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CUTTING EDGE

# Cutting Edge: TGF-β Signaling Is Required for the In Vivo Expansion and Immunosuppressive Capacity of Regulatory $CD4^+CD25^+$ T Cells[1]

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***Data regarding the role of TGF-β for the in vivo function of regulatory $CD4^+$ $CD25^+$ T cells (Treg) are controversial. A transgenic mouse model with impaired TGF-β signaling specifically in T cells was used to assess the role of endogenous TGF-β for the in vivo function of $CD4^+$ $CD25^+$ Treg in a murine model of colitis induced by dextran sulfate. Transfer of wild-type, but not transgenic $CD4^+$ $CD25^+$ Treg was found to suppress colitis in wild-type mice. In addition, by transferring CFSE-labeled $CD4^+CD25^+$ Treg we could demonstrate that endogenous TGF-β promotes the expansion of $CD4^+$ $CD25^+$ Treg in vivo. Transgenic mice themselves developed reduced numbers of peripheral $CD4^+$ $CD25^+$ Treg and were more susceptible to the induction of colitis, which could be prevented by the transfer of wild-type Treg. These data indicate that TGF-β signaling in $CD4^+$ $CD25^+$ Treg is required for their in vivo expansion and suppressive capacity. The Journal of Immunology*, 2004, 173: 6526–6531.**

A prominent role for TGF-β1 in the homeostatic regulation of the immune system was suggested by the phenotype of TGF-$\beta 1^{-/-}$ mice. These mice develop a rapid wasting syndrome, which leads to death at the age of 3–4 wk (1). The autoimmune phenotype of TGF-$\beta 1^{-/-}$ mice has been largely ascribed to the presence of activated $CD4^+$ T cells (2). However, the precise mechanism of spontaneous T cell activation in the absence of TGF-β1 remains to be elucidated. To analyze the specific effects of TGF-β on T cells, we and others previously created transgenic (TG)[4] mouse models with an impaired TGF-β signaling pathway in T cells by overexpressing a truncated version of the TGF-β type II receptor (ΔuTβRII) under control of T cell-specific promoters (3–5). In one of these models, an autoimmune-like phenotype was observed, although these mice survived at least until 3–4 mo of age without developing overt disease (4). The TG mice generated in our group did not develop any spontaneous autoimmune disease, but showed an increased susceptibility to the induction of allergic and autoimmune diseases (3, 6, 7).

Regulatory T cells (Treg) have been defined, for the lack of a more specific marker, as small $CD4^+CD25^+$ T cells. The presence of these cells has been shown to be essential for the control of various autoreactive T cell responses (8). Ablating the generation of Treg by neonatal thymectomy on day 3 led to the development of autoimmune disease, suggesting the thymus as the site of $CD4^+CD25^+$ Treg generation (9). Recent evidence suggests that $CD4^+CD25^+$ Treg may also be induced in the periphery (10). Of note, TGF-β has been implicated in the conversion of naive $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T cells by the induction of Foxp3 (11, 12). However, it was reported that in TGF-$\beta 1^{-/-}$ mice, $CD4^+CD25^+$ Treg were able to develop up to an age of 2 wk and that autocrine TGF-β1 production was not essential for these cells to exhibit suppressive activity in vivo (13). Furthermore, because of the rapid onset of inflammation in TGF-$\beta 1^{-/-}$ mice (1), it is difficult to study the development of Treg function over time.

Several experimental colitis models have been developed to enhance our understanding of mucosal immune regulation (14, 15). In dextran sulfate sodium (DSS)-induced colitis, mice ingest DSS for 6–10 days and develop an acute colitis that is characterized by diarrhea, gross rectal bleeding, and weight loss. Histologically, the model resembles some features of ulcerative colitis. Macrophages and neutrophils have been identified as the main effector cells of acute DSS colitis (16).

The data presented demonstrate that TGF-β promotes the in vivo expansion and suppressive function of $CD4^+CD25^+$ Treg. These findings indicate that the impairment of TGF-β

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[4] Abbreviations used in this paper: TG, transgenic; Treg, regulatory T cell; DSS, dextran sulfate sodium; LPMN, lamina propria mononuclear; MLN, mesenteric lymph node; WT, wild type.

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signaling in CD4$^+$CD25$^+$ Treg might contribute to the increased susceptibility to autoimmune disease found in hCD2-ΔkT$\beta$RII mice.

## Materials and Methods

### *Animals*

The generation and characterization of TG hCD2-ΔkT$\beta$ RII mice is described elsewhere (3). All TG lines were established and maintained as heterozygotes on a FVB/N background. For colitis induction, age- and sex-matched mice between 8 and 10 wk of age were used. Age-matched nontransgenic littermates were used as controls. Animal care was in accordance with the governmental and institutional guidelines.

### *Induction of DSS colitis*

DSS (ICN Biochemicals, Cleveland, OH) with a molecular weight of 36–40 kDa was dissolved in deionized autoclaved water to provide a working solution of 2.25 or 3% (w/v). Mice were given free access to water substituted with DSS for 9 days.

### *Collection of colonic tissue*

A 0.75-cm segment from the distal colon was removed and embedded in paraffin. Five-micrometer sections were stained with H&E.

### *Histological and endoscopic scoring*

Histological scoring was performed by a pathologist in a blinded fashion as described before (17). The experimental setup for endoscopic scoring consisted of a miniendoscope, a light source, and an air pump to inflate the mouse colon. Scoring was performed in a blinded fashion, giving 0–3 points for vascularity, translucency, and stool.

### *Lymphocyte separation and adoptive cell transfer*

CD25$^+$ lymphocytes from spleens of 7- to 9-wk-old mice were separated with MACS beads. In a second step, CD4$^+$CD25$^+$ cells were sorted with a FACS-Vantage cell sorter (BD Biosciences, Heidelberg, Germany). Purity of cell separation ranged around 95% as assessed by flow cytometry. For some experiments, $7.5 \times 10^5$ CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ T cells were injected i.p. 8 h before colitis induction. For CFSE labeling, cells were incubated in 2 $\mu$M CFSE (Molecular Probes, Leiden, The Netherlands) in PBS at 37°C for 10 min. Isolation of lamina propria mononuclear (LPMN) cells was performed essentially as described previously (14).

### *Flow cytometry*

For flow cytometric analysis, cells were stained with CD25-PE (Miltenyi Biotec, Auburn, CA) and CD4-PE-Cy7 (Caltag Laboratories, Burlingame, CA). Flow cytometry was performed with a FACSCalibur using CellQuest software (BD Biosciences). At least $1 \times 10^6$ cells were analyzed.

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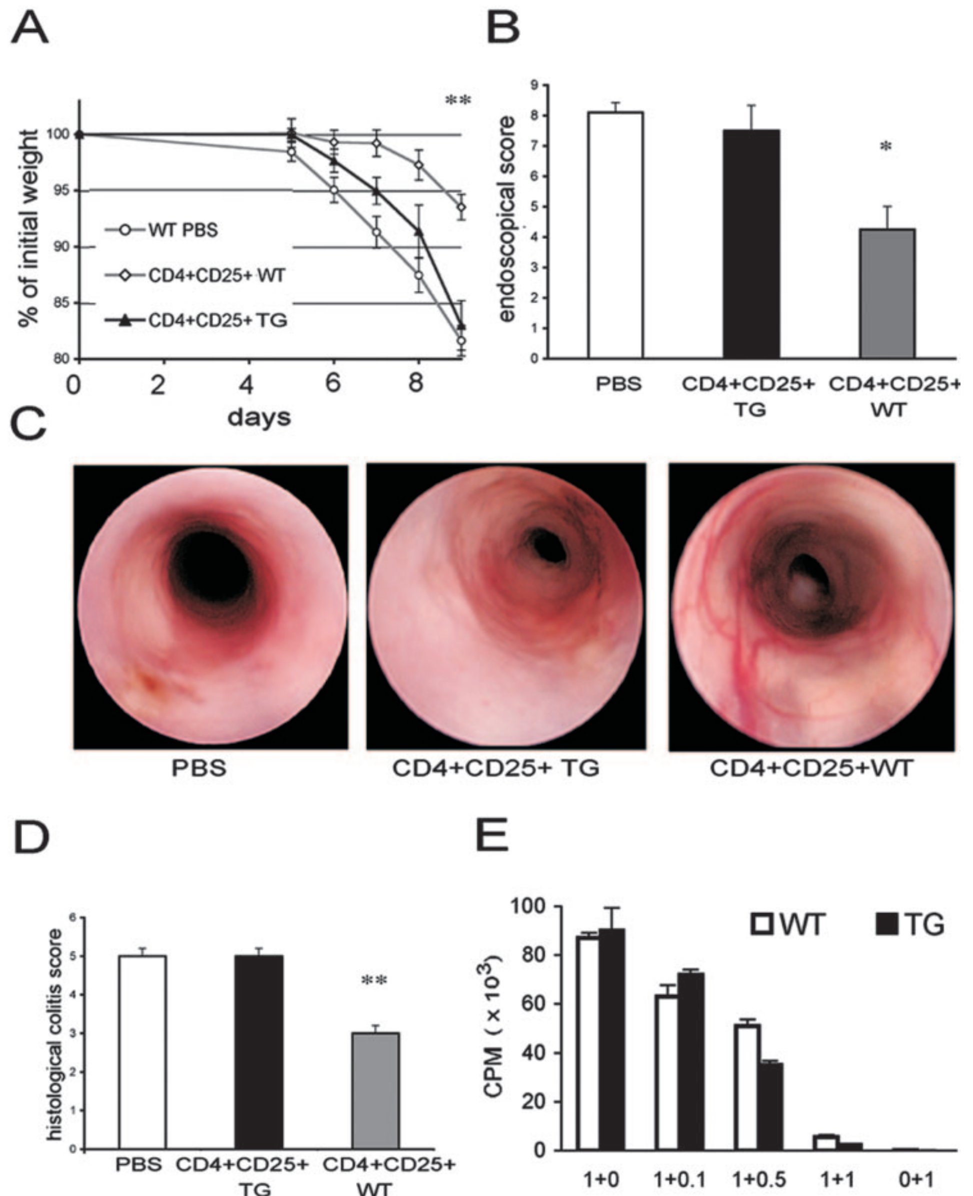


**FIGURE 1.** The suppressive capacity of TG CD4$^+$CD25$^+$ T cells is decreased in vivo. In brief, $7.5 \times 10^5$ WT or hCD2-ΔkT$\beta$RII TG CD4$^+$CD25$^+$ T cells or PBS were injected i.p. 8 h before the induction of colitis in WT mice. *A,* Weight loss relative to the initial weight on day 0 (**, $p < 0.01$). *B,* The endoscopic colitis score was assessed in at least four mice per group (*, $p < 0.03$). *C,* Representative endoscopic photographs demonstrating inflammatory changes, including loss of vascularization in mice injected with PBS or TG CD4$^+$CD25$^+$ T cells. *D,* The histological colitis score was determined in the distal colon (**, $p < 0.01$). At least five animals per group were analyzed in three independent experiments. *E,* The in vitro suppressive capacity of TG and WT CD4$^+$CD25$^+$ T cells is similar. CD4$^+$CD25$^+$ T cells were added to CD25$^-$ WT responder cells at ratios as indicated.

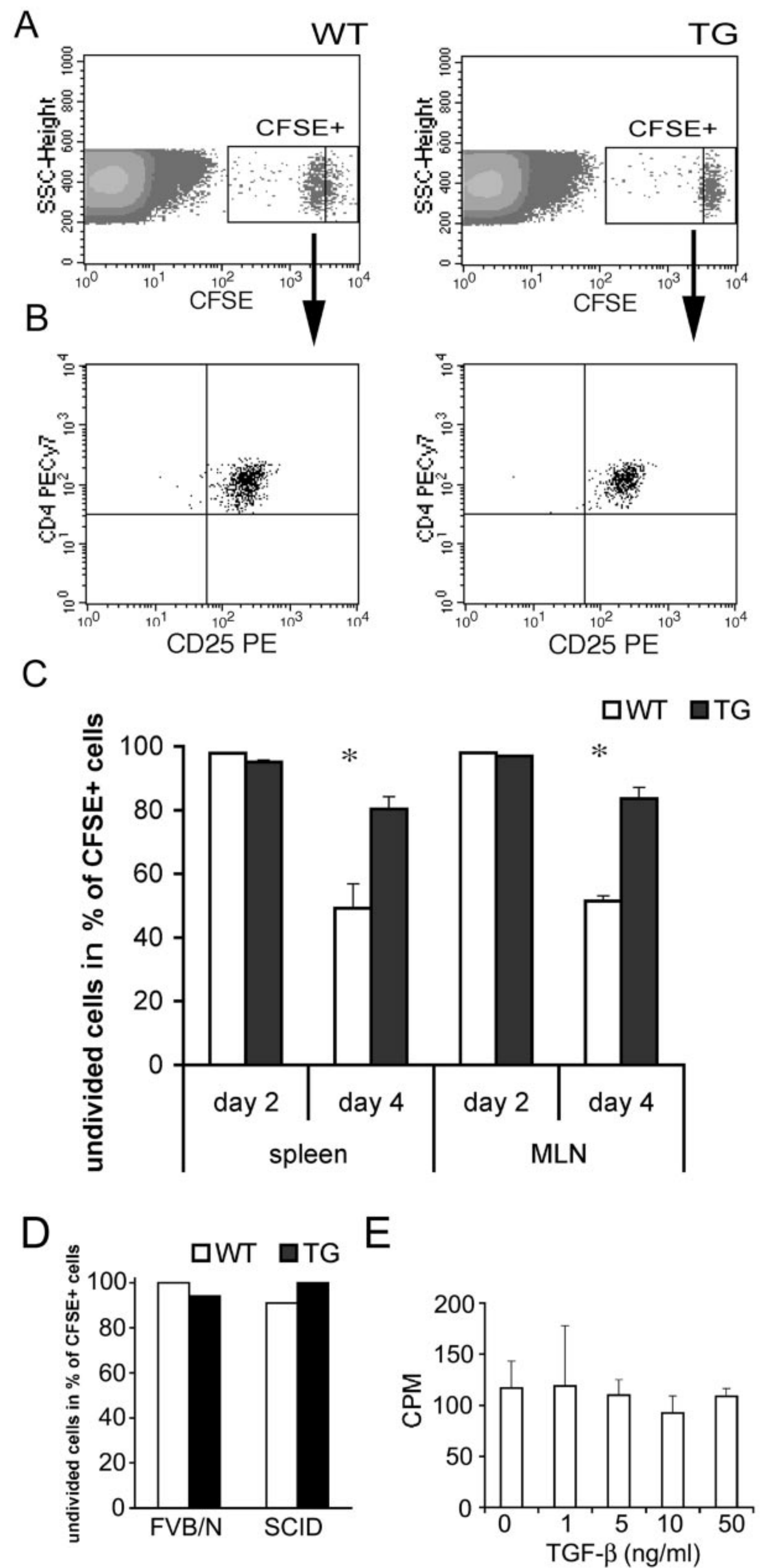


**FIGURE 2.** TGF-β promotes the expansion of CD4+ CD25+ T cells in vivo. WT mice were fed 3% DSS in drinking water (*A–C*) or water only (*D*) over a period of 2 or 4 days. CD4+CD25+ T cells were labeled with CFSE and $1.5 \times 10^6$ cells were injected i.p. 8 h before the induction of colitis. The spleen and MLN were analyzed. *A,* Representative analysis of splenic cells on day 4 of colitis. *B,* The gate is set on CFSE+ cells to confirm that they are CD4+ and CD25+. *C,* Significantly more TG CFSE+CD4+CD25+ Treg remained undivided as compared with WT (*, $p < 0.04$). *D,* Analysis of splenic cells on day 4 after transfer into WT FVB/N or SCID mice. *E,* Effect of TGF-β1 on the in vitro proliferation of allogenically stimulated CD4+CD25+ T cells.

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*Cell culture and cell proliferation assays*

Cell culture was performed essentially as described elsewhere (3). Supernatants were collected after 60 h and frozen in liquid nitrogen. Suppressor assays were performed essentially as described previously (18).

*Cytokine assays*

Concentrations of IFN-γ, IL-5, IL-6, IL-10 (Mouse BD OptEIA ELISA Sets; BD Pharmingen, San Diego, CA), and TNF-α (R&D Systems, Minneapolis, MN) in cell culture supernatants were measured according to the manufacturer's instructions.

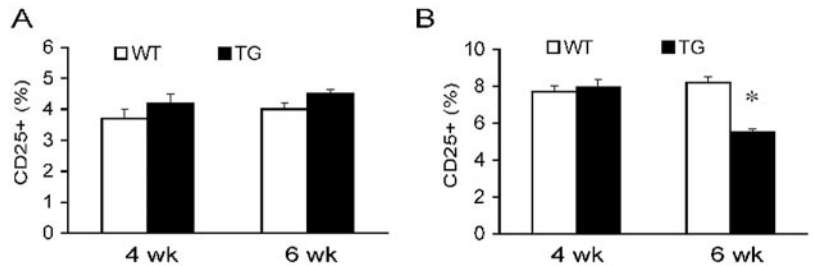


**FIGURE 3.** $CD4^+CD25^+$ T cell numbers are reduced in adult hCD2-ΔkTβRII mice. The relative numbers of $CD25^+$ cells from thymus (*A*) and spleen (*B*) were determined by flow cytometry. $CD4^+CD8^-$ cells were gated. There was no difference in the total cell numbers between WT and TG mice. At least nine littermates were analyzed at each time point (*, $p < 0.03$).

*Statistical analysis*

Mean ± SEM are given. For the comparison of groups, the two-sided Wilcoxon rank sum test was applied and a $p < 0.05$ was considered to be significant. Experiments were repeated at least twice with similar results.

## Results

### *$CD4^+CD25^+$ Treg require TGF-β signaling for their in vivo suppressive capacity*

TGF-β has been suggested as an effector cytokine of $CD4^+CD25^+$ Treg function (19–21). However, the effects of TGF-β on $CD4^+CD25^+$ Treg themselves are largely unknown. Therefore, the suppressive capacity of $CD4^+CD25^+$ Treg isolated from wild-type (WT) and from hCD2-ΔkTβRII TG mice was compared in a transfer model. $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells isolated from spleens of WT or hCD2-ΔkTβRII mice were transferred into WT mice before colitis induction with 3% DSS. WT $CD4^+CD25^+$ Treg mediated a significant protection from colitis, which was characterized by a reduced weight loss and a lower endoscopic and histological colitis score (Fig. 1,*A–D*). In contrast, $CD4^+CD25^+$ Treg isolated from hCD2-ΔkTβRII mice lacked a protective effect (Fig. 1, *A–D*). No in vivo suppressive capacity was observed for $CD4^+CD25^-$ T cells isolated either from WT or TG mice (percentage of initial weight: WT $CD4^+CD25^-$, 85 ± 3.3; TG $CD4^+CD25^-$, 85 ± 2.3; WT PBS, 82 ± 1.4; histological colitis score WT $CD4^+CD25^-$, 5 ± 0; TG $CD4^+CD25^-$, 5 ± 0; WT PBS, 5 ± 0.1; mean ± SEM from at least four animals). Of note, the in vitro suppressive capacity of TG $CD4^+CD25^+$ Treg was not decreased as compared with WT $CD4^+CD25^+$ Treg (Fig. 1*E*), underlining that the in vitro suppressor assays do not adequately reflect the in vivo situation.

### *Endogenous TGF-β promotes the expansion of $CD4^+CD25^+$ Treg in vivo*

To analyze whether the reduced suppressive capacity of TG $CD4^+CD25^+$ Treg was attributable to a defective in vivo migration or proliferation, WT and TG $CD4^+CD25^+$ Treg were labeled with CFSE before transfer into WT mice and colitis induction. On day 2 of colitis, $CD4^+CD25^+CFSE^+$ cells could be found in mesenteric lymph nodes (MLN) and to a lesser extent in the spleen, but not in the blood (data not shown). There was no difference between WT and TG $CD4^+CD25^+CFSE^+$ cell numbers in MLN and spleen at this time point, arguing against a defect in migration induced by the impairment of TGF-β signaling ($CD4^+CD25^+CFSE^+$ cells of total MLN cells: TG, 0.013% vs WT, 0.012%). On day 2 of colitis, most $CD4^+CD25^+CFSE^+$ cells were undivided, but on day 4, only 40–50% of WT, but 80–90% of TG $CD4^+CD25^+CFSE^+$ cells remained undivided (Fig. 2,*A* and *C*). The $CFSE^+$ cells were confirmed to be $CD4^+$ and $CD25^+$ by flow cytometry (Fig. 2*B*). Upon transfer of $CFSE^+CD4^+CD25^+$ cells into healthy WT mice or immunodeficient SCID mice, no difference in proliferation was observed between TG and WT $CD4^+CD25^+$ Treg until day 4 after transfer (Fig. 2*D*). In vitro, TGF-β alone or in combination with IL-2 was not sufficient to induce proliferation of $CD4^+CD25^+$ T cells (Fig. 2*E* and data not shown). These results demonstrate that endogenous TGF-β significantly contributes to the expansion of $CD4^+CD25^+$ Treg in vivo, but indicates the involvement of other mediators of inflammation yet to be identified.

### *Adult hCD2-ΔkTβRII mice develop decreased numbers of peripheral $CD4^+CD25^+$ Treg*

Since the expansion of $CD4^+CD25^+$ Treg was reduced upon transfer and colitis induction, we aimed to analyze $CD4^+CD25^+$ Treg numbers in hCD2-ΔkTβRII TG mice. No difference was observed in thymic $CD4^+CD8^-CD25^+$ T cell numbers between TG and WT mice of various ages (Fig. 3*A*). In peripheral lymphoid organs of BALB/c mice, the numbers of $CD4^+CD25^+$ Treg seem to be maintained from an age of 2 wk onward (22). Accordingly, in FVB/N WT mice between 3 and 7 wk of age, the numbers of peripheral $CD4^+CD25^+$ Treg were relatively stable (Fig. 3*B*). However, in TG mice the numbers of $CD4^+CD25^+$ Treg decreased between the age of 4–6 wk, but remained stable thereafter (Fig. 3*B*).

### *The susceptibility to the induction of colitis is increased in hCD2-ΔkTβRII mice and can be completely reversed by the transfer of WT $CD4^+CD25^+$ Treg*

To support a role for endogenous TGF-β for the functional development of $CD4^+CD25^+$ Treg in vivo, an acute colitis was induced in hCD2-ΔkTβRII mice by feeding 2.25% DSS in drinking water over a period of 9 days. TG mice developed a severe colitis, whereas WT mice developed only a mild colitis at this concentration of DSS (Fig. 4,*A–C*). Furthermore, mononuclear cells isolated from the lamina propria of TG coli showed an increased cytokine production, mainly of IL-6, IL-10, and IFN-γ as compared with WT (Fig. 4*D*).

Since the number and the functional capacity of TG $CD4^+CD25^+$ Treg was reduced, we tested whether the transfer of WT $CD4^+CD25^+$ Treg into TG mice was able to ameliorate colitis. The increased susceptibility of TG mice could be completely reversed by the transfer of WT $CD4^+CD25^+$ Treg 8 h before colitis induction (Fig. 4, *A–C*). The increased cytokine production of TG LPMN cells was markedly reduced by the transfer of $CD4^+CD25^+$ Treg, although not reaching WT levels (Fig. 4*D*). In contrast, no protection was seen after the transfer of $CD4^+CD25^-$ T cells (data not shown).

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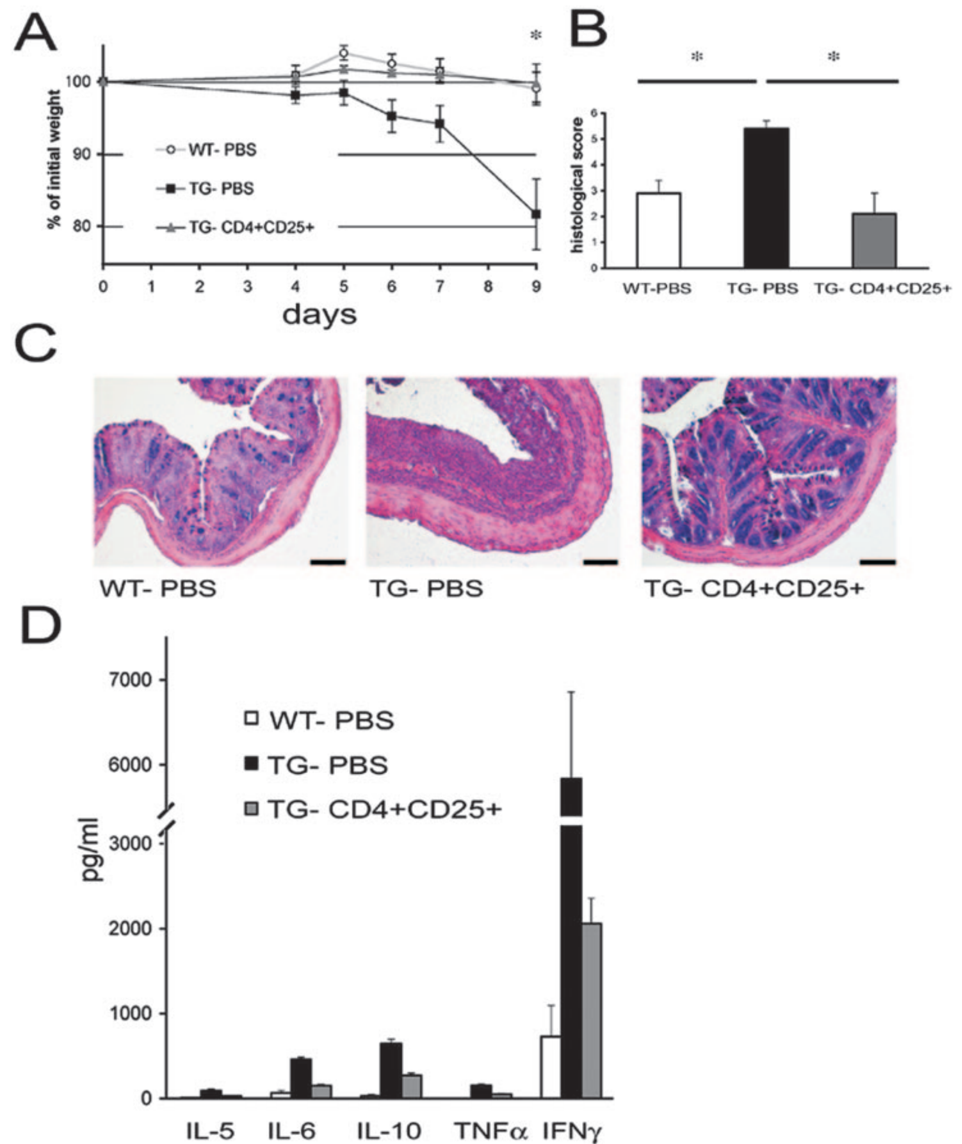


**FIGURE 4.** The increased susceptibility of hCD2-ΔkTβRII mice to DSS-induced colitis is reversed by the transfer of WT $CD4^+CD25^+$ T cells. In brief, $7.5 \times 10^5$ WT $CD4^+CD25^+$ T cells or PBS were injected i.p. 8 h before the induction of colitis. *A,* Weight loss relative to the initial weight on day 0 (*, $p < 0.03$). *B,* Histological score (*, $p < 0.03$). *C,* Representative histological sections of the distal colon are shown. Bars, 100 μm. *D,* Cytokine production of LPMN cells.

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# Discussion

Data regarding the role of TGF-β for the in vivo function of $CD4^+CD25^+$ Treg are controversial. Whereas it was reported that $CD4^+CD25^+$ Treg isolated from TGF-$\beta1^{-/-}$ mice do not protect recipient mice from colitis in the SCID transfer colitis model (20) and that the suppression of islet-specific $CD8^+$ T cells by $CD4^+CD25^+$ Treg is mediated by TGF-β (21), it was also reported that TGF-β1 expression is not essential for the in vivo function of $CD4^+CD25^+$ Treg (13). In the colitis model described, TGF-β signaling in effector T cells was not essential for suppression mediated by $CD4^+CD25^+$ Treg. However, significant differences exist in the TG mice, disease models, and the population of effector cells analyzed. In other experiments, the suppression of inflammation mediated by $CD4^+CD25^+$ Treg could be abrogated by injecting neutralizing TGF-β Ab (23–26).

However, from the experiments using TGF$\beta1^{-/-}$ mice or anti-TGF-β treatment, it remains unclear whether TGF-β is required as an effector cytokine or whether $CD4^+CD25^+$ Treg themselves require TGF-β for their proper in vivo suppressive function. We therefore investigated the role of TGF-β signaling for the function of $CD4^+CD25^+$ Treg. The decreased in vivo suppressive capacity of TG $CD4^+CD25^+$ Treg demonstrates that the effect of endogenous TGF-β on $CD4^+CD25^+$ Treg themselves is essential for their proper in vivo suppressive capacity. It has previously been demonstrated that the expression of Foxp3, a transcription factor essential for the development and function of $CD4^+CD25^+$ Treg, is modulated by TGF-β in vitro (11, 12) and in vivo (18). Therefore, Foxp3 might mediate some of the effects induced by TGF-β.

It was recently reported that TGF-$\beta1^{-/-}$ mice have normal numbers of $CD4^+CD25^+$ Treg after birth, indicating that $CD4^+CD25^+$ Treg are able to develop in complete absence of endogenous TGF-β1 expression (13, 27). However, there is increasing evidence that TGF-β might regulate the peripheral pool of $CD4^+CD25^+$ Treg. In vitro experiments with human naive T cells suggested that TGF-β may expand precommitted $CD4^+CD25^+$ Treg (28). In vivo, it was shown that a transient pulse of TGF-β in the islets during the priming phase of diabetes protected mice from disease and increased the number of intraislet $CD4^+CD25^+$ T cells (29). TGF-β was shown to convert naive $CD4^+CD25^-$ responder T cells to $CD4^+CD25^+$ T

cells in the presence of TCR stimulation (11, 12). We here describe a reduction in the numbers of peripheral $CD4^+CD25^+$ Treg in mice with impaired TGF-$\beta$ signaling in T cells after the age of 4 wk. This could either be due to a defect in the conversion of $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ Treg or to a decreased expansion of preformed $CD4^+CD25^+$ Treg in vivo. We therefore analyzed the proliferative capacity of $CD4^+CD25^+$ Treg with impaired TGF-$\beta$ signaling in a transfer model and could demonstrate that endogenous TGF-$\beta$ promotes the in vivo expansion of $CD4^+CD25^+$ Treg in the setting of inflammation. No difference in the proliferative capacity of TG and WT Treg was observed in vitro or in vivo upon transfer into immunocompetent healthy and immunodeficient SCID mice. This indicates that TGF-$\beta$ signaling alone is not sufficient to induce proliferation in Treg and that in vivo factors induced by inflammation might cooperate with TGF-$\beta$ to induce proliferation.

In conclusion, we demonstrate here for the first time, that the effect of endogenous TGF-$\beta$ on Treg themselves is required for their proper in vivo expansion and in vivo suppressive capacity.

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