE model. 4cre-f/n mice were highly resistant to the induction of EAE (Figure 6A). Six out of eight 4cre-f/n mice did not develop disease, and two of them developed mild disease in contrast to the severe disease developed in all control mice (Figure 6A). To investigate the function of T cell-produced TGF-β1 in the regulation of effector T cell differentiation, we assessed the production of IFN-γ and IL-17 in $CD4^+$ T cells that had infiltrated spinal cords. As expected, populations of $CD4^+$ T cells producing IFN-γ or IL-17 were observed in control n/+ mice (Figures 6B and 6C). However, the number of IL-17-producing T cells was greatly reduced in 4cre-f/n mice (Figures 6B and 6C), whereas IFN-γ-positive T cells were not significantly different between the two groups (Figure 6C). Interestingly, in the two 4cre-f/n mice that developed mild EAE, infiltrating $CD4^+$ T cells still produced minimal amounts of IL-17 but great amounts of IFN-γ (Figures 6B and 6C). These observations demonstrate an essential role for T cell-produced TGF-β1 in the differentiation of Th17 cells in the EAE model.

## DISCUSSION

TGF-β1 is a regulatory cytokine with pleiotropic roles in T cell development, tolerance, and homeostasis (Li et al., 2006a; Marie et al., 2006). Here, we reported a mouse model in which the first exon of the *Tgfb1* gene and the fourth exon of the hypothetical gene LOC232987 are flanked with *loxP* sites. By breeding these mice to CD4-Cre transgenic mice, we specifically deleted the *loxP*-encompassed genomic region in T cells. Deletion of *Tgfb1* exon 1 inactivated the *Tgfb1* gene. The potential effect of removal of LOC232987 exon 4 was controlled for by the introduction of a *Tgfb1* null allele that has an intact LOC232987 gene. Therefore, T cells from 4cre-f/n mice are heterozygous for the exon 4 of LOC232987 gene. 4cre-f/n mice but none of the control mice developed an inflammatory disorder. With this model, we further showed that T cell-produced TGF-β1 inhibited the expansion of thymic Treg cells and the proliferation, activation, and differentiation of T cells in peripheral lymphoid organs. T cell-produced TGF-β1 was dispensable for the maintenance of peripheral Treg cells but was required for Treg-cell-mediated suppression of Th1-cell differentiation and colitis in a transfer model. T cells were also vital sources of TGF-β1 that promoted Th17-cell differentiation

and the development of EAE. These findings reveal important functions for T cell-produced TGF-β1 in the regulation of T cell tolerance and effector T cell differentiation in vivo.

We have recently reported that TGF-β signaling in T cells promotes thymic $CD8^+$ T cell maturation and inhibits thymic Treg cell expansion (Li et al., 2006a). Here, we show that T cell-produced TGF-β1 is required for the inhibition of Treg cells but is dispensable for $CD8^+$ T cell development. These observations suggested that TGF-β1 produced by cells other than T cells is involved in $CD8^+$ T cell maturation or that other isoforms of TGF-β (TGF-β2 and TGF-β3) are important. Previous studies showed that thymic epithelial cells produce TGF-β1 (Takahama et al., 1994). The functions of TGF-β1 produced by these cells in T cell development are under investigation.

We and others found that TGF-β signaling maintains peripheral Treg cells and inhibits T cell activation and differentiation (Li et al., 2006a; Marie et al., 2006). In this report, we showed that T cell-produced TGF-β1 suppresses T cell proliferation, activation, and differentiation but is dispensable for the maintenance of peripheral Treg cells. In contrast, Treg cells expanded in the absence of T cell-produced TGF-β1. Increased Treg-cell proliferation was also observed in T cell-specific TGFβRII-deficient mice, although the cell number was reduced (Li et al., 2006a). These findings suggested that Treg-cell maintenance is likely to depend on other cellular sources of TGF-β1. To determine the status of Treg cells in inflamed tissues, we compared colonic Treg cells to mesenteric-lymph-node Treg cells of 4cre-f/n mice. Interestingly, we observed reduced Foxp3 levels in colonic Treg cells. We do not know whether this is a consequence of the inflammatory environment in the colon or is an intrinsic deficiency due to lack of T cell-produced TGF-β1. It is however noteworthy that we observed reduced Foxp3 levels in Treg cells from the islets of type I diabetic NOD mice (Wan and Flavell, 2007).

Although T cell-produced TGF-β1 was dispensable for the maintenance of Treg cells, Treg-cell-produced TGF-β1 was essential for Treg cell function in vivo. We found that TGF-β1-deficient Treg cells were defective in inhibiting colitis induced by transferred naive T cells, and this defect was associated with the failure to inhibit naive T cell differentiation into Th1 cells. Previous studies with Treg cells isolated from TGF-β1-deficient mice have generated contrasting results (Fahlen et al., 2005; Kullberg et al., 2005; Nakamura et al., 2004). The different findings on the function of Treg-cell-produced TGF-β1 in colitis might be explained by different gut flora present in different institutions. In addition, one complication of the earlier studies is that CD25 was used as a marker for Treg cells, and activated T cells may contaminate the Treg-cell population. This variable was controlled for in our study, because we isolated Treg cells on the basis of the expression of Foxp3. A previous study from our laboratory showed that TGF-β signaling in $CD8^+$ T cells is required for Treg-cell-mediated protection of diabetes (Green et al., 2003). Blockade of TGF-β signaling in $CD8^+$ T cells also alleviated the inhibition of tumor immunity or cytotoxic T cell function by Treg cells (Chen et al., 2005; Mempel et al., 2006). These studies reveal that TGF-β1 is also essential for Treg-cell-mediated inhibition of $CD8^+$ T cell activity. The functions of Treg-cell-produced TGF-β1 in these disease models are under investigation.

Nonetheless, TGF-β1-deficient Treg cells possess normal suppressive activity in vitro. In addition, mice devoid of Treg cells developed a more severe phenotype than did 4cre-f/n mice (Fontenot et al., 2003). These observations suggested that secretion of TGF-β1 is only one mechanism of Treg-cell-mediated immune tolerance. Treg cells may secrete other suppressive cytokines such as IL-10. The interaction between CTLA-4 on Treg cells and CD80/CD86 on effector T cells can induce "outside-in" signaling by CD80 and CD86 ligands and result in suppression (Paust et al., 2004; Taylor et al., 2004). The functions of these pathways in vivo remain to be fully established.

Results presented in this study demonstrate an essential role for T cell-produced TGF-β1 in T cell tolerance. Although we currently do not know the nature of the activating stimuli that drive the activation of T cells in 4cre-f/n mice, the prevalent development of colitis suggests that T cell responses are directed against commensal bacterium antigens. Colitis in these mice was associated with enhanced Th1 and Th2, but diminished Th17-cell differentiation. It is interesting to note that colitis that developed in IL-10-deficient mice is dependent on the innate cytokine IL-23, which promotes Th17-cell differentiation (Yen et al., 2006). Recent studies have also revealed a pivotal role for IL-23 in several models of intestinal inflammation, including *Helicobacter hepaticus*-triggered T cell-dependent colitis (Kullberg et al., 2006), naive T cell-induced colitis upon transfer to *Rag1*$^{-/-}$ mice (Hue et al., 2006), and mucosal inflammation mediated by innate immune cells (Hue et al., 2006; Uhlig et al., 2006). Interestingly, these studies show that IL-23 can additionally promote IL-17 production by innate immune cells (Hue et al., 2006; Uhlig et al., 2006) and is required for T cell production of IFN-γ in the transfer model (Hue et al., 2006; Kullberg et al., 2006). Importantly, IFN-γ precipitates naive T cell-induced colitis (Powrie et al., 1994) and the colitis triggered by *Helicobacter hepaticus* infection (Kullberg et al., 2006). On the basis of these observations, it has been proposed that IL-23 induces the production of both IFN-γ and IL-17 that synergistically induce intestinal inflammation (Kullberg et al., 2006). Because we observed reduced Th17 cells in 4cre-f/n mice, colitis that developed in these mice may be independent of Th17 cells. Nonetheless, it is possible that IL-17 produced by Th17 cells that remained in 4cre-f/n mice or by other cell types contributes to the disease pathology. The definitive functions of IFN-γ, IL-17, and Th2 cytokines in colitis that developed in 4cre-f/n mice remain topics for future investigation. A recent study showed that colitis developed in IL-10- or IL-2-deficient mice is differentially regulated by Toll-like receptor pathways (Rakoff-Nahoum et al., 2006). The function of Toll-like receptors in colitis developed in 4cre-f/n mice remains to be determined.

An important observation made in this study is that T cell-produced TGF-β1 is essential for the differentiation

of adjuvant-induced Th17 cells and the development of EAE. This is in contrast to the spontaneous colitis phenotype that developed in these mice. These observations suggest that colitis and EAE can be triggered by different effector mechanisms. These results are in line with the recent findings that TGF-β promotes Th17-cell differentiation (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006a) and with the observations that inhibition of TGF-β signaling in T cells prevents EAE (Veldhoen et al., 2006b), whereas overexpression of TGF-β1 in T cells exaggerates the disease (Bettelli et al., 2006). We do not know whether TGF-β1 produced by Treg cells, non-Treg cells, or both are important for Th17-cell differentiation. Interestingly, Treg cells promote Th17-cell differentiation in the presence of LPS in vitro (Bettelli et al., 2006; Veldhoen et al., 2006a). A recent study showed that cotransfer of Treg cells enhances IL-17 but inhibits IFN-γ production (Lohr et al., 2006). These observations suggest a proinflammatory role for Treg cells in Th17-cell differentiation through the production of TGF-β1.

In conclusion, in this report we have uncovered a regulatory circuit whereby T cell-produced TGF-β1 essentially controls T cell tolerance. The finding of a dual role for T cell TGF-β1 in inhibiting Th1 and promoting Th17-cell differentiation can also be exploited for the immunotherapy of diseases mediated by these effector T cells.

## EXPERIMENTAL PROCEDURES

### Mice

Mouse genomic DNA of the *Tgfb1* gene was isolated from a 129SV BAC library (Genome Systems). We constructed the targeting vector by cloning three genomic fragments into plasmid pEasy-Flox. Linearized targeting vector was transfected into ES cells (TC1). Homologous recombinants were identified by Southern-blot analysis and were transfected with a pPGK-Cre plasmid for deletion of neomycin selection marker in vitro. Clones carrying the mutated allele of the *Tgfb1* gene TGF-β1$^{f}$ (f) were injected into blastocysts and were implanted into foster mothers. Chimeric mice were bred to C57BL/6 mice, and the F1 generation was screened for germline transmission of the mutated allele. GFP knockin mice were similarly generated (M.O.L. and R.A.F., unpublished data). All mice were backcrossed to C57BL/6 background for eight to nine generations and were maintained under specific pathogen-free conditions. CD4-Cre transgenic mice and Foxp3-RFP knockin mice were described previously (Lee et al., 2001; Wan and Flavell, 2005). All animal experimentation was conducted in accordance with institutional guidelines.

### PCR Typing and RT-PCR Analysis

For detection of the wild-type, floxed, and deleted alleles, DNA isolated from different cell types was analyzed by PCR with the following primer set: 5′-CTTCCTAACCCCAGAGGTGGA-3′, 5′-CACATTAAGTC GTGGCTAGGG-3′, and 5′-CCCAGGCTAGCCTTGAACTTCT-3′. For the analysis of LOC232987 expression, RNA was purified from FACS sorted CD4$^{+}$ cells with RNeasy Mini Kit (Qiagen), and were reverse transcribed into cDNA with proSTAR First-Strand RT-PCR Kit (Stratagene). cDNA was amplified with the following primer set: 5′-CCTGCA CTTCGCTACTAAAGG-3′, and 5′-CTAGGCACATGGCAAAAGCCA-3′.

### Histopathology and Immunohistochemistry

Tissues from sacrificed animals were fixed in Streck Tissue Fixative (Streck Laboratories) and embedded in paraffin. Five micrometer sections were stained with hematoxylin and eosin. For detection of IgG deposition in kidney glomeruli, kidneys were frozen in OCT media, and eight micrometer cryostat sections were cut and fixed in acetone for 10 min. Kidney sections were stained with Alexa-Fluor-488-labeled goat anti-mouse IgG antibody (Molecular Probes).

### ELISA

Cytokine amounts in tissue-culture supernatants were assayed with ELISA antibody pairs for IL-4, IFN-γ (BD Biosciences PharMingen), and IL-17 (SouthernBiotech) in accordance with the manufacturer's recommendations. For detecting TGF-β1, latent TGF-β1 in the culture supernatant was activated by acid treatment and was assayed with antibody pairs from R&D Systems (BAF240 and MAB1835). The amounts of dsDNA antibody and nuclear antibody in mouse sera were determined with ELISA kits from Alpha Diagnostic International. Sera from eight 4cre-f/n and eight control f/f, +/n, f/n, or 4cre-f/+ mice aged 4–7 months were assayed individually with 1:100 dilution of sera in 1% BSA in PBS.

### FACS

Cells from the spleen, lymph nodes, or thymus were depleted of erythrocytes by hypotonic lysis. Cells were preincubated with 2.4G2 mAb to block FcγR for 15 min, and this was followed by the incubation with specific antibodies for 30 min on ice. All samples were analyzed with FACSCalibur or LSRII (Becton Dickinson) machines. FACS data were analyzed with FloJo (Tree Star) software. All antibodies except Foxp3 and BrdU antibodies were obtained from BD Biosciences PharMingen. For intracellular cytokine staining, single-cell suspensions were stimulated with 50 ng/ml PMA (Sigma) and 1 μM ionomycin (Sigma) for 4 hr; GolgiStop (BD Biosciences PharMingen) was added for the final 2 hr of culture. After stimulation, cells were first stained with CD4, CD8, CD45.1, and CD45.2 antibodies, fixed and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences PharMingen), and finally stained with IFN-γ, IL-4, and IL-17 antibodies in accordance with the manufacturer's instructions.

### BrdU Labeling

Eight-week-old 4cre-f/n or littermate control mice were given 50 mg/kg BrdU by i.p. injection. Thymic, splenic, and lymph-node cells were prepared and stained for cell surface markers and Foxp3 (eBioscience) 16 hr later. Cells were incubated overnight in 1% PFA and 0.01% Tween 20, and this incubation was followed by Dnase I (250 u/ml, Sigma) treatment and staining with BrdU antibody (Beckton Dickinson).

### Cell Purification and Culture

CD4$^{+}$ T cells were enriched from spleen and lymph-node cells by positive selection with anti-CD4 microbeads (Miltenyi Biotec). Enriched T cells were further purified with a cell sorter (Becton Dickinson) by gating on CD4$^{+}$Foxp3$^{+}$ (Treg) and CD4$^{+}$Foxp3$^{-}$CD62L$^{hi}$CD45Rb$^{hi}$ (naive) cells. Spleen cells were also used to purify CD4$^{+}$TCR-β$^{+}$ (CD4$^{+}$ T cells), CD8$^{+}$TCR-β$^{+}$ (CD8$^{+}$ T cells), B220$^{+}$ (B cells), and CD11c$^{+}$ (dendritic cells) by FACS sorting. The purity of these cells was >95%. We derived bone-marrow macrophages by growing bone-marrow precursor cells with L929-cell conditional medium.

For Treg-cell suppression assays, 5 × 10$^{4}$ naive CD4$^{+}$ T cells were cultured in 96-well plates with 10$^{5}$ irradiated splenocytes, 2 μg/ml CD3 antibody, and indicated numbers of Treg cells for 72 hr. Proliferation of T cells was determined by thymidine incorporation for the final 12–16 hr of the culture. For cytokine-production measurements, 2 × 10$^{6}$ spleen cells were stimulated with 0.1 μg/ml CD3 and 0.5 μg/ml CD28 antibodies in 2 ml complete medium in 24-well plates. After 24 hr, supernatants were collected and assayed for cytokine amounts by ELISA. For detecting TGF-β1 production, total CD4$^{+}$ T cells were cultured in plates coated with CD3 (5 μg/ml) and CD28 (2 μg/ml) antibodies for 3 days in complete medium supplemented with IL-2 (100 u/ml, BD Biosciences Pharmingen) and were restimulated in plates coated with CD3 (10 μg/ml) antibody for 24 hr in X-vivo medium supplemented with Nutridoma-SP (Roche). Sorted CD4$^{+}$Foxp3$^{+}$ or CD4$^{+}$Foxp3$^{-}$ T cells were also cultured in plates coated with CD3

(10 μg/ml) and CD28 (4 μg/ml) antibodies for 24 hr in X-vivo medium supplemented with Nutridoma-SP and IL-2 ($10^6$ u/ml).

### T Cell Transfer Model of Colitis

A total of $4 \times 10^5$ naive T cells and $1 \times 10^5$ Treg cells were transferred alone or in combination to *Rag1*$^{-/-}$ mice as described previously (Powrie et al., 1996). After T cell reconstitution, mice were weighted weekly and monitored for signs of disease. Naive T cells were also purified from CD45.1$^+$ C57BL/6 congenic mice, labeled with CFSE, and transferred alone or in combination with CD45.2$^+$ Treg cells to *Rag1*$^{-/-}$ mice.

### EAE Induction and Disease Scoring

Mice were immunized subcutaneously with 50 μg/mouse MOG 35-55 peptide in 200 μl emulsion of CFA (IFA supplemented with 2.5 mg/ml *Mycobacterium tuberculosis*) and were injected on days 0 and 2 with 200 ng/mouse pertussis toxin (List Biological Laboratories). The scoring system used was as follows: 1, limp tail; 2, partial hind-limb paralysis; 3, total hind-limb paralysis; 4, hind-limb paralysis and 75% body paralysis; and 5, complete body paralysis/moribund.

### Isolation of Mononuclear Cells from Spinal Cords

Mononuclear cells from spinal cords were isolated as described previously (Ivanov et al., 2006). In brief, mice were perfused with 30 ml PBS with 2 mM EDTA. Spinal cords were dissected, cut into pieces, and digested in PBS supplemented with 10 mg/ml Collagenase D (Roche). The digested spinal cords were vortexed intensively and passed through a 40 μm cell strainer. Cells were washed in PBS, resuspended in 6 ml 38% Percoll solution (GE Healthcare), and pelleted for 20 min at 2000 rpm. Cells were washed in PBS and used in experiments.

### Statistical Analyses

The Student's t test was used to calculate statistical significance for difference in a particular measurement between groups. A p value of $\leq 0.05$ was considered statistically significant.

### Supplemental Data

Four figures are available online at http://www.immunity.com/cgi/content/full/26/5/579/DC1/.

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## REFERENCES

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T.B., Oukka, M., Weiner, H.L., and Kuchroo, V.K. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature *441*, 235–238.

Chen, M.L., Pittet, M.J., Gorelik, L., Flavell, R.A., Weissleder, R., von Boehmer, H., and Khazaie, K. (2005). Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. Proc. Natl. Acad. Sci. USA *102*, 419–424.

Chen, W., Jin, W., Hardegen, N., Lei, K.J., Li, L., Marinos, N., McGrady, G., and Wahl, S.M. (2003). Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J. Exp. Med. *198*, 1875–1886.

Cua, D.J., Sherlock, J., Chen, Y., Murphy, C.A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., et al. (2003). Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature *421*, 744–748.

Dang, H., Geiser, A.G., Letterio, J.J., Nakabayashi, T., Kong, L., Fernandes, G., and Talal, N. (1995). SLE-like autoantibodies and Sjogren's syndrome-like lymphoproliferation in TGF-beta knockout mice. J. Immunol. *155*, 3205–3212.

Fahlen, L., Read, S., Gorelik, L., Hurst, S.D., Coffman, R.L., Flavell, R.A., and Powrie, F. (2005). T cells that cannot respond to TGF-{beta} escape control by CD4+CD25+ regulatory T cells. J. Exp. Med. *201*, 737–746.

Fantini, M.C., Becker, C., Monteleone, G., Pallone, F., Galle, P.R., and Neurath, M.F. (2004). Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. J. Immunol. *172*, 5149–5153.

Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat. Immunol. *4*, 330–336.

Fontenot, J.D., and Rudensky, A.Y. (2005). A well adapted regulatory contrivance: Regulatory T cell development and the forkhead family transcription factor Foxp3. Nat. Immunol. *6*, 331–337.

Gorelik, L., and Flavell, R.A. (2000). Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. Immunity *12*, 171–181.

Green, E.A., Gorelik, L., McGregor, C.M., Tran, E.H., and Flavell, R.A. (2003). CD4+CD25+ T regulatory cells control anti-islet CD8+ T cells through TGF-beta-TGF-beta receptor interactions in type 1 diabetes. Proc. Natl. Acad. Sci. USA *100*, 10878–10883.

Hue, S., Ahern, P., Buonocore, S., Kullberg, M.C., Cua, D.J., McKenzie, B.S., Powrie, F., and Maloy, K.J. (2006). Interleukin-23 drives innate and T cell-mediated intestinal inflammation. J. Exp. Med. *203*, 2473–2483.

Intlekofer, A.M., Takemoto, N., Wherry, E.J., Longworth, S.A., Northrup, J.T., Palanivel, V.R., Mullen, A.C., Gasink, C.R., Kaech, S.M., Miller, J.D., et al. (2005). Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. Nat. Immunol. *6*, 1236–1244.

Ivanov, I.I., McKenzie, B.S., Zhou, L., Tadokoro, C.E., Lepelley, A., Lafaille, J.J., Cua, D.J., and Littman, D.R. (2006). The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell *126*, 1121–1133.

Kehrl, J.H., Wakefield, L.M., Roberts, A.B., Jakowlew, S., Alvarez-Mon, M., Derynck, R., Sporn, M.B., and Fauci, A.S. (1986). Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. J. Exp. Med. *163*, 1037–1050.

Kulkarni, A.B., Huh, C.G., Becker, D., Geiser, A., Lyght, M., Flanders, K.C., Roberts, A.B., Sporn, M.B., Ward, J.M., and Karlsson, S. (1993). Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. Proc. Natl. Acad. Sci. USA *90*, 770–774.

Kullberg, M.C., Hay, V., Cheever, A.W., Mamura, M., Sher, A., Letterio, J.J., Shevach, E.M., and Piccirillo, C.A. (2005). TGF-beta1 production by CD4+ CD25+ regulatory T cells is not essential for suppression of intestinal inflammation. Eur. J. Immunol. *35*, 2886–2895.

Kullberg, M.C., Jankovic, D., Feng, C.G., Hue, S., Gorelick, P.L., McKenzie, B.S., Cua, D.J., Powrie, F., Cheever, A.W., Maloy, K.J., and Sher, A. (2006). IL-23 plays a key role in Helicobacter hepaticus-induced T cell-dependent colitis. J. Exp. Med. *203*, 2485–2494.

Langrish, C.L., Chen, Y., Blumenschein, W.M., Mattson, J., Basham, B., Sedgwick, J.D., McClanahan, T., Kastelein, R.A., and Cua, D.J. (2005). IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J. Exp. Med. *201*, 233–240.

Lee, P.P., Fitzpatrick, D.R., Beard, C., Jessup, H.K., Lehar, S., Makar, K.W., Perez-Melgosa, M., Sweetser, M.T., Schlissel, M.S., Nguyen, S., et al. (2001). A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. Immunity *15*, 763–774.

Li, M.O., Sanjabi, S., and Flavell, R.A. (2006a). Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. Immunity *25*, 455–471.

Li, M.O., Wan, Y.Y., Sanjabi, S., Robertson, A.K., and Flavell, R.A. (2006b). Transforming growth factor-beta regulation of immune responses. Annu. Rev. Immunol. *24*, 99–146.

Lohr, J., Knoechel, B., Wang, J.J., Villarino, A.V., and Abbas, A.K. (2006). Role of IL-17 and regulatory T lymphocytes in a systemic autoimmune disease. J. Exp. Med. *203*, 2785–2791.

Longenecker, G., Thyagarajan, T., Nagineni, C.N., Flanders, K.C., Factor, V., Miller, G., Ward, J.M., Nalca, A., Rangnekar, V.M., Thorgeirsson, S., and Kulkarni, A.B. (2002). Endocrine expression of the active form of TGF-beta1 in the TGF-beta1 null mice fails to ameliorate lethal phenotype. Cytokine *18*, 43–50.

Lucas, P.J., Kim, S.J., Melby, S.J., and Gress, R.E. (2000). Disruption of T cell homeostasis in mice expressing a T cell-specific dominant negative transforming growth factor beta II receptor. J. Exp. Med. *191*, 1187–1196.

Mangan, P.R., Harrington, L.E., O'Quinn, D.B., Helms, W.S., Bullard, D.C., Elson, C.O., Hatton, R.D., Wahl, S.M., Schoeb, T.R., and Weaver, C.T. (2006). Transforming growth factor-beta induces development of the T(H)17 lineage. Nature *441*, 231–234.

Marie, J.C., Letterio, J.J., Gavin, M., and Rudensky, A.Y. (2005). TGF-{beta}1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. J. Exp. Med. *201*, 1061–1067.

Marie, J.C., Liggitt, D., and Rudensky, A.Y. (2006). Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. Immunity *25*, 441–454.

Mempel, T.R., Pittet, M.J., Khazaie, K., Weninger, W., Weissleder, R., von Boehmer, H., and von Andrian, U.H. (2006). Regulatory T cells reversibly suppress cytotoxic T cell function independent of effector differentiation. Immunity *25*, 129–141.

Murphy, C.A., Langrish, C.L., Chen, Y., Blumenschein, W., McClanahan, T., Kastelein, R.A., Sedgwick, J.D., and Cua, D.J. (2003). Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. J. Exp. Med. *198*, 1951–1957.

Nakamura, K., Kitani, A., Fuss, I., Pedersen, A., Harada, N., Nawata, H., and Strober, W. (2004). TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice. J. Immunol. *172*, 834–842.

Nakamura, K., Kitani, A., and Strober, W. (2001). Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J. Exp. Med. *194*, 629–644.

Paust, S., Lu, L., McCarty, N., and Cantor, H. (2004). Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. Proc. Natl. Acad. Sci. USA *101*, 10398–10403.

Piccirillo, C.A., Letterio, J.J., Thornton, A.M., McHugh, R.S., Mamura, M., Mizuhara, H., and Shevach, E.M. (2002). CD4(+)CD25(+) regulatory T cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness. J. Exp. Med. *196*, 237–246.

Powrie, F., Carlino, J., Leach, M.W., Mauze, S., and Coffman, R.L. (1996). A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. J. Exp. Med. *183*, 2669–2674.

Powrie, F., Leach, M.W., Mauze, S., Menon, S., Caddle, L.B., and Coffman, R.L. (1994). Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. Immunity *1*, 553–562.

Rajewsky, K., Gu, H., Kuhn, R., Betz, U.A., Muller, W., Roes, J., and Schwenk, F. (1996). Conditional gene targeting. J. Clin. Invest. *98*, 600–603.

Rakoff-Nahoum, S., Hao, L., and Medzhitov, R. (2006). Role of toll-like receptors in spontaneous commensal-dependent colitis. Immunity *25*, 319–329.

Sakaguchi, S. (2004). Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu. Rev. Immunol. *22*, 531–562.

Shull, M.M., Ormsby, I., Kier, A.B., Pawlowski, S., Diebold, R.J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., et al. (1992). Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature *359*, 693–699.

Takahama, Y., Letterio, J.J., Suzuki, H., Farr, A.G., and Singer, A. (1994). Early progression of thymocytes along the CD4/CD8 developmental pathway is regulated by a subset of thymic epithelial cells expressing transforming growth factor beta. J. Exp. Med. *179*, 1495–1506.

Taylor, P.A., Lees, C.J., Fournier, S., Allison, J.P., Sharpe, A.H., and Blazar, B.R. (2004). B7 expression on T cells down-regulates immune responses through CTLA-4 ligation via T-T interactions. J. Immunol. *172*, 34–39.

Uhlig, H.H., McKenzie, B.S., Hue, S., Thompson, C., Joyce-Shaikh, B., Stepankova, R., Robinson, N., Buonocore, S., Tlaskalova-Hogenova, H., Cua, D.J., and Powrie, F. (2006). Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. Immunity *25*, 309–318.

Veldhoen, M., Hocking, R.J., Atkins, C.J., Locksley, R.M., and Stockinger, B. (2006a). TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity *24*, 179–189.

Veldhoen, M., Hocking, R.J., Flavell, R.A., and Stockinger, B. (2006b). Signals mediated by transforming growth factor-beta initiate autoimmune encephalomyelitis, but chronic inflammation is needed to sustain disease. Nat. Immunol. *7*, 1151–1156.

Wan, Y.Y., and Flavell, R.A. (2005). Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. Proc. Natl. Acad. Sci. USA *102*, 5126–5131.

Wan, Y.Y., and Flavell, R.A. (2007). Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. Nature *445*, 766–770.

Yaswen, L., Kulkarni, A.B., Fredrickson, T., Mittleman, B., Schiffman, R., Payne, S., Longenecker, G., Mozes, E., and Karlsson, S. (1996). Autoimmune manifestations in the transforming growth factor-beta 1 knockout mouse. Blood *87*, 1439–1445.

Yen, D., Cheung, J., Scheerens, H., Poulet, F., McClanahan, T., McKenzie, B., Kleinschek, M.A., Owyang, A., Mattson, J., Blumenschein, W., et al. (2006). IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. J. Clin. Invest. *116*, 1310–1316.