# Th3 Cells in Peripheral Tolerance. I. Induction of Foxp3-Positive Regulatory T Cells by Th3 Cells Derived from TGF-$\beta$ T Cell-Transgenic Mice[1]

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**TGF-$\beta$ has been shown to be critical in the generation of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Tregs). Because Th3 cells produce large amounts of TGF-$\beta$, we asked whether induction of Th3 cells in the periphery was a mechanism by which CD4$^+$CD25$^+$ Tregs were induced in the peripheral immune compartment. To address this issue, we generated a TGF-$\beta$1-transgenic (Tg) mouse in which TGF-$\beta$ is linked to the IL-2 promoter and T cells transiently overexpress TGF-$\beta$ upon TCR stimulation but produce little or no IL-2, IL-4, IL-10, IL-13, or IFN-$\gamma$. Naive TGF-$\beta$-Tg mice are phenotypically normal with comparable numbers of lymphocytes and thymic-derived Tregs. We found that repeated antigenic stimulation of pathogenic myelin oligodendrocyte glycoprotein (MOG)-specific CD4$^+$CD25$^-$ T cells from TGF-$\beta$ Tg mice crossed to MOG TCR-Tg mice induced Foxp3 expression in both CD25$^+$ and CD25$^-$ populations. Both CD25 subsets were anergic and had potent suppressive properties in vitro and in vivo. Furthermore, adoptive transfer of these induced regulatory CD25$^{+/-}$ T cells suppressed experimental autoimmune encephalomyelitis when administrated before disease induction or during ongoing experimental autoimmune encephalomyelitis. The suppressive effect of TGF-$\beta$ on T cell responses was due to the induction of Tregs and not to the direct inhibition of cell proliferation. The differentiation of Th3 cells in vitro was TGF-$\beta$ dependent as anti-TGF-$\beta$ abrogated their development. Thus, Ag-specific TGF-$\beta$-producing Th3 cells play a crucial role in inducing and maintaining peripheral tolerance by driving the differentiation of Ag-specific Foxp3$^+$ regulatory cells in the periphery.** ***The Journal of Immunology,*** **2007, 178: 179–185.**

The concept that peripheral immune tolerance is actively maintained has long been recognized using animal models of both transplantation and autoimmune diseases in which tolerance can be transferred by T cells from a tolerant animal to a naive host (1–3). Specialized T cells that inhibit the proliferation and activation of effector T cells are known as suppressor or regulatory T cells (Tregs)[3] and have been best characterized in the CD4$^+$ T cell subset (4–8).

One of the major classes of Tregs is CD4$^+$CD25$^+$ T cells which emerge from the thymus (7, 9) and play a crucial role in the maintenance of self tolerance. CD4$^+$CD25$^+$ Tregs express the transcription factor *Foxp3* gene at relatively high levels and mutations of the *Foxp3* gene in both mice and humans are associated with pronounced immunopathology (10–12). Forced expression of the *Foxp3* gene in CD4$^+$CD25$^-$ non-Tregs induces a suppressive phenotype and Treg-associated markers in these cells (13, 14), indicating that suppressive functions are instructively programmed and that it is possible to convert nonregulatory CD25$^-$ cells into CD25$^+$ regulatory cells as a result of *Foxp3* expression. Such conversion has been demonstrated in vivo in thymectomized mice whose CD25$^-$ T cells in the periphery can be converted into Foxp3$^+$CD25$^+$ T cells by continuous low-dose Ag stimulation (15). TGF-$\beta$ has been suggested as the primary factor that induces *Foxp3* expression from a naive CD4$^+$CD25$^-$ population in vitro (16). Yet, it is not clear whether TGF-$\beta$ is the factor that induces and maintains *Foxp3* expression in the periphery.

TGF-$\beta$ is one of the key molecules contributing to peripheral tolerance (17). TGF-$\beta$-deficient mice have marked inflammation in multiple organs (18) and abrogation of TGF-$\beta$ signaling in T cells alone results in spontaneous T cell differentiation and autoimmune disease indicating an essential role for TGF-$\beta$ signaling in T cell homeostasis and the prevention of inflammatory autoimmunity (19). T cells that produce TGF-$\beta$ are important regulators that control unwanted immune responses in vivo in several disease models in a TGF-$\beta$-dependent fashion (6, 20). We reported a crucial role for TGF-$\beta$ in regulatory T cell function in the rat experimental autoimmune encephalomyelitis (EAE) model (4). Th3 cells that we cloned from orally tolerized mice are class II-restricted T cells that have the identical $\alpha\beta$TCR as Th1 and Th2 cells; they produce a high amount of TGF-$\beta$, low amounts of IL-4 and IL-10, and no IFN-$\gamma$ or IL-2 upon TCR ligation and suppressed EAE in a TGF-$\beta$-dependent fashion (6).

Because several lines of evidence point to TGF-$\beta$ as an important molecule in the induction and maintenance of CD25$^+$ Tregs, we wonder whether in vivo activation of Th3 cells could induce CD25$^+$ Tregs in the periphery. However, it has been extremely difficult to systematically study Th3 cells as they have inherently poor growth characteristics due to the large amounts of TGF-$\beta$ they secrete. To investigate the biology of Th3 cells in terms of their differentiation, phenotype, and function, we created a TGF-$\beta$-transgenic (Tg) mouse in which the *tgf-$\beta$1* gene is under the control of the IL-2 promoter. Previous attempts in which tgf-$\beta$1

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[3] Abbreviations used in this paper: Treg, regulatory T cell; EAE, experimental autoimmune encephalomyelitis; Tg, transgenic; MOG, myelin oligodendrocyte glycoprotein; 7-AAD, 7-aminoactinomycin D; WT, wild type.

was expressed under the control of the human CD2 promoter resulted in the constitutive expression of biologically active TGF-β in T cells, but these animals had abnormal T cell development that included increased T cell apoptosis, reduced proliferation, and thymic atrophy (21). By placing the *tgf-β* gene under the control of the IL-2 promoter which is then subject to negative regulation by TGF-β (22), we succeeded in creating a TGF-β1-Tg mouse that was phenotypically normal and in which the active form of TGF-β-1 was transiently induced in T cells upon TCR ligation. By crossing these mice to myelin oligodendrocyte glycoprotein (MOG)-specific TCR-Tg (2D2) mice, we previously showed that during acute inflammation, TGF-β and IL-6 together induce the differentiation of pathogenic Th17 cells from naive T cells (23). In this study, we demonstrate that in the absence of inflammation, TGF-β alone induces the differentiation of Foxp3$^+$ regulatory cells from the same MOG-specific naive T cells. Thus, Th3 cells induce and establish immune tolerance in the periphery.

## Materials and Methods

### *Generation of genetic mutant mice*

The mouse IL-2 promoter-enhancer region from the *Xho*I site at ~8.4 kb to the *Pst*I site at +45 bp (24), relative to the transcriptional start site, was cloned upstream of a mutated porcine TGF-β1 precursor protein sequence. The porcine TGF-β1 sequence used in the construct corresponds to positions 436-1625 of the 1750-bp cDNA and contains serine residues at amino acid positions 223 and 225, which enables secretion of active TGF-β1 molecules without acid treatment (25). The IL-2 promoter-TGF-β1 sequence was then cut to replace the *GFP* gene in the modified EGFP-1 plasmid (BD Clontech), which contains the 3′ splice and poly(A) site from human β-globin (24). The fragment cut with *Xho*I was isolated (see Fig. 1*a*) and microinjected into the pronuclei of fertilized C57BL/6 oocytes by standard methods at the Brigham and Women's Hospital Transgenic Mouse Core Facility (Boston, MA). Transgenic founders were identified by Southern blot analysis with $^{32}$P-labeled probes using the Stratagene Prime-it II kit (Stratagene). The transgenic founder mice were bred with wild-type (WT) C57BL/6 mice (The Jackson Laboratory). Routine screening to identify the TGF-β1-Tg mice was performed by PCR genotyping of tail DNA using primers specific for intersection of the *TGF-β1* gene with vector (CCAGAGAGTCGTCAGAAGA and GGCGGCCGGCCGGCCA).

To generate Ag-specific T cells that overexpress TGF-β1, TGF-β1-Tg mice were intercrossed with MOG TCR-Tg (2D2) mice (26); this generated 2D2 × TgTGFβ double-Tg mice. The presence of the $MOG_{35–55}$ TCR transgene was confirmed by FACS analysis from tail blood using specific Abs to Vβ11 and/or Vα3.2 (26). All mice were used between age 6–12 wk when >90% of CD3$^+$ cells are CD4$^+$Vβ11$^+$. No spontaneous EAE or optic neuritis was observed in the 2D2 × TgTGFβ mice.

All mice were housed in a specific pathogen/viral-free animal facility at the Harvard Institutes of Medicine. All breeding and experiments were performed in accordance with the guidelines of the committee on Animal of Harvard Medical School.

### *Abs and reagents*

The peptide MOG 35–55 (MEVGWYRSPFSRVVHLYRNGK) were synthesized by D. Teplow (Biopolymer Facility, Center for Neurologic Diseases, Boston, MA). The following reagents were obtained from BD Biosciences: purified anti-mouse CD3 (NA/LETM) Ab, purified anti-mouse CD16/CD32 Ab, and FITC-conjugated anti-mouse TCR Vα3.2 and CD4, PE-conjugated anti-mouse Vβ11 and CD25, and the respective isotypic control mAbs. The following reagents were purchased from R&D Systems: DuoSet ELISA Development system for mouse IL-13, anti-TGF-β1,2,3 mAb, biotinylated chicken anti-TGF-β1, and the respective isotypic control Abs. 7-Aminoactinomycin D (7-AAD) was purchased from Calbiochem.

### *T cell proliferation assay and cytokine ELISA*

For proliferation assays, cells were grown in DMEM 10% FCS supplemented with $5 \times 10^{-5}$ M 2-ME (Sigma-Aldrich), 2 μM L-glutamine, and 100 U/μg penicillin/streptomycin (BioWhittaker) at 37°C and 5% $CO_2$ for 60 h. RBC-lysed whole spleen cells (irradiated, 4000 rad) of C57BL/6 mice were used as APCs as indicated. Cells were pulsed with 1 μCi [$^3$H]thymidine for the last 16 h of incubation and thymidine incorporation was measured using a microbeta liquid scintillation and luminescence counter (PerkinElmer). Data are presented as mean ± SD of triplicate wells.

For cytokine production, T cells ($1.5 \times 10^6$ cells/ml) were grown in X-VIVO 20 medium (BioWhittaker) with soluble anti-CD3 in the presence of APCs ($3 \times 10^6$ cells/ml) in 96-well plates (0.2 ml/well). Cell-free supernatants were collected at 48 h for the determination of IL-2, -4, -5, -10, -13, and IFN-γ production by ELISA using paired mAbs specific for the corresponding cytokines (BD Biosciences). Alternatively, supernatants were acidified before proceed to the measurement of total TGF-β production by ELISA. A standard curve was generated using known amounts of the respective purified recombinant murine cytokines.

### *Cell cultures*

T cells ($1 \times 10^6$ cells/ml) from 2D2 and 2D2 × TgTGFβ mice that stimulated with 20 μg/ml $MOG_{35–55}$ in the presence of APCs ($1.25 \times 10^6$ cells/ml) were either tested for proliferation at 60 h or kept in culture for 5~7 days. Live cells were then harvested and were either restimulated with 20 μg/ml $MOG_{35–55}$ in the presence of APCs or subject to adoptive transfer into WT B6 mice. Alternatively, CD4$^+$CD25$^-$ populations that were separated using a FACSAria Cell Sorter (BD Biosciences; the purity of sorted cells was ~99%) were primed the same way, and restimulated ($0.25 \times 10^6$ cells/ml) with 20 μg/ml $MOG_{35–55}$ in the presence of 40 U/ml recombinant human IL-2 and APCs for 3 days before being rested in fresh medium for an additional 7–10 days. The stimulation and resting cycles were repeated up to three times. In some studies, anti-TGF-β1,2,3 mAb and its isotypic Ab control were added in the primary cultures as indicated.

### *Suppression assay*

Freshly isolated CD4 T cells from 2D2 mice were used as responder cells and cocultured with either naive and primed 2D2 or 2D2 × TgTGFβ T cells or the individual CD25$^+$, CD25$^-$CD4$^+$ subsets that were sorted from 2D2 or 2D2 × TgTGFβ cell lines. Proliferations to 20 μg/ml $MOG_{35–55}$ in the presence of APCs were measured at 60 h.

### *Induction and assessment of EAE in the adoptive transferred recipients*

T cells from 2D2 or 2D2 × TgTGFβ mice were primed as described above. Purified live T cells ($1 \times 10^6$ cells/mouse), or PBS as control, were injected into WT B6 mice i.v. either 2 days before or 10 days after EAE induction. For EAE induction, all recipients were immunized with 100 μg of MOG peptide 35–55 emulsified in CFA (Difco CFA supplemented with 400 μg of *Mycobacterium tuberculosis*) s.c. and injected i.v. on days 0 and 2 with 150 ng of pertussis toxin. Clinical assessment of EAE was performed daily after disease induction according to the following criteria: 0, no disease; 1, decreased tail tone; 2, hind limb weakness or partial paralysis; 3, complete hind limb paralysis; 4, front and hind limb paralysis; and 5, moribund state.

### *Flow cytometry analysis*

T cells were resuspended in PBS containing 1% BSA and 0.1% sodium azide (Sigma-Aldrich). For the staining of surface Ags, cells were incubated with FITC- or PE-conjugated mAbs or their isotype control mAbs as indicated for 20 min on ice. After washing, stained cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences) and quantified with FlowJo (Tree Star) software. Cells were depicted for the florescence intensity of the indicated surface marker.

### *Quantitative RT-PCR*

Naive CD4$^+$CD25$^-$ cells purified from 2D2 or 2D2 × TgTGFβ mice were cultured with 20 μg/ml $MOG_{35–55}$ in the presence of APCs for 0, 48, and 120 h before RNA extraction (TRIzol; Invitrogen Life Technologies) according to the manufacturer's protocol. CD4$^+$CD25$^+$ subsets without stimulation were used as controls. In other experiments, CD25$^+$ and CD25$^-$CD4$^+$ subsets from rested 2D2 or 2D2 × TgTGFβ cell lines were subject to RNA extraction without restimulation. DNase digestion was applied to all samples before RT and quantitative PCR using TaqMan kits (Applied Biosystems). GAPDH mRNA levels was measured (Applied Biosystems) as an internal control. Relative *Foxp* 3 expression ($2^{-\Delta CT\ (Foxp3\ -\ GAPDH)} \times 1{,}000$) was presented as the mean ± SD of triplicate samples, where CT is the cycle threshold.

### *Statistical analysis*

Student's t tests were used for the significance of data comparison in the proliferation assays. Linear regression analysis was used for comparing EAE disease courses. One-way ANOVA analysis followed by a Dunnett's multiple comparison as posttest was used for comparing disease onset and mean clinical scores.

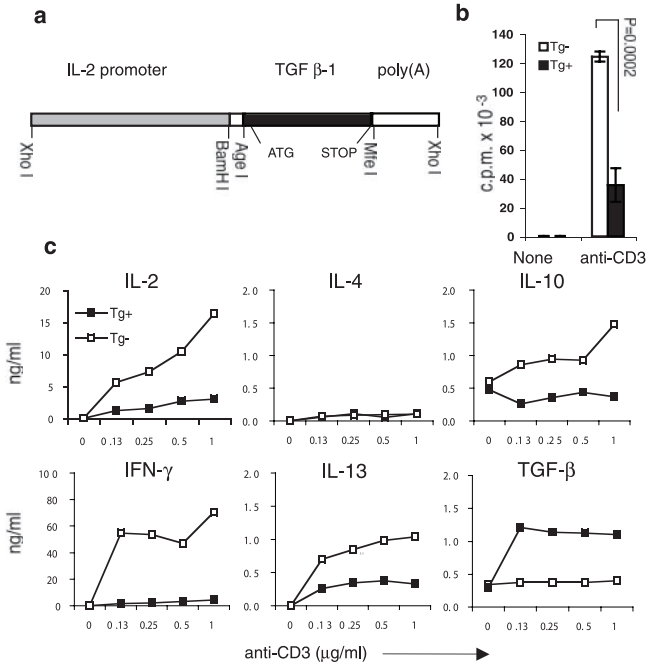


**FIGURE 1.** Reduced proliferation and cytokine production by TGF-$\beta$-Tg T cells. *a*, DNA construct used for production of Tg mice. *b*, Proliferations of T cells ($1 \times 10^6$ cells/ml) from naive $Tg^-$ or $Tg^+$ mice that were stimulated by 0.5 $\mu$g/ml soluble anti-CD3 mAb in the presence of syngenic APCs ($n = 3$/group). *c*, Cytokine productions of these naive $Tg^-$ or $Tg^+$ T cells when stimulated by graded concentrations of soluble anti-CD3 mAb as in *b*. IL-5 could not be detected (data not shown). Data represent one of three independent experiments.

## Results

*Altered cytokine secretion profile in T cells from TGF-β-Tg mice*

Naive mice bearing TGF-$\beta$1 transgene ($Tg^+$) under the control of the IL-2 promoter (Fig. 1*a*) appear normal compared with the Tg-negative ($Tg^-$) littermates. Because there is evidence for IL-2 and IL-2R expression in the thymus, it was possible that T cell development would be impaired in the TGF-$\beta$-$Tg^+$ mice we generated. Nonetheless, T cell development in the thymus was normal as measured by numbers and percentage of CD4, CD8, $CD4^+CD8^+$ and $CD4^+CD25^+$ T cell subsets in the thymus (Table I). Furthermore, the numbers and percentage of each T cell subset as well as B cells, macrophages, and dendritic cells in the peripheral lymphoid organs (spleen and lymph nodes) of naive $Tg^+$ mice were identical with those of normal WT mice (Table I). In addition, we did not observe differences in total serum Ig levels or serum cytokine levels (IL-2, IFN-$\gamma$, IL-4, IL-10, TGF-$\beta$) in Tg vs WT mice.

In contrast to the characteristics of naive T cells from $Tg^+$ mice, when T cells from $Tg^+$ mice were stimulated with anti-CD3, proliferative responses and cytokine production were markedly altered compared with WT mice. As shown in Fig. 1*b*, the proliferative response of $Tg^+$ T cells to soluble anti-CD3 in the presence of APC was markedly decreased compared with the proliferative response in $Tg^-$ littermates. Consistent with this, CSFE staining of $Tg^+$ T cells after anti-CD3 stimulation showed fewer cells entering the cell cycle (data not shown). In addition, as shown in Fig. 1*c*, we observed a decrease in IL-2, IFN-$\gamma$, IL-13, and IL-10 production and an increase in TGF-$\beta$ production in $Tg^+$ as compared with $Tg^-$ T cells. No differences were observed in IL-4 production and IL-5 was not detected in either group. Thus, the increased production of TGF-$\beta$ by $Tg^+$ T cells results in both an impaired proliferative response and a decrease of IL-10 and both Th1 (IFN-$\gamma$) and Th2 (IL-13) cytokines, suggesting that Th0 cells that predominantly produce TGF-$\beta$ are unable to differentiate into Th1-, Th2-, or IL-10-producing cells. These properties of $Tg^+$ T cells are identical with those we observed in myelin basic protein-specific Th3 clones isolated from the mesenteric lymph nodes of orally tolerized SJL mice (6).

*Expression of TGF-β converts Ag-specific T cells into anergic cells with regulatory properties*

To study the differentiation, phenotype, and function of T cells with an identical TCR but with or without the TGF-$\beta$ transgene, we further crossed the TGF-$\beta$-Tg mouse to a TCR-Tg mouse (2D2) in which the majority of the peripheral $CD4^+$ T cells express a clonotypic TCR (V$\alpha$3.2 and V$\beta$11) which recognizes the MOG peptide 35–55/I-$A^b$ (26). Greater than 90% of T cells in the 2D2 $\times$ TgTGF$\beta$ double-Tg mouse are $CD4^+V\alpha3.2^+V\beta11^+$ and thus are specific for $MOG_{35-55}$ peptide.

As we have previously shown, during acute inflammation, TGF-$\beta$ and IL-6 together induce the differentiation of pathogenic Th17 cells from naive 2D2 $\times$ TgTGF$\beta$ cells (23). In the present study, we tested the differentiation of the 2D2 $\times$ TgTGF$\beta$ T cells in the absence of inflammation to examine the effect of TGF$\beta$ alone on the differentiation of T cells. We compared the function of 2D2 $\times$ TgTGF$\beta$ cells before (in the primary culture) and after (in the secondary culture) cell differentiation in vitro with $MOG_{35-55}$ for 5–7 days. 2D2 $\times$ TgTGF$\beta$ T cells that were freshly isolated from naive mice or had been primed in vitro were challenged with $MOG_{35-55}$ in the presence of syngenic APCs. Their proliferative responses were measured and compared with fresh T cells or equally primed T cells from single transgenic 2D2 control mice. Consistent with what we observed above with anti-CD3

Table I. *Lymphocyte subsets in naive TGFβ $Tg^-$ and $Tg^+$ littermates*[a]

| | Thymus | | Spleen | |
|---|---|---|---|---|
| | $Tg^-$ | $Tg^+$ | $Tg^-$ | $Tg^+$ |
| Percent of total lymphocytes | | | | |
| $CD4^+$ | 4.5 ± 2.3 | 6.3 ± 2.5 | 22.8 ± 6.2 | 20.7 ± 3.1 |
| $CD8^+$ | 1.5 ± 0.9 | 2.2 ± 1.4 | 15.6 ± 3.6 | 13.6 ± 2.3 |
| $CD4^+CD8^+$ | 79.0 ± 11.0 | 82.5 ± 3.9 | 0.4 ± 0.2 | 0.2 ± 0.0 |
| $CD19^+$ | | | 56.4 ± 10.5 | 60.6 ± 6.1 |
| $CD11b^+$ | | | 4.0 ± 1.6 | 3.4 ± 0.2 |
| $CD11c^+$ | | | 0.7 ± 0.2 | 0.7 ± 0.3 |
| Percent of $CD4^+$ T cells | | | | |
| $CD25^+$ | | | 8.2 ± 2.2 | 7.1 ± 1.8 |

[a] Data are shown as mean ± SD ($n = 4$). Significance examined by Student's *t* test: no significant difference found in any comparable groups.

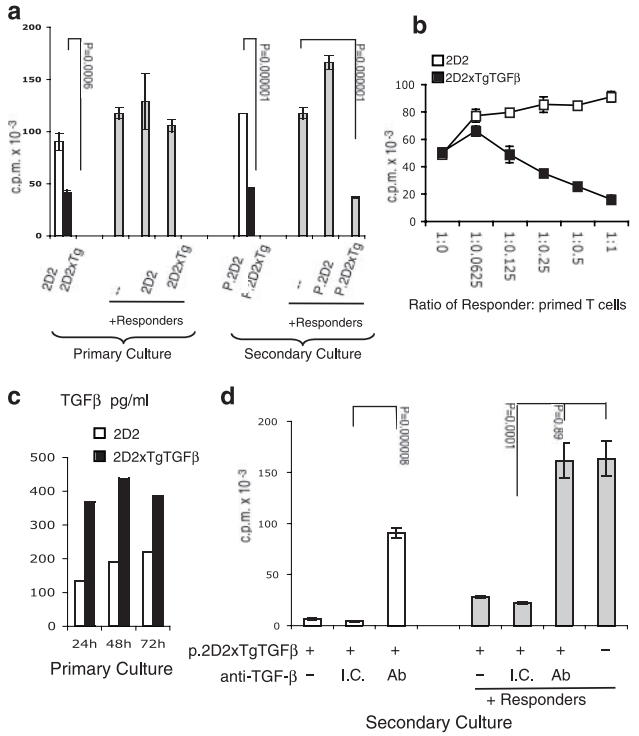


**FIGURE 2.** TGF-$\beta$ overexpression converted naive T cells into hyporeactive/suppressor cells in vitro. *a*, In the primary culture, freshly isolated T cells from naive 2D2 or 2D2 × TgTGF$\beta$ mice were used for self proliferation or in the suppression assay with responder cells. T cells that were primed with 20 $\mu$g/ml $MOG_{35-55}$ in the presence of syngenic APC for 5~7 days were isolated and used as primed cells (p.2D2 or p.2D2 × TgTGF$\beta$) in the secondary culture. For the suppression assay, naive or primed T cells were cocultured with equal amounts of responder cells (freshly isolated 2D2 T cells). All cultures were stimulated with 20 $\mu$g/ml $MOG_{35-55}$plus APC and proliferations were measured at 60 h. The data represent mean of triplicate wells in proliferation assay and the experiment was repeated six times with similar results obtained. *b*, Proliferation of responder cells that were cultured alone or with titrated amounts of primed 2D2 (p.2D2) or 2D2 × TgTGF$\beta$ (p.2D2 × TgTGF$\beta$) T cells. *c*, TGF-$\beta$ secretion in the supernatants of 2D2 and 2D2 × TgTGF$\beta$ T cell primary cultures in response to 10 $\mu$g/ml $MOG_{35-55}$ plus APC was compared at three time points. The data represent mean of duplicated wells in TGF-$\beta$ ELISA and the experiment was repeated three times with similar results. *d*, 2D2 × TgTGF$\beta$ T cells (p.2D2 × TgTGF$\beta$) that had been primed in the presence of no Ab, 10 $\mu$g/ml isotype control (I.C.) or anti-TGF-$\beta$ Ab for 5~7 days were recovered and restimulated either alone or with equal amount of responder cells in the secondary cultures. Proliferation to $MOG_{35-55}$ was measured by 60 h. Data represent one of two independent experiments with similar results.

stimulation of $Tg^+$ T cells (Fig. 1*b*), as shown in Fig. 2*a*, there was a reduction of proliferation of 2D2 × TgTGF$\beta$ T cells compared with the control 2D2 T cells in the primary culture, and the Ag-specific hyporesponsiveness of 2D2 × TgTGF$\beta$ T cells was maintained after a second stimulation (in secondary culture).

To investigate whether the hyporesponsiveness of 2D2 × TgTGF$\beta$ T cells reflected functional paralysis or their immunoregulatory properties, we mixed the MOG-specific responder T cells with 2D2 × TgTGF$\beta$ T cells before and after the 5–7 day in vitro-priming process. As shown in Fig. 2*a* primary culture, when we mixed freshly isolated 2D2 × TgTGF$\beta$ T cells with freshly isolated $CD4^+$ T cells from 2D2 mice (responder cells) and measured proliferation, there was no suppressive effect of freshly isolated 2D2 × TgTGF$\beta$ T cells on the proliferation of responder cells. However, when we tested primed 2D2 × TgTGF$\beta$ T cells that had been differentiated in vitro with $MOG_{35-55}$ for 5–7 days in the same assay (secondary culture), p.2D2 × TgTGF$\beta$ cells suppressed the proliferation of responder cells in a dose-dependent fashion and the inhibitory effect was seen at low responder-suppressor ratios (1: 0.25) (Fig. 2, *a* and *b*). Identically primed 2D2 T (p.2D2) cells proliferated well in the secondary culture and did not have a suppressive effect on responder cells.

It was unexpected that increased amounts of TGF-$\beta$ produced by 2D2 × TgTGF$\beta$ cells in the primary culture would not have suppressive effect on responder cells (Fig. 2*c*). We thus tested whether the acquisition of suppressive properties in the secondary culture was dependent upon the secretion of TGF-$\beta$ in the primary culture. As shown in Fig. 2*d*, priming in the presence of neutralizing anti-TGF-$\beta$ Ab completely abrogated both the anergic and suppressive properties of 2D2 × TgTGF$\beta$ T cells in the secondary culture. These results demonstrate that TGF-$\beta$ present in the primary culture plays an essential role in the maturation and differentiation of Th0 cells into anergic/suppressive T cells in vitro. Such differentiation does not appear to require either IL-4 or IL-10, because minimal amounts of these cytokines were detected in the primary cultures of TGF-$\beta$ $Tg^+$ T cells (Fig. 1*c*).

### *TGF-β induces $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^-Foxp3^+$ Tregs from naive Ag-specific $CD4^+CD25^-Foxp3^-$ peripheral T cells*

TGF-$\beta$ has been suggested to play a major role in inducing *Foxp3* expression in peripheral T cells upon TCR stimulation. Therefore, we asked, upon stimulation of Th3 cells, whether TGF-$\beta$ production induces the expression of transcriptional factor Foxp3 in T cells that differentiate into regulatory cells. We first examined the expression of the *Foxp3* by quantitative real-time PCR in splenic $CD4^+CD25^-$ T cells from both 2D2 × TgTGF$\beta$ and 2D2 mice following activation. *Foxp3* expression was markedly increased when the $CD4^+CD25^-$ 2D2 × TgTGF$\beta$ T cells were activated by $MOG_{33-35}$ in the presence of syngeneic APC, a magnitude that was ~45 times greater than that of $CD4^+CD25^-$ 2D2 T cells at 120 h after activation (Fig. 3*a*). Furthermore, if $CD4^+CD25^-$ 2D2 × TgTGF$\beta$ cells and 2D2 T cells were repeatedly stimulated and rested for 13 days in between, the ratio of *Foxp3* mRNA in the resting 2D2 × TgTGF$\beta$ T cells vs 2D2 T cells increased with each round of stimulation (Fig. 3*b*). These data demonstrate that TGF-$\beta$ secreted by T cells upon activation induced *Foxp3* gene expression in these cells and repeated Ag stimulation in the presence of TGF-$\beta$ further committed these cells to the regulatory lineage as defined by *Foxp3* expression.

Because acquisition of Foxp3 is often associated with surface expression of CD25 and is essential for the development and function of Tregs (13, 14), we asked whether the expression of TGF-$\beta$ in T cells would convert $CD4^+CD25^-$ peripheral T cells into $CD4^+CD25^+$ Tregs. We found no increase in the expression pattern or intensity of surface hallmarks of Tregs, such as CD25, glucocorticoid-induced TNFR, CTLA-4, and OX-40 on either naive, activated, or resting 2D2 × TgTGF$\beta$ T cells that had been stimulated for several rounds as compared with the 2D2 controls (data not shown). Fig. 4*a* is one example of CD25 expression on 2D2 and 2D2 × TgTGF$\beta$ T cells that underwent three rounds of stimulation and resting. There was a higher percentage of $CD25^+$ vs $CD25^-$ T cells in the 2D2 lines, whereas there were equal numbers of $CD25^-$ and $CD25^+$ T cells in the 2D2 × TgTGF$\beta$ cell line. Nevertheless, when we separated these two subsets by FACS sorting and tested them both for regulatory function in vitro and for *Foxp3* expression, we found both $CD25^-$ and $CD25^+$ subsets of 2D2 × TgTGF$\beta$ $CD4^+$ T cells were anergic and both suppressed the proliferation of responder cells (Fig. 4*b*). *Foxp3* mRNA was constitutively higher in the $CD25^-$ and $CD25^+$ subsets of the 2D2 × TgTGF$\beta$ cells (resting stage) when compared with the same subsets

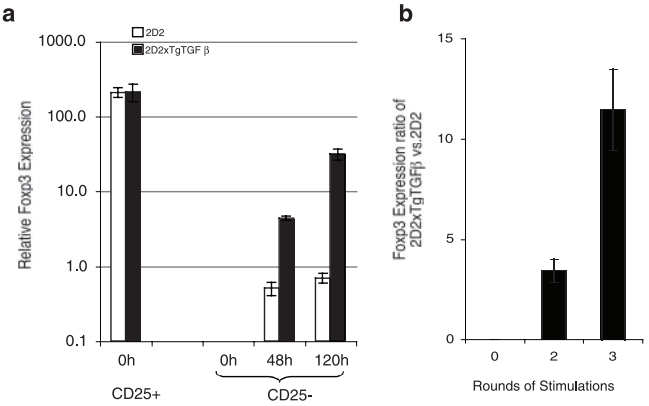


**FIGURE 3.** TGF-$\beta$ mediates *Foxp3* induction in CD4$^+$CD25$^-$ Th3 cells. *a*, cDNA from 2D2 and 2D2 $\times$ TgTGF$\beta$ CD4$^+$ CD25$^+$ or CD25$^-$ T subsets with or without stimulation with 20 $\mu$g/ml $MOG_{35-55}$ in the presence of APC for indicated hours were subjected to real-time quantitative PCR using *Foxp3* or GAPDH-specific primers and probes. Relative *Foxp* 3 expression (compared with GAPDH) is presented as the mean $\pm$ SD of triplicate samples. The data are representative of three experiments. *b*, Relative expression of *Foxp* 3 in resting 2D2 or 2D2 $\times$ TgTGF$\beta$ T cells that had been stimulated for zero, two, or three rounds were measured by quantitative PCR. The ratio of relative gene expression of 2D2 $\times$ TgTGF$\beta$ vs 2D2 T cells is shown. The data are representative of three independent experiments.

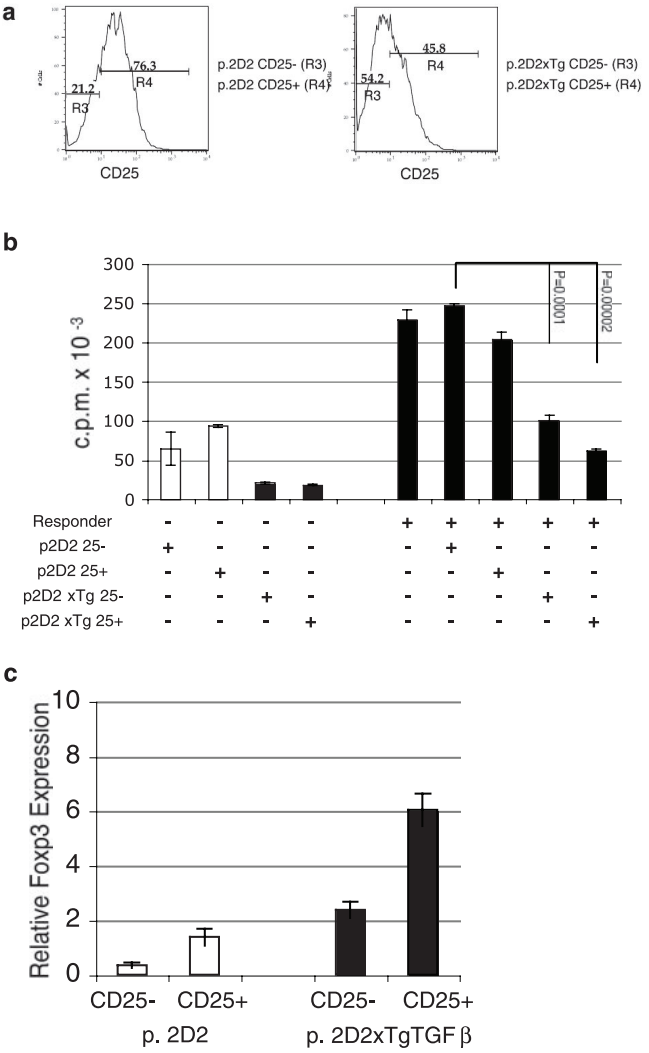


**FIGURE 4.** Both CD25$^+$ or CD25$^-$ CD4$^+$ Th3 cells are suppressors and express *Foxp3* mRNA. *a*, CD4$^+$CD25$^-$ T cells from 2D2 and 2D2 $\times$ TgTGF$\beta$ mice were repetitively stimulated with $MOG_{35-55}$ and rested for three rounds. At day 13 after last stimulation, cells were stained with FITC-anti-CD4, PE-anti-CD25 mAb, and 7-AAD and gated on CD4$^+$7-AAD$^-$ cells. Four populations of cells (CD25$^-$ and CD25$^+$ of p.2D2, CD25$^-$ and CD25$^+$ of p.2D2 $\times$ TgTGF$\beta$) were isolated by FACS sorting and the percentage of each population before sorting is depicted in the graph. *b*, The individual populations ($6.25 \times 10^4$) were restimulated with 20 $\mu$g/ml $MOG_{35-55}$ and APCs ($2.5 \times 10^5$) in 0.2 ml of culture alone or with responder cells ($1.25 \times 10^5$) and proliferative response to $MOG_{35-55}$ were measured. *c*, Relative *Foxp* 3 expression of the CD25$^-$ and CD25$^+$ subsets that were sorted from resting p.2D2 or p. 2D2 $\times$ TgTGF$\beta$ after three rounds of stimulations. The data are representative of three independent experiments.

of 2D2 T cells (Fig. 4*c*). Taken together, these results suggest that TGF-$\beta$ secreting 2D2 $\times$ TgTGF$\beta$ cells differentiate into a unique regulatory population in which both the CD25$^-$ and CD25$^+$ subset have suppressive properties.

### *TGF-$\beta$ induced autoreactive Th3 cells ameliorate EAE upon adoptive transfer*

To investigate whether, as demonstrated by the in vitro assay, differentiated 2D2 $\times$ TgTGF$\beta$ T cells could suppress the activation of pathogenic T cells that are specific for the encephalitogenic MOG peptide, their in vivo regulatory properties were examined in the MOG-induced EAE model. Primed 2D2 or primed 2D2 $\times$ TgTGF$\beta$ T cells were transferred into naive WT B6 mice ($1 \times 10^6$ cells/mouse) 2 days before s.c. immunization with $MOG_{35-55}$/CFA followed by pertussis toxin. Recipients of p.2D2 $\times$ TgTGF$\beta$ cells had a lower disease incidence, a delay in disease onset, and reduced mean clinical scores compared with the PBS or p.2D2 control (Fig 5*a* and Table II). We then tested whether in vitro-differentiated 2D2 $\times$ TgTGF$\beta$ T cells could suppress EAE when given after encephalitogenic cells were induced and again compared them to the control-primed 2D2 population. As shown in Fig. 5*b*, transfer of as few as $1 \times 10^6$ primed 2D2 $\times$ TgTGF$\beta$ cells on day 10 after immunization reduced disease incidence, day of onset, and severity as compare with PBS and the control-primed 2D2 cells. Further analysis of immune responses in recipients showed decreased proliferative and IFN-$\gamma$ production by host CD4$^+$ T cells upon rechallenge by $MOG_{35-55}$ ex vivo (data not shown), confirming that the regulatory activity of primed 2D2 $\times$ TgTGF$\beta$T cells was on autologous, Ag-reactive CD4$^+$ T cells. These data demonstrate that differentiated 2D2 $\times$ TgTGF$\beta$ cells could not only prevent the induction of EAE but also control the progression of the disease after it was induced.

## Discussion

TGF-$\beta$, a cytokine with marked immunosuppressive properties, has recently been reported to have an essential role in both inducing and maintaining *Foxp3* in CD25$^+$ Tregs (16, 27). The observation that the number of CD25$^+$ Tregs is increased in the periphery by the tissue-specific expression of TGF-$\beta$ in the pancreas (28) or following oral tolerance (29, 30) raises the question about the relationship of CD25$^+$ Tregs and Th3 cells in the peripheral T cell repertoire. To answer this question and to obtain a homogenous source of Th3 cells, we generated a transgenic model that dominantly, but transiently, produces TGF-$\beta$ following TCR ligation. A similar transgenic model has been used to successfully elucidate the role of T cell-derived IL-10 in immune responses (31). We found that repeated antigenic-specific stimulation of naive peripheral CD4$^+$CD25$^-$ T cells from TGF-$\beta$-Tg$^+$ mice in vitro induces *Foxp3* expression in both the CD25$^-$ and the CD25$^+$ fractions of

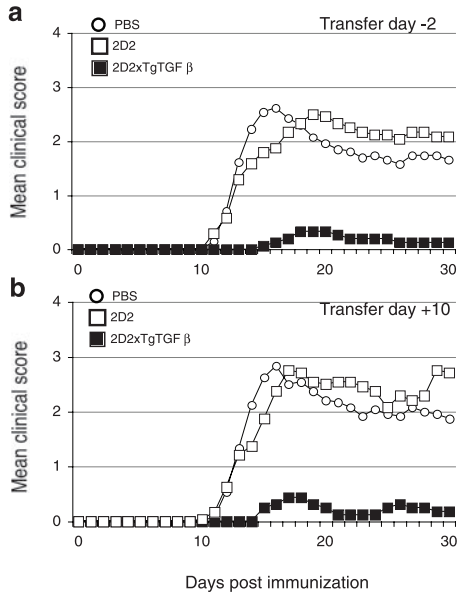


**FIGURE 5.** EAE clinical courses of WT recipients of regulatory Th3 cells. p.2D2 and p.2D2 × TgTGFβ T cells were injected i.v. into WT B6 mice 2 days before (*a*) or 10 days after (*b*) EAE induction. The third group received PBS and served as controls. Mice were monitored for clinical signs from the day of EAE induction. Accumulated results from three to four independent experiments are depicted as mean clinical score of each group in the graphs. Linear regression analysis of the individual disease curves was used. ($p < 0.0001$: p.2D2 × TgTGFβ vs PBS or vs p.2D2; $p > 0.05$: p.2D2 vs PBS).

T cells, which in turn programs both subsets to become anergic with potent suppressive activities in vitro and in vivo. In contrast, naive $CD4^+CD25^-$ T cells from non-TGF-β-Tg littermates with identical MOG-specific αβTCR differentiated into effector cells that enhance EAE after adoptive transfer. The generation of $Foxp3^+$ Th3 cells was further demonstrated in vivo by using the IL-2-deficient mouse model (accompanying article to this report (32)), in which the immune deficiency of these mice was corrected by crossing TGF-β transgene into the IL-2-deficient mice. Therefore, TGF-β is a key element that influences T cells during the maturation phase and instructs cells to differentiate into $Foxp3^+$ Tregs.

Although TGF-β is a well-recognized immunoregulatory cytokine, the mechanism by which TGF-β-secreting T cells regulate immune responses is not well-defined. In our system, the high level of TGF-β production by $Tg^+$ T cells in the primary culture inhibits these cells from entering the cell cycle (33, 34) but had no direct inhibitory effect on the proliferation of other cells (responders) in primary coculture (Fig. 2*a*). This indicates that one of the mechanisms by which T cell-derived TGF-β regulates the immune response is by inhibiting cell proliferation in an autocrine fashion, as opposed to a paracrine fashion that requires a high level of exogenous TGF-β (35). However, after T cell differentiation, under the influence of TGF-β, primed $Tg^+$ T cells demonstrated regulatory capacity in vitro and in vivo. Of note, because the TGF-β transgene is under the control of IL-2 promoter and TGF-β negatively regulates the IL-2 promoter (22), the secretion of TGF-β from the transgene is transient. After priming, $Tg^+$ T cells no longer produce more TGF-β than $Tg^-$ cells and suppression of responders is not due to excessive TGF-β. Therefore, the suppressive effect of TGF-β on T cell responses is primarily due to the induction of Tregs, but not direct inhibition in a paracrine fashion.

The crucial role of TGF-β in the differentiation of regulatory Th3 cells is supported by the observation that TGF-β is the only cytokine that is produced in significant amounts by $Tg^+$ T cells and the presence of neutralizing anti-TGF-β Ab during priming abrogated the anergic/suppressor property of $Tg^+$ T cells upon rechallenge with Ag. $Tg^-$ T cells that have the identical TCR but only produced low levels of TGF-β from their endogenous gene differentiated into effector cells indicating that a critical amount of TGF-β is needed to reach a threshold for Th3 regulatory cell differentiation. Time was required for the maturation and differentiation of $Tg^+$ T cells into Tregs in vitro, as it required >5 days of antigenic stimulation for the suppressive phenotype to develop. It has been reported that a small percentage of $CD25^+$ cells, with similar regulatory function and phenotype as thymic originating $CD25^+$ Tregs, could arise from donor $CD25^-$ cells upon homeostatic proliferation in recipient (36). However, it is unclear what signals these cells received during their peripheral expansion to distinguish themselves from the 90% of donor $CD25^-$ cells that differentiated into effector cells with an activated phenotype. After T cell activation and clonal expansion, the majority of T cells undergo apoptosis, a phenomenon long recognized as activation-induced cell death. We postulate that if the TGF-β signal is present during the expansion phase, transcription factor *Foxp3* is induced by TGF-β which then rescues cells from apoptosis and keeps the cells in an anergic stage. At the same time, these cells mature and differentiate into cells with regulatory function. Therefore, some may maintain their activation markers, such as CD25, glucocorticoid-induced TNFR, and CTLA-4. This suggests that TGF-β-producing Th3 cells are different from the thymic-derived $CD4^+$ $CD25^+$ Tregs, one represents a separate cell lineage. Different

Table II. *Inhibition of EAE by in vitro-differentiated Th3 cells*[a]

| Day of Transfer | Incidence | Mortality | Mean Day of Onset[b] (±SD) | Mean Clinical Score[b] (±SD) |
|---|---|---|---|---|
| Day −2 | | | | |
| PBS | 11/13 (84.60%) | 0.00 | 12.3 ± 0.90 | 2.73 ± 1.28 |
| p.2D2 | 10/12 (83.30%) | 0.25 | 12.7 ± 2.45[c] | 2.62 ± 1.89[c] |
| p.2D2 × Tg | 3/15 (20.00%) | 0.00 | 17.0 ± 1.73[d] | 0.33 ± 0.82[d] |
| Day +10 | | | | |
| PBS | 11/12 (91.70%) | 0.17 | 12.5 ± 0.93 | 3.10 ± 1.26 |
| p.2D2 | 12/12 (100.0%) | 0.08 | 13.7 ± 2.19[e] | 2.62 ± 1.90[e] |
| p.2D2 × Tg | 3/8 (37.50%) | 0.00 | 18.7 ± 6.35[f] | 0.60 ± 1.06[f] |

[a] In vitro-primed T cells were injected i.v. into WT B6 mice 2 days before or 10 days after EAE induction. Mice were monitored for the development of EAE starting the day of induction.

[b] Statistical analysis was performed by comparing three groups using one-way ANOVA followed by a Dunnett's multiple comparison test.

[c,e] p.2D2 vs PBS: $p > 0.05$.

[d,f] p.2D2 × Tg vs PBS: $p < 0.01$; p.2D2 × Tg vs 2D2: $p < 0.01$.

from thymic-derived Tregs, Th3 cells induced in the periphery are Ag specific and do not always have the Treg-specific surface markers.

An important phenotype that is often associated with the regulatory function of T cells is anergy upon rechallenge. Elevated expression of the transcription factor *Foxp3* or the E3 ubiquitin ligase, gene related to anergy in lymphocytes (37) has been suggested to be responsible for the anergic stage, hence the regulatory function of $CD25^+$ Tregs and $CD25^-$ Tregs, respectively. If the prevention of programmed cell death is related to the anergic/regulatory pathway, it is possible that TGF-$\beta$, which previously has been shown to be related to the mitochondrial antiapoptotic protein Bcl-$x_L$ (38), could also arrest cells in a anergic stage and instruct cells to the same pathway. Experiments are underway to evaluate the role of TGF-$\beta$ in the induction of regulatory cells in the periphery independent of *Foxp3* gene up-regulation.

In summary, using a transgenic model we have been able to elucidate the role of Th3 cells in immune regulation. As originally described, Th3 cells are class II-restricted T cells that have the identical $\alpha\beta$TCR as Th1 and Th2 cells; they produce high amount of TGF-$\beta$, low amounts of IL-4 and IL-10 and no IFN-$\gamma$ or IL-2 upon TCR ligation (6). In the absence of inflammation, the secretion of TGF-$\beta$ up-regulates the expression of the *Foxp3* gene in activated T cells during T cell expansion which induces the differentiation of Tregs in the peripheral repertoire in the absence of thymic $CD25^+$ Tregs. Thus, Th3 cells appear to be central mediators of peripheral immune tolerance both by direct effects and indirectly by the induction of $Foxp3^+$ Tregs.

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## Disclosures

The authors have no financial conflict of interest.

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