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Abrogation of TGFβ Signaling in T Cells Leads to Spontaneous T Cell Differentiation and Autoimmune Disease

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Summary

Targeted mutation of TGFβ1 in mice demonstrated that TGFβ1 is one of the key negative regulators of immune homeostasis, as its absence leads to activation of a self-targeted immune response. Nevertheless, because of the highly pleiotropic properties of TGFβ and the presence of TGFβ receptors on most cell types, its biologic role in the regulation of immune homeostasis is not yet understood. To limit the consequences of TGFβ effects to a single cell type, we developed a transgenic approach to abrogate the TGFβ response in key immune cells. Specifically, we expressed a dominant-negative TGFβ receptor type II under a T cell–specific promoter and created a mouse model where signaling by TGFβ is blocked specifically in T cells. Using this transgenic model, we show that T cell homeostasis requires TGFβ signaling in T cells.

Introduction

TGFβ is one of the most widely distributed cytokines that acts on virtually all cell types and mediates highly pleiotropic functions. All three isoforms of TGFβ in mammals share a high level of homology and use the same receptor complex for signaling to exert a seemingly redundant set of functions in vitro (Arai et al., 1990; Massague, 1990). TGFβ1 is a predominant TGFβ isoform in lymphoid organs and is the major TGFβ species constitutively present in serum. Conversely, TGFβ2 and 3 are predominantly expressed in mesenchymal tissues and bones (Pelton et al., 1989; Millan et al., 1991; Schmid et al., 1991). Differential tissue distribution of different isoforms of TGFβ as well as different affinity of these isoforms for the TGFβ receptor (Miyazono et al., 1994) may account for different physiological roles of TGFβ isoforms in vivo. Accordingly, TGFβ1 knockout (KO) mice are able to survive only until 3–4 weeks of age, when they experience spontaneous activation of a self-targeted immune response that leads to their death (Shull et al., 1992; Kulkarni et al., 1993). Since only approximately half of the TGFβ1 knockout mice are born (Kulkarni et al., 1995), TGFβ1 is, apparently, also required for a number of developmental processes, in particular, for hematopoiesis (Martin et al., 1995). TGFβ2 and 3 knockout animals experience a different set of major developmental defects resulting in malformation of bones and internal organs, which leads to embryonic lethality (Kaartinen et al., 1995; Proetzel et al., 1995; Sanford et al., 1997), thus making the study of the role of TGFβ2 and 3 in immune regulation in vivo very difficult.

While an autoimmune phenotype in TGFβ1 knockout mice has been ascribed largely to the presence of activated $CD4^+$ T cells (Christ et al., 1994; Diebold et al., 1995; Letterio et al., 1996), the mechanism leading to the activation of these T cells and the acquisition of their effector function (e.g., cytokine production) has not yet been identified. Spontaneous upregulation of MHC class II and I on multiple tissues was observed to precede lymphocyte infiltration into these tissues and was suggested to play a crucial role in the initiation of the autoimmune response in TGFβ1 knockout mice (Geiser et al., 1993). However, it was unclear whether uncontrolled activation of T cells and autoimmune disease in TGFβ1 knockout mice arises from the lack of control by TGFβ1 on the expression of MHC molecules, thereby leading to improper antigen presentation and T cell activation, or from the loss of direct control by TGFβ1 on the maintenance of T cell homeostasis.

Numerous in vitro studies have also delineated a complicated and in some aspects controversial role of TGFβ in immune regulation (Letterio and Roberts, 1998). One of the major difficulties in defining a specific role for TGFβ in immune regulation comes from the widespread expression of TGFβ and its receptors (Miyazono et al., 1994), which makes it difficult to separate the effects of TGFβ on T cells from its effects on antigen-presenting cells or on nonlymphoid cells. In order to study a role of TGFβ signaling in the regulation of T cells in vivo and in vitro, we have overcome the shortcomings of the above mentioned studies by creating a model in which signaling by all three endogenously produced isoforms of TGFβ can be blocked selectively in T cells. This strategy employs the expression of a dominant-negative form of TGFβ receptor type II (dnTGFβRII). This molecule has a truncated intracellular kinase domain, and although it binds all three isoforms of TGFβ, it is incapable of mediating any signal transduction (Wieser et al., 1993; Wrana et al., 1994). By the use of different cell-specific promoters, this approach has been successfully used to selectively block TGFβ signaling in a variety of cell types (e.g., mammary cells [Bottinger et al., 1997], osteoblasts [Erlebacher et al., 1998], and skin cells [Amendt et al., 1998]). In order to address the role of TGFβ in the regulation of T cell differentiation and activation, we adopted this strategy and created a mouse transgenic for the expression of a dominant-negative form of TGFβ receptor type II expressed under the control of the murine CD4 promoter. This particular CD4 transgenic construct lacks the CD8 silencer, which therefore allows the expression of the transgene in $CD4^+$ as well as $CD8^+$ T cells (Sawada et al., 1994). The resulting transgenic mice have impaired TGFβ signaling specifically and exclusively in T cells. These mice developed autoimmune disease characterized by inflammatory infiltration in several organs and the presence of circulating autoimmune antibodies. In addition, in the absence of TGFβ signaling, most of the T cells differentiate spontaneously into Type 1/Type 2 cytokine secreting cells. We conclude that T

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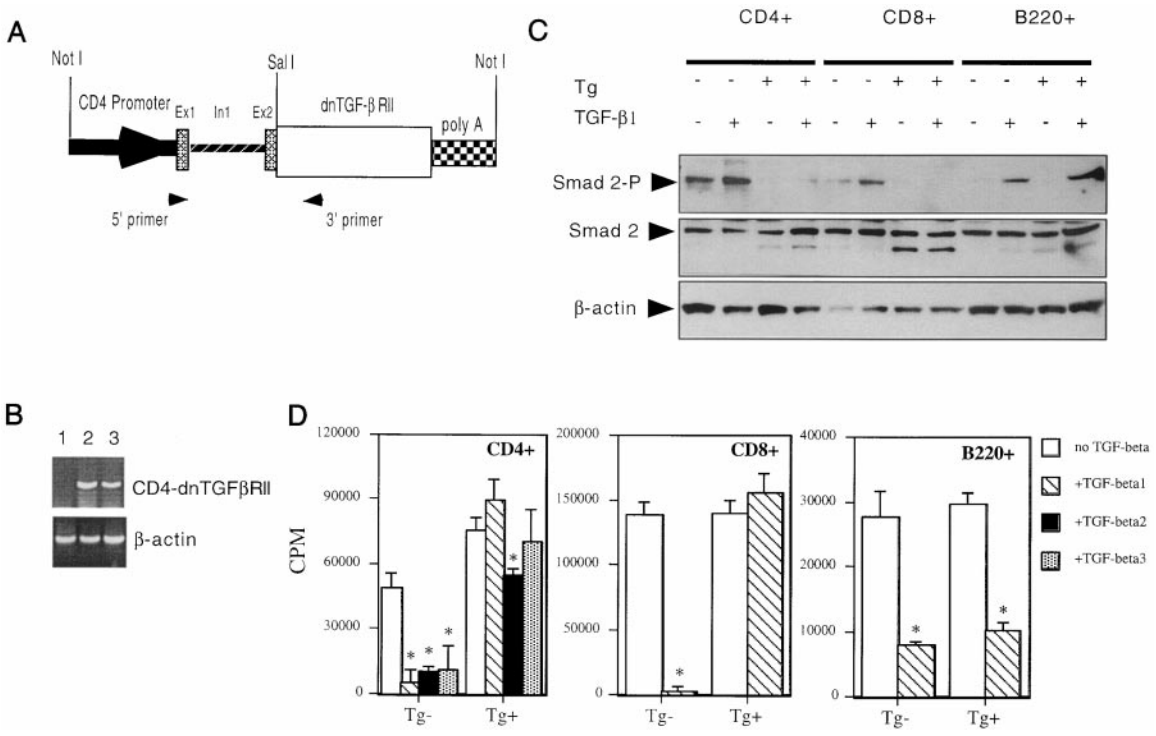

Figure 1. Generation and Characterization of CD4-dnTGFβRII Transgenic Mice

(A) Schematic representation of CD4-dnTGFβRII construct. The open box represents extracellular and transmembrane portions of human TGFβ receptor type II between nucleotides −7 and +573 followed by a stop codon. Primers used for RT–PCR analysis of the transgene expression in transgene-positive mice are also shown.

(B) RT–PCR was performed using the primers indicated in (A) to show the spliced mRNA species of the transgene. Total RNA was extracted from the thymuses of (1) transgene-negative mouse and (2 and 3) progeny of different transgenic founder mice.

(C) Magnetic bead–purified $CD4^+$, $CD8^+$ or unfractionated spleen cells from Tg^+ or Tg^- littermates were stimulated with either anti-CD3/anti-CD28 mAbs (5 μg/ml each) (for $CD4^+$ and $CD8^+$ cells) or LPS (10 μg/ml) (for total splenocytes) in the presence (plus) or absence (minus) of huTGFβ1 (3 ng/ml). After 30 min of culture, total cell lysates were prepared and subjected to gel electrophoresis followed by Western blotting with anti-phospho-Smad2-specific Ab, and blots were stripped and subsequently reprobed with anti-Smad2 mAb and β-actin Ab.

(D) Purified $CD4^+$ or $CD8^+$ from 6-week-old Tg^+ and Tg^- mice were stimulated with anti-CD3 mAb (5 μg/ml) and anti-CD28 (10 μg/ml) precoated plastic wells in 96-well plates for 72 hr in the presence or absence of 3 ng/ml of TGFβ1, TGFβ2, or TGFβ3 (for $CD4^+$), or TGFβ1 for $CD8^+$ or $B220^+$ cells. $B220^+$ cells were stimulated with 10 μg/ml of LPS for 48 hr. One microcurie per well of [^{3}H]thymidine was added for the last 8 hr of culture. One representative experiment out of six experiments is shown. An asterisk indicates statistically significant decrease ($p < 0.01$) in [^{3}H]thymidine incorporation in the presence of exogenously added TGFβ.

cell homeostasis and prevention of inflammatory infiltration require TGFβ signaling in T cells.

Results

Generation of the CD4-dnTGFβRII Transgenic Mice

Dominant-negative TGFβ receptor type II has been previously shown to block TGFβ signaling when expressed at a sufficiently high level by interfering with the assembly of a functional signaling complex consisting of TGFβ and type II and type I TGFβ receptors (Chen et al., 1993; Wieser et al., 1993). In order to study the role of TGFβ in the regulation of T lymphocytes exclusively, we created transgenic mice expressing dominant-negative TGFβ receptor type II (dnTGFβRII) under the control of the CD4 promoter (Figure 1A). This particular CD4 promoter construct lacks the CD8 silencer, thus allowing the expression of a particular transgene in $CD8^+$ as well as $CD4^+$ T cells but not in B cells (Sawada et al., 1994).

Using DNA microinjection into (C57BL/6xC3H)F_1 fertilized oocytes, we obtained six transgene-positive mice. All pups appeared normal at 2 weeks of age, but two of the transgene-positive pups died suddenly between 2 and 3 weeks of age, thus not allowing us to examine causes of their death. One more transgene-positive mouse died at 4 weeks of age and postmortem examination revealed severe multiorgan mononuclear infiltration; the expedited death of that animal did not allow us to investigate the pattern of transgene expression. Three remaining transgene-positive mice appeared healthy and were bred; their progeny was used to identify transgenic lines expressing a functional dominant-negative TGFβ receptor type II. Using RT–PCR with primers spanning the ∼2.4 kb intron region of the CD4 promoter construct, we were able to identify two lines that expressed spliced mRNA encoding the dnTGFβRII (Figure 1B). Since both of these transgenic lines demonstrated a very similar phenotype, we present the results obtained only from one of these transgenic lines. We tested

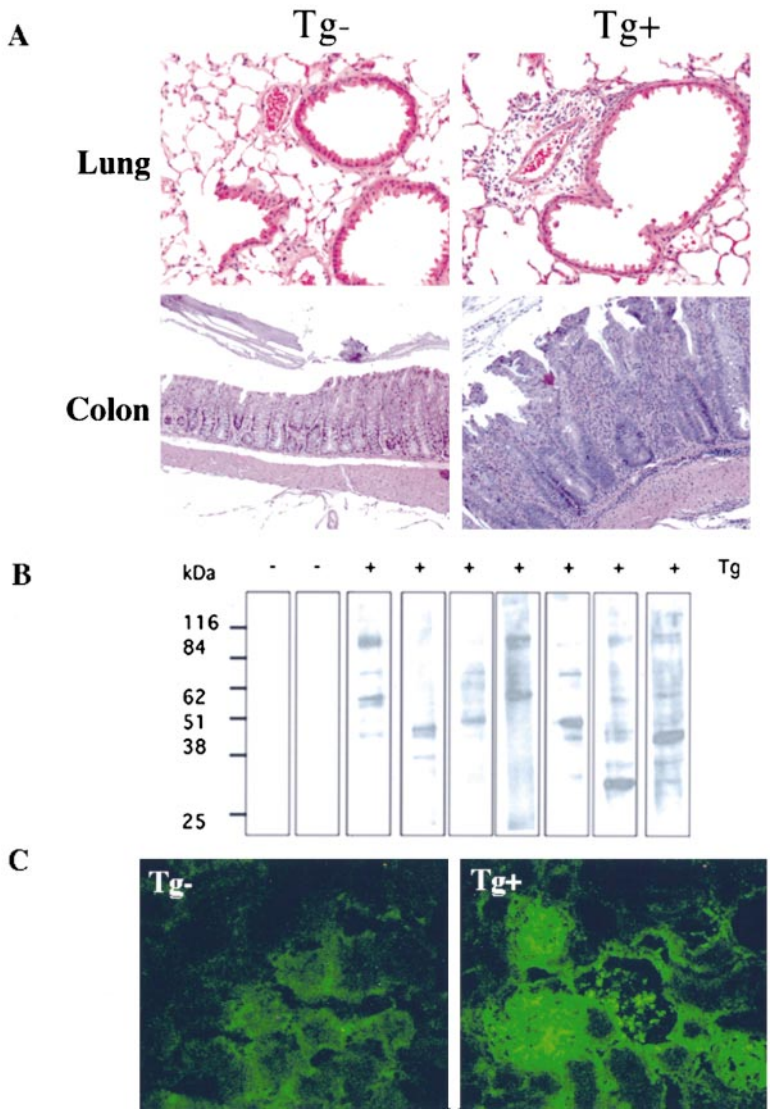

Figure 2. Autoimmune Manifestations in CD4-dnTGFβRII Tg^+ Mice

(A) Lung sections (top panel, 200× magnification) reveal perivascular infiltration of mononuclear cells in lungs of 5-month-old CD4-dnTGFβRII transgenic mice (right panel). Colon sections (bottom panel, 100×) exhibit accumulation of inflammatory cells in lamina propria and intestinal glands. Hematoxylin and eosin staining of tissues from Tg^- littermate mice are shown as a control (left panel).

(B) Nuclear protein extract from murine T lymphocytes was separated on a 10% SDS gel, transferred to the PVDF membrane, and cut into strips; each strip was blotted with 1:150 dilution of serum from an individual 5-month-old mouse. After washing, strips were developed using mouse IgG-specific HRP-labeled rabbit Abs. Sera from two Tg^- and seven Tg^+ mice have been analyzed.

(C) Frozen kidney sections from transgene-negative (left) or transgene-positive mice (right) were stained with FITC-labeled goat anti-mouse IgG. Three mice per group have been analyzed and the representative microscopic view (300× magnification) is presented.

whether the transgenic lines expressed dnTGFβRII on T cells at a level sufficient to block all TGFβ signaling. To do this we used an approach based on the established TGFβ signaling mechanism in which binding of TGFβ to the functional TGFβ receptor type II leads to the phosphorylation of the signaling molecule Smad2 on Ser465/467 residues (Abdollah et al., 1997). As can be seen from Figure 1C, there is a constitutive level of phosphorylated Smad2 present in $CD4^+$ and $CD8^+$ cells from transgene-negative littermates without any addition of exogenous TGFβ to culture medium, presumably as a result of TGFβ present in serum, stimulating these cells. The level of phosphorylation of Smad2 molecule is however increased further after culture with 3 ng/ml of TGFβ1 for 30 min. In contrast, there was no detectable phospho–Smad2 present in $CD4^+$ and $CD8^+$ T cells from transgene-positive animals with or without addition of TGFβ1 to the culture medium. As expected, since the transgene is only T cell specific, there was no change in the levels of phosphorylated Smad2 in B cells from CD4-dnTGFβRII mice relative to B cells from wild-type mice. These results indicated that the level of dnTGFβRII expressed was sufficient to block TGFβ signaling through functional TGFβ receptor in T cells exclusively. We next tested whether the dnTGFβRII transgene could interfere with the known ability of TGFβ to inhibit T cell functions such as proliferation. As can be seen from Figure 1D, as expected (Kehrl et al., 1986), proliferation of $CD4^+$ and $CD8^+$ T cells in response to CD3 and CD28 cross-linking was dramatically inhibited by TGFβ1; at the same time, both T cell subsets from CD4-dnTGFβRII mice were refractory to the inhibitory effects of TGFβ1. On the other hand, LPS-induced proliferation of B cells from CD4-dnTGFβRII mice was inhibited by TGFβ1 to the same extent as proliferation of B cells from transgene-negative littermates. Thus, as expected B lymphocytes not expressing dnTGFβRII are susceptible to the inhibitory properties of TGFβ. We also tested whether T cells from CD4-dnTGFβRII mice are resistant to other isoforms of TGFβ, which could be present in tissues

Table 1. Phenotype of Mononuclear Cells in the Peripheral Lymphoid Organs of 5-month-old CD4-dnTGFβRII Transgene-Positive and -Negative Mice

	Spleen		Lymph Node	
	Tg^-	Tg^+	Tg^-	Tg^+
Total Cell Number	51.3 ± 18.6	85.5 ± 12.0*	11.5 ± 6.1	39.0 ± 12.7*
$CD4^+$	16.4 ± 2.6	12.7 ± 1.8	26.7 ± 2.1	23.0 ± 1.4
$CD8^+$	13.1 ± 0.8	11.0 ± 1.4	22.0 ± 1.7	26.5 ± 2.1
$B220^+$	59.0 ± 1.7	40.0 ± 1.4*	49.0 ± 4.6	49.0 ± 1.4
$Mac\text{-}1^+Gr\text{-}1^-$	1.4 ± 0.8	1.3 ± 0.4	1.0 ± 0.0	1.0 ± 0.0
$NK1.1^+/CD4^+$	9.2 ± 2.8	15.0 ± 1.4	15.0 ± 1.7	18.5 ± 2.1
$CD62L^{high}CD44^{low}/CD4^+$	54.3 ± 4.5	12.5 ± 2.1*	57.0 ± 14.7	16.5 ± 0.7*
$CD25^+/CD4^+$	5.3 ± 1.5	15.0 ± 2.8*	7.7 ± 2.9	15.0 ± 7.1*
$CD62L^{high}CD44^{low}/CD8^+$	37.7 ± 7.8	3.0 ± 2.8*	55.7 ± 19.1	3.0 ± 1.4*
$CD25^+/CD8^+$	18.7 ± 5.1	26.5 ± 13.4	3.7 ± 2.1	6.0 ± 2.8

* Indicates statistically significant ($p < 0.05$) difference between the particular measurement for the Tg^+ versus Tg^- mice. Five 5-month-old mice per group have been analyzed and data are presented as an average ± SD for each group.

infiltrated by T cells. As could be seen from the same figure, again as expected (Cousins et al., 1991; Qian et al., 1996), proliferation of $CD4^+$ cells from wild-type mice was inhibited by TGFβ2 and 3 to the same extent as by TGFβ1. At the same time, transgenic $CD4^+$ cells were completely resistant to inhibition by TGFβ3. There was, however, slight inhibition of proliferation by TGFβ2, probably due to the 10-fold lower affinity of TGFβ2 for TGFβRII than of TGFβ1 or TGFβ3 for the same receptor (Miyazono et al., 1994). These experiments further confirm that there is a sufficient level of dnTGFβRII expressed in T cells from transgenic animals to block the action of TGFβ.

Development of Autoimmune Inflammatory Disease in CD4-dnTGFβRII Mice in the Absence of TGFβ-Mediated Regulation of T Cells

Progeny of mice from two transgenic lines that expressed functional dnTGFβRII lived without any detectable problems until 3–4 months of age, when they began displaying signs of sickness, wasting, and diarrhea. Upon histological examination of tissues from these mice, we observed mononuclear cell infiltration of multiple organs. Specifically, as can be seen from Figure 2A, marked-to-severe inflammatory bowel disease was present in all transgene-positive but not transgene-negative mice analyzed. There was moderate-to-severe infiltration of mucosal propria, subglandular propria, and to a lesser extent muscularis and perivascular subserosa with dense infiltrates of lymphocytes, macrophages, and plasma cells. In addition, there was associated distortion of crypt architecture, crypt abscesses, and superficial thinning and compensatory hyperplasia in crypt bases. Lungs of transgenic animals had focal perivascular and intraalveolar infiltration of lymphocytes and macrophages. In the liver there were clusters of macrophages and lymphocytes located in parenchyma and adjacent to blood vessels. Mild infiltration was also observed in stomach, duodenum, pancreas, and kidney of transgene-positive mice (data not shown). None of these changes were observed in transgene-negative littermates.

TGFβ KO mice have circulating autoreactive antibodies (Yaswen et al., 1996), but because TGFβ is so pleiotropic, it was not clear whether disregulation of T cells or both T cells and B cells was the cause of autoantibody secretion. In order to test whether lack of TGFβ signaling in T cells is sufficient for induction of autoantibody secretion, we tested the serum of CD4-dnTGFβRII mice for the presence of autoantibodies. As can be seen from Figure 2B, Western blots of nuclear protein extract using serum from 5-month-old transgene-positive and -negative littermates revealed the presence of autoreactive antibodies in sera of Tg^+ but not Tg^- mice. Antinuclear antibody reactivity varied from mouse to mouse, indicating that the absence of TGFβ signaling in T cells did not lead to the appearance of a specific immune response to a dominant antigen. Since the presence of autoimmune antibodies frequently leads to formation of immune complexes and their deposition in kidney glomeruli (Dixon et al., 1978), we tested whether this process occurred in CD4-dnTGFβRII transgenic mice. As can be seen from Figure 2C, specific staining of frozen kidney sections with FITC-labeled anti-mouse IgG serum revealed IgG deposits in kidney glomeruli of transgene positive mice but not of their wild-type littermates.

Spontaneous Activation of T Cells in CD4-dnTGFβRII Mice In Vivo

In order to understand how all these histopathological changes are brought about, we focused on the analyses of T cells, that is, the cells rendered insensitive to control of TGFβ in these mice. Phenotypic analyses of cells in the lymphoid organs of these animals revealed an increase in total cell numbers with an especially dramatic increase observed in lymph nodes, which exhibited a 3-fold increase (Table 1). Thymuses of 5-month-old mice were completely involuted as judged by cellular composition and histological appearance, and only thymic lymph nodes were detectable in the area where the thymus is normally present (data not shown). Nevertheless, cellular composition of lymphoid organs from CD4-dnTGFβRII mice was not much different from that of wild-type littermates (Table 1). The percentage of B cells was decreased in the spleen, although the total number of splenic B cells was in fact not changed. The most dramatic alteration in the phenotype of the lymphoid compartment could be seen in the phenotype of T cells; as can be seen from Figure 3, there were almost no $CD62L^{high}CD44^{low}$ naive T cells remaining in the lymphoid

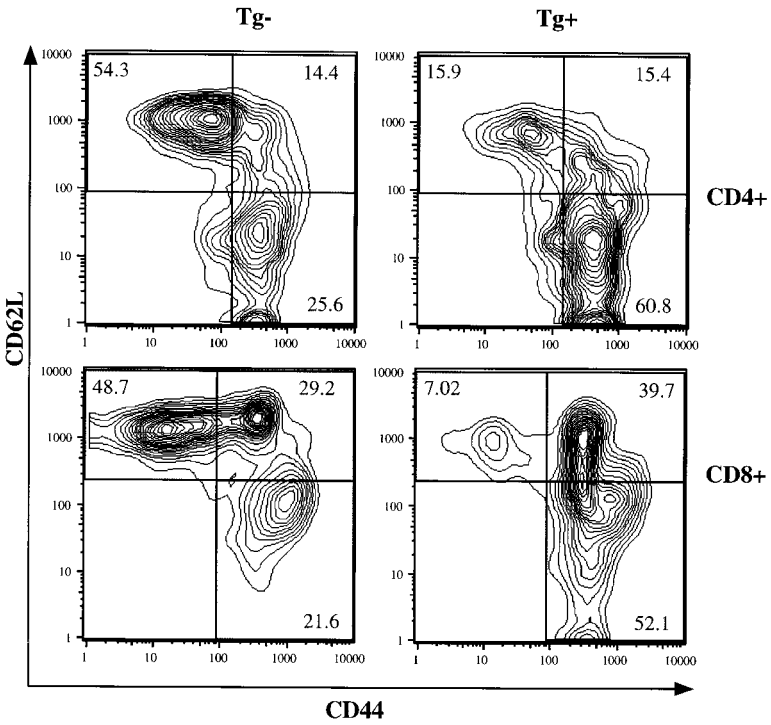

Figure 3. T Lymphocytes in Old CD4-dnTGFβRII Tg^+ Mice Are of Activated/Memory Phenotype

Spleen cells from 5-month-old Tg^- (left panel) or Tg^+ (right panel) littermates were analyzed by flow cytometry for the CD62L and CD44 expression on either $CD4^+$ (top panel) or $CD8^+$ (bottom panel) T cells. Five mice per group have been analyzed individually and results of the analyses of a representative individual mouse per group are presented. Similar results were obtained using lymph node cells.

organs of Tg^+ animals. As judged by the staining for CD25 and CD69, only ~20% of T cells in CD4-dnTGFβRII mice were positive for these markers (Table 1) and hence were activated; since more then 90% of the T cells were $CD44^{high}$, we conclude that the most of the T cells were memory cells.

In order to understand the reason for such changes in the phenotype of T cells, we first had to rule out the possibility that T cell development was abnormal in the absence of TGFβ signaling. As can be seen from Figure 4A, the analysis of very young 4-week-old transgenic mice revealed normal thymic cellularity and subset distribution, and in the periphery there were also no changes in the distribution of cellular subsets. Still, even at this age $CD4^+$ and $CD8^+$ T cells from Tg^+ mice already displayed a decrease in the percentage of cells with a naive $CD62^{high}CD44^{low}$ phenotype (Figure 4B), relative to T cells from Tg^- littermates (e.g., 53.7% ± 4.2% versus 70.6% ± 2.2% for $CD4^+$ T cells in lymph nodes of Tg^+ versus Tg^- mice). This process of loss of naive cells and acceleration in appearance of cells with the activated/memory phenotype was a gradual process in CD4-dnTGFβRII mice and was observed to accelerate as a function of age in these mice (data not shown).

T Cells from CD4-dnTGFβRII Mice Spontaneously Differentiate In Vivo into Effector Cytokine–Producing Cells

We wanted to determine next whether the increase in the percentage of cells with the activated/memory phenotype in transgenic animals was also associated with differentiation of these cells into functional Type 1/2 cells. As can be seen from Figure 5A, activation of T cells from 10-week-old mice with anti-CD3/28 mAbs demonstrated that while cells from Tg^- mice produced mostly IL-2 in a 24 hr period, cells from Tg^+ mice produced both IFNγ and IL-4. In order to determine the proportion of T cells capable of cytokine production, we performed intracellular cytokine staining of T cells from 5-month-old mice. As Figure 5B demonstrates, in contrast to T cells from Tg^- mice, which had only few $CD4^+$ and $CD8^+$ cells capable of IFNγ and IL-4 production, all $CD8^+$ T cells from Tg^+ mice were capable of IFNγ secretion, and the majority of $CD4^+$ T cells were capable of Th1 and/or Th2 cytokine production. It is interesting to note that under experimental conditions, the percentage of T cells from Tg^- mice that was capable of effector cytokine secretion was smaller than the percentage of T cells that carries activated/memory markers. On the other hand, most of the cells from Tg^+ mice were capable of effector cytokine secretion. These observations indicated increased activation/differentiation of T cells in the absence of TGFβ signaling.

Increase in the Level of T Cell–Dependent Immunoglobulin Secretion in CD4-dnTGFβRII Mice

Since CD4-dnTGFβRII mice develop the ability to secret autoreactive IgG antibodies and display T cells with an acquired Th1 and Th2 phenotype, it suggests that there could be an increase in the levels of T cell–dependent antibody secretion. We therefore assessed how the abrogation of TGFβ signaling in T cells affected overall serum immunoglobulin levels. As can be seen from Figure 6, while serum levels of T cell–independent immunoglobulins IgM and IgG3 were not significantly different between transgene-positive and -negative mice, the serum levels of T cell–dependent antibodies were much higher in CD4-dnTGFβRII mice than in wild-type mice. Specifically, the levels of Th2-dependent IgG1 increased

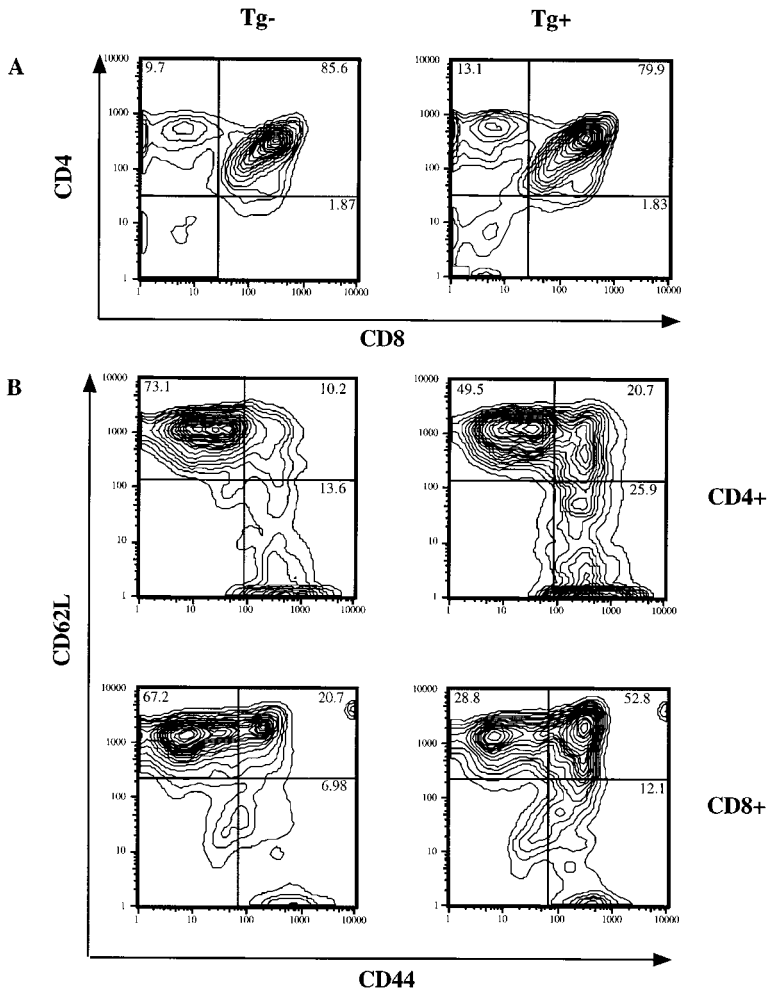

Figure 4. Normal T Cell Development in Young CD4-dnTGFβRII Tg$^+$ Mice

(A) CD4 and CD8 expression on the thymocytes from 4-week-old transgene-positive (right panel) or transgene-negative (left panel) littermates as analyzed by flowcytometry.

(B) Analyses of CD62L and CD44 expression on CD4$^+$ (top panel) and CD8$^+$ (bottom panel) T cells in the lymph nodes of 4-week-old mice. Six mice per group have been analyzed individually, and analyses of a representative individual mouse per group are presented.

8-fold, Th1-dependent IgG2a increased 4-fold, and TGFβ/IL-5-dependent IgA increased 16-fold. These data show that even in the presence of TGFβ signaling in B cells, the absence of TGFβ signaling in T cells leads to disregulation of B cell homeostasis and increased immunoglobulin secretion.

Discussion

The results presented in this paper demonstrate that TGFβ is directly required to maintain T cell homeostasis and that the abrogation of TGFβ signaling in T cells leads to T cell differentiation into effector T cells. Furthermore, we showed that the lack of TGFβ signaling in T cells leads to inflammatory infiltration in multiple organs, immunopathology, and secretion of autoimmune antibodies.

The important role of TGFβ in immune homeostasis has been previously suggested by the observation of multifocal immune-mediated inflammation in TGFβ1 KO mice (Shull et al., 1992; Kulkarni et al., 1993) similar to that which we describe in CD4-dnTGFβRII transgenic mice. Although the phenotype of TGFβ1 KO mice demonstrated the importance of TGFβ in the maintenance of T cell homeostasis, the mechanisms whereby TGFβ prevents "spontaneous" T cell activation have been unclear. Since TGFβ plays an important role in the regulation of MHC levels (Panek et al., 1995; Lee et al., 1997), lack of TGFβ1 can lead to the spontaneous upregulation of MHC class I and II on multiple tissues observed in the TGFβ1 KO (Geiser et al., 1993). Upregulated expression of MHC might, in turn, be responsible for abnormal antigen presentation to T cells and the initiation of self-mediated immune response in these mice. Furthermore, embryonic lethality of more than 50% in the mice (reaching 100% in C57BL/6 mice) indicates that TGFβ1 is crucial for embryonic development. The requirement for TGFβ1 in the development of the hematopoietic system (Martin et al., 1995), and disregulation of hematopoiesis in adult mice (Letterio et al., 1996; Yaswen et al., 1996), makes it very difficult to study the role of TGFβ in regulation of the immune system using TGFβ1 KO animals. Another complicating factor in studying the role of TGFβ in immune regulation using TGFβ1 KO mice is the possibility that TGFβ1 plays an important role in mitochondrial metabolism, as demonstrated by the increase in the number of mitochondria and the decrease in mitochondrial membrane potential in cells of TGFβ1 KO mice (Williams et al., 1996). The reduced mitochondrial potential could adversely effect cellular metabolism and make

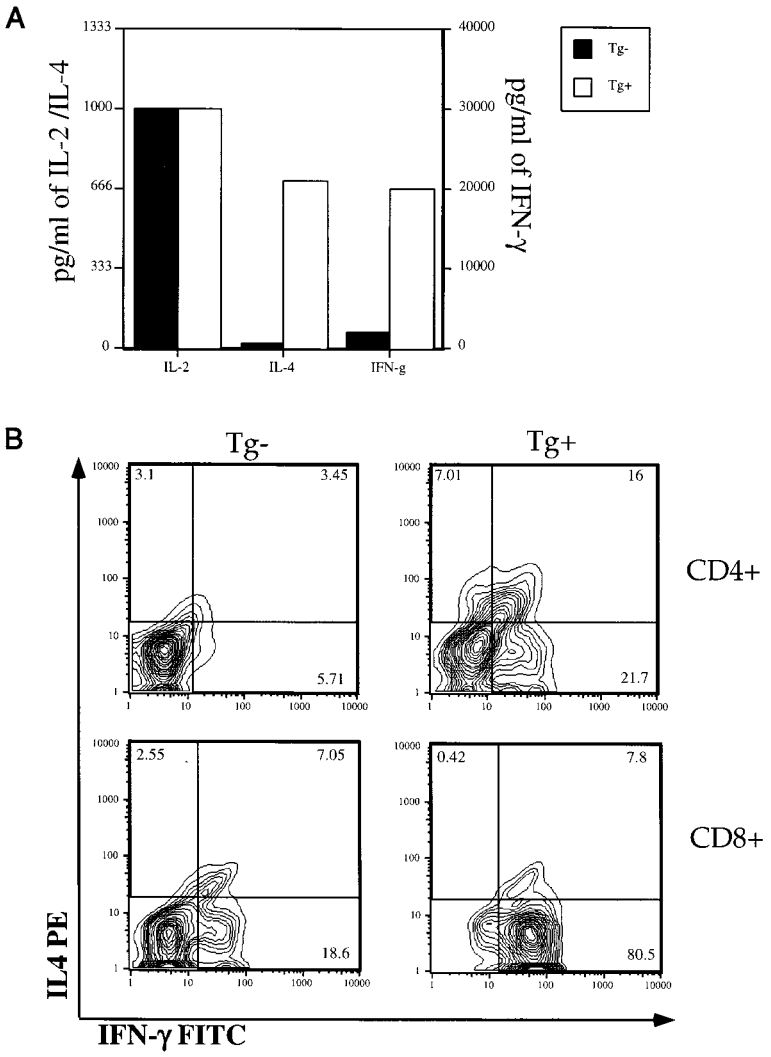

Figure 5. Most T Lymphocytes in Old CD4-dnTGFβRII Tg^+ Mice Differentiate into Type 1 or 2 Cytokine-Secreting Cells

(A) Total spleen cells from 3-month-old Tg^+ or Tg^- animals were stimulated for 24 hr with anti-CD3 (0.1 μg/ml) and anti-CD28 (0.5 μg/ml) mAbs. Cytokines in culture supernatants were assayed using ELISA.

(B) Spleen cells from 5-month-old Tg^- (left panel) or Tg^+ (right panel) littermates were stimulated with PMA/ionomycin for 4 hr, with monensin added for the last 2 hr of stimulation culture. Afterward cells were stained for CD4 and CD8 and fixed/permeabilized prior to the staining with fluorescent anti-IFNγ and anti-IL-4 mAbs. Cells were analyzed by flow cytometry for the IFNγ and IL-4 expression in either $CD4^+$ (top panel) or $CD8^+$ (bottom panel) T cells. Five mice per group have been analyzed individually, and representative results of the analyses of lymphocytes from an individual mouse in each group are presented. Very similar results were obtained using lymph node cells.

cells more susceptible to apoptosis, thus contributing to the lethality of mice. It is also important to note that TGFβ1 KO mice still express TGFβ2 and TGFβ3. These cytokines could provide some compensation for the lack of TGFβ1, thus further compounding the interpretation of the role of TGFβ in regulation of T cells based on the results obtained from TGFβ1 KO mice. The above mentioned drawbacks of the TGFβ1 KO mice make them an impractical model to study TGFβ regulation of the immune response in general and of T cells in particular.

Since TGFβ signaling in CD4-dnTGFβRII mice is affected only in T cells and is normal in all other cell types, these mice lack most of the problems associated with the use of TGFβ1 KO mice as a model for dissecting the role of TGFβ signaling in T cells. It is therefore possible to conclude from our results that disregulation of TGFβ signaling in T cells is sufficient to break their homeostasis and cause their activation. Furthermore, we show that T cell–specific blockade of TGFβ signaling leads to autoimmune disease characterized by multifocal inflammatory infiltration and autoantibody secretion. The importance of T and B lymphocytes in autoimmune disease caused by the lack of TGFβ1 has been previously demonstrated using TGFβ1 KO SCID mice (Diebold et al., 1995). Further studies using MHC class II KO narrowed down $CD4^+$ T cells as mediators of multifocal inflammatory infiltration and autoantibody secretion (Letterio et al., 1996). Although this study did not address the role of TGFβ in regulation of $CD8^+$ T cells, the substantially longer life span of SCID TGFβ1 KO mice versus MHC class II/TGFβ1 double-KO mice indicates that there may be some involvement of $CD8^+$ T cells in mediating the lethality of TGFβ1 KO mice. While both of those studies established the causative role of T cells in mediating pathological inflammatory lesions developed as a result of TGFβ1 deficiency, due to the limitations of TGFβ1 KO system delineated above, both of these studies were incapable of demonstrating the causative role of T cells in initiating the pathological inflammatory response in that system. Using transgenic mice with T cell–specific ablation of TGFβ signaling, we conclude that lack of TGFβ signaling in T cells alone is sufficient to induce T cell–mediated multifocal inflammatory lesions and autoantibody secretion. Although both $CD4^+$ and $CD8^+$ cells are refractory to TGFβ signaling in CD4-dnTGFβRII mice, the results of Letterio et al. (1996) indicating that $CD4^+$ T cells are the major mediators of inflammation in TGFβ1 KO mice, taken together with our results, suggest

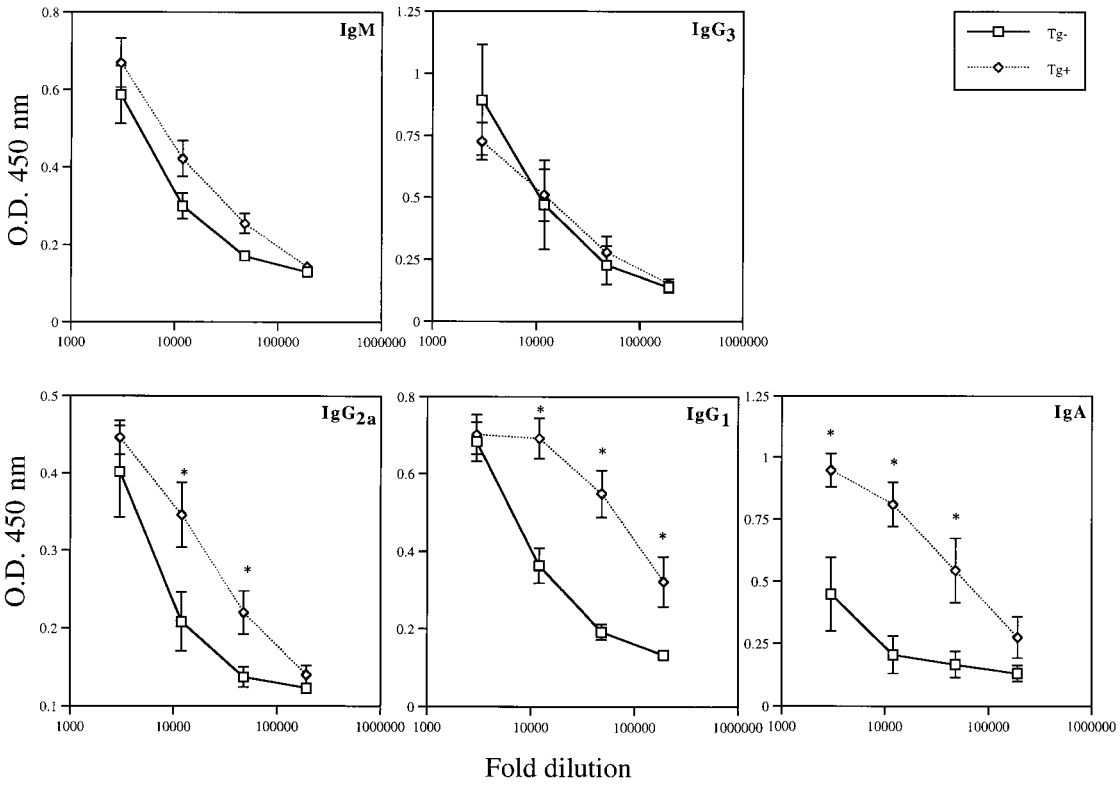

Figure 6. Increase in the Levels of T Cell–Dependent Classes of Immunoglobulins in Sera of CD4-dnTGFβRII Mice

Four 4-fold dilutions of sera from seven 5- to 6-month-old CD4-dnTGFβRII mice or five wild-type littermates were assayed for the levels of IgM, IgG3, IgG1, IgG2, and IgA using ELISA. The results are presented as an average value for all mice in a group ± SE. An asterisk indicates statistically significant increase ($p < 0.01$) in the immunoglobulin level in transgene-positive mice.

that $CD4^+$ cells but perhaps not $CD8^+$ cells are the likely mediators and also initiators of the immune-mediated inflammation in the absence of TGFβ signaling in T cells. Nevertheless, to address this issue unequivocally, we will need to cross CD4-dnTGFβRII mice with various KO mice.

TGFβ1 KO mice develop multifocal inflammation at a much faster rate and severity than CD4-dnTGFβRII mice. The more severe autoimmune phenotype observed in TGFβ1 KO mice relative to that in CD4-dnTGFβRII mice could be caused by the abnormal upregulation of MHC class I and II molecules in tissues of these mice as a consequence of lack of TGFβ control on MHC expression. Furthermore, disregulated homeostasis of multiple tissues in the absence of TGFβ1 could result in exposure and presentation of self-antigens that are normally not presented. The abnormal self-antigen presentation to T cells, together with the lack of TGFβ control of T cells, could lead to the accelerated and more severe autoimmune response in those mice relative to CD4-dnTGFβRII mice, where TGFβ signaling is blocked only in T cells.

Alternatively, it is possible that we do not achieve complete blockade of TGFβ signaling in T cells of both transgenic lines that we derived and studied. Although the signal transduction and T cell activation studies that we performed did not reveal any "leakage" of dominant-negative TGFβRII, we cannot exclude the possibility that our tests are not sensitive enough to detect some residual TGFβ signaling that may still play a role in preventing spontaneous activation of self-reactive T cells, thereby slowing down the development of autoimmune disease in these mice. Nevertheless, even if such low-level TGFβ signaling does occur in T cells from transgenic mice, that still would not change our conclusions, which are based on the finding in these mice that TGFβ signaling in T cells is required for maintenance of T cell homeostasis.

The presence of TGFβ in the lymphocyte environment is essential for maintenance of T cell homeostasis, as we demonstrate that in the absence of TGFβ signaling, T cells spontaneously differentiate into effector cells capable of effector cytokine secretion and the provision of help to B cells. Our results illustrate that even though adult wild-type mice contain a substantial percentage of effector/memory T cells (as determined by CD44 and CD62L expression), it appears that a far smaller percentage of cells is capable of secreting effector cytokines such as IL-4 and IFNγ (as determined by intracellular cytokine staining analysis). These results indicate that in normal mice not all T cells that have undergone the activation process under normal conditions have differentiated into effector T cells. On the other hand, in the absence of TGFβ signaling, most of the T cells from CD4-dnTGFβRII mice have differentiated in vivo into effector T cells capable of secreting IL-4 and/or IFNγ. In addition to the acquisition of cytokine secreting properties, these T cells acquired effector function as indicated by our finding of increased levels of T cell–dependent immunoglobulin levels. Indeed, CD4-dnTGFβRII mice demonstrated elevated serum immunoglobulin levels for both Th1- and Th2-dependent IgG

subclasses relative to that in wild-type mice. It is also interesting to note that CD4-dnTGFβRII mice exhibit an *elevated* level of serum IgA. This contrasts with the TGFβ1 KO mice, which in agreement with the requirement for TGFβ in IgA class switching (Kim and Kagnoff, 1990; McIntyre et al., 1995) exhibit decreased levels of IgA relative to wild-type mice (van Ginkel et al., 1999). This result confirms that B cells in CD4-dnTGFβRII mice are responsive to TGFβ and indicates that there is probably an increase in T cell production of TGFβ in these animals.

Although we currently do not know the nature of the activating stimuli that drive activation and differentiation of T cells in the absence of TGFβ signaling in CD4-dnTGFβRII mice, the presence of autoimmune IgG antibodies in these mice (Figure 2B) indicates that at least some of the T cell response is directed against self-antigens. Since TGFβ signaling is not affected in B cells in CD4-dnTGFβRII mice, the presence of T cell–dependent subclasses of autoantibodies indicates that B cell tolerance depends on T cell tolerance. This suggests that B cells do recognize and present self-antigen under normal nonpathological conditions, and that these B cells will get activated and produce autoantibodies provided they encounter autoreactive T cells such as those in our transgenic mice. Although B cell tolerance to self-antigen has been demonstrated in several systems and was proposed to be an important mechanism for maintenance of self-tolerance, our data support the hypothesis that T cells are more sensitive than B cells to tolerance induction to self-antigens and that breaking of T cell tolerance to self-antigen is sufficient to break B cell tolerance and induce a B cell–mediated self-directed immune-response (Adelstein et al., 1991; Akkaraju et al., 1997). Indeed, it seems that much higher doses of antigen are required to induce B cell tolerance directly (Adelstein et al., 1991).

The results of this paper demonstrate the important role of TGFβ in the regulation of T cell homeostasis and prevention of immune inflammation in many organs. We also provide evidence that TGFβ is an important anti-differentiation factor for T cells under physiological conditions in vivo. In addition, CD4-dnTGFβRII mice provide a good model to study the regulation of various immune responses in the absence of TGFβ signaling in T cells.

Experimental Procedures

Plasmids and Transgenic Mice

The human TGFβ type II receptor sequence between nucleotides −7 and +573, which encodes the extracellular and transmembrane portions of the TGFβ type II receptor (Bottinger et al., 1997), was a generous gift from L. Wakefield. The dnTGFβRII fragment was amplified using primers with flanking SalI restriction sites and cloned into the SalI site of plasmid CD4 promoter vector p37.1 (a gift of D. Littman). The sequence of dnTGFβRII was confirmed by sequencing. In order to generate transgenic mice, the CD4-dnTGFβRII fragment containing CD4 promoter, dnTGFβRII and polyadenylation sequence was excised by NotI, purified, and injected into (C57BL/6xC3H)F_1 fertilized eggs. Founder mice were identified using PCR and backcrossed at least three times onto B10.BR background for further experiments.

RNA Analysis

Total spleen cell RNA was extracted using TRIzol (GIBCO–BRL) and reverse transcribed using Superscript II (GIBCO–BRL). PCR was performed using forward primer from CD4 exon 1 and reverse primer from dnTGFβRII. This primer pair amplifies an approximately 300 bp product from the cDNA sequence; the genomic DNA sequence is 2400 bp longer because of an intron sequence. β-actin primers were used as a positive control for cDNA loading; the sequence of β-actin primers was as described (Gorelik and Mokyr, 1995).

Cell Purification and Culture

$CD4^+$ or $CD8^+$ T cells were enriched from spleen and lymph node cells by negative selection through the depletion using anti-MHC class II (M1/115), anti-NK1.1 (HB191), anti-FcRII (2.4G2), and anti-CD8 (53-6.7) or anti-CD4 (GK1.5) mAbs, respectively, followed by depletion with a mixture of magnetic beads conjugated to anti-rat Ig and anti-mouse Ig antibodies (PerSeptive Biosystems). The purity of cells was usually greater than 90%. For some experiments cells were purified using a cell sorter (Becton Dickinson) by gating on $B220^+TCR\alpha\beta^-$ ($B220^+$ cells), $CD4^+CD44^{low}$ ($CD4^+$ cells) or $CD8^+CD44^{low}$ ($CD8^+$ cells); in that case, the purity of cells was >99%. All mAbs used for staining were from PharMingen. Essentially the same results were obtained using cells enriched by magnetic beads or cells sorted by FACS.

For analyses of Smad2 phosphorylation, 2×10^6 cells of indicated cell populations were incubated with 3 ng/ml TGFβ1 (R&D Systems) in 1 ml complete culture media for 30 min. Afterward, Na_3VO_4 was added to culture medium, and cells were harvested, pelleted, and lysed in lysis buffer (300 mM Tris, 50 mM NaCl, 0.5% Triton X) containing Complete protease inhibitor cocktail (Boehringer-Manheim) and 1 mM Na_3VO_4 (Sigma).

For the cell proliferation assay, 96-well plates (Falcon) were precoated with 5 μg/ml anti-CD3 mAb (2C11) and 10 μg/ml anti-CD28 mAb (37.51) in 20 ml PBS per well for at least 1 hr at 37°C or overnight at 4°C. The antibody solution was aspirated, and 10^5 $CD4^+$ or $CD8^+$ cells per well were placed in 200 μl complete media per well with or without 3 ng/ml recombinant human TGFβ1, TGFβ2, or TGFβ3 (all three cytokines are from R&D Systems). For B cell proliferation analyses, we stimulated 2×10^5 total spleen cells or $B220^+$ cells per well with 10 μg/ml LPS (Sigma) in 200 μl complete culture media with or without 3 ng/ml TGFβ1. Cells were stimulated for 72 hr (for T cells) or for 48 hr (for B cells), 1 μCi/well of [^{3}H]thymidine (Amersham) was added for the last 8–10 hr of cell culture. Background counts of cells alone without any stimulation were lower then 800 cpm.

For cytokine production measurements, 2×10^6 spleen cells were stimulated by addition of 0.1 μg/ml anti-CD3 mAb and 0.5 μg/ml anti-CD28 mAb in 2 ml complete media per well in 24-well plates (Falcon). After 24 hr, supernatants were collected and assayed for cytokine levels by ELISA.

Histopathology and Immunohistochemistry

Animals were sacrificed and selected tissues were fixed in 10% formalin (Sigma) and embedded in paraffin. Five micrometer sections were stained with hematoxylin and eosin and analyzed. For detection of IgG deposits in kidney glomeruli, kidneys were frozen in OCT media and stored at −70°C. Four micrometer cryostat sections were cut and fixed in acetone for 10 min. Kidney sections were stained with FITC-labeled goat anti-mouse IgG (SBA) or FITC-labeled goat anti-rabbit IgG (Santa Cruz) as a negative control. Pictures were taken using a Zeiss Axiovert 25 microscope and a Sony DXC-9000 3CCD color camera.

Western Blot

For the analysis of Smad2 expression, total cell lysates of indicated cell populations were separated on 10% SDS gel transferred to PVDF membrane (Millipore) and probed with anti-phospho-Smad2 (Ser465/467) Ab (Upstate Biotechnology). Amounts of protein were determined by Bio-Rad protein assay to ensure equal protein loading for the analysis. Subsequently, the membrane was striped with 0.1 M Glycine (pH 2.5) for 30 min at room temperature and reprobed with antibodies to total Smad2 (Transduction Laboratories) and β-actin (Santa Cruz).

For the analysis of the presence of anti-ribonuclear protein antibodies in the serum of mice, nuclear extracts were prepared from the purified $CD4^+$ T cells as described (Schreiber et al., 1989). Ten micrograms of nuclear extract per lane was separated on 12% SDS gel, transferred to PVDF membrane, and individual strips were

probed with serum (1:150 dilution) from 5-month-old transgene-negative and -positive mice. Nuclear protein–specific antibodies were visualized with rabbit HRP-conjugated anti-mouse IgG Ab (Amersham). The immune complexes were detected using ECL system (Pierce).

FACS Analyses

Cells (10^6) from spleen, lymph nodes, or thymus were first preincubated with 2.4G2 mAb to block FcγR and then incubated with indicated antibodies for 30 min on ice. The samples were washed and analyzed on FACSCalibur (Becton Dickinson), and 20,000 live cell events were collected. The analyses were conducted using CELLquest (Becton Dickinson) or FloJo (Tree Star) analysis software. All antibodies used for staining were obtained from PharMingen.

For intracellular cytokine staining, single-cell suspensions of spleen or lymph node cells were stimulated with 50 ng/ml PMA (Sigma) and 1 μM ionomycine (Sigma) for 4 hr; monensin (PharMingen) was added for the last 2 hr of stimulation culture. After stimulation, cells were stained with a mixture of Cy-Chrome-labeled anti-CD4 and biotinylated anti-CD8 mAbs, subsequently fixed and permeabilized using a Cytofix/Cytoperm kit (PharMingen), and then stained using FITC-labeled anti-IFNγ mAb and PE-labeled anti-IL-4 mAb and streptavidin-APC (all from PharMingen) according to manufacture's recommendations. Isotype matched FITC- and PE-labeled mAb were used as negative control for intracellular cytokine staining. Fifty-thousand events were collected, and after gating on $CD4^+$ or $CD8^+$ cells, intracellular cytokine staining was analyzed. Gates for cytokine staining were set using isotype-matched control antibody staining.

ELISA

Cytokine levels in tissue culture supernatants were assayed using ELISA antibody pairs for IL-2, IL-4, and IFNγ (PharMingen) according to manufacture's recommendations.

The levels of different immunoglobulin subclasses in mice sera were assayed using immunoglobulin clonotyping system (Southern Biotechnology Associates) according to manufacture's recommendations. Sera from five transgene-negative and seven transgene-positive 5-month-old mice were assayed individually and at the same time using four 4-fold dilutions of sera in PBS with a starting dilution of 1:3000.

Statistical Analyses

Where indicated Student's t test was used to calculate statistical significance for difference in a particular measurement between different groups. P value of <0.05 was considered to be statistically significant.

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