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CUTTING EDGE

Cutting Edge: TGF-β Induces a Regulatory Phenotype in $CD4^+CD25^-$ T Cells through Foxp3 Induction and Down-Regulation of Smad7

Massimo C. Fantini, Christoph Becker,* Giovanni Monteleone,† Francesco Pallone,† Peter R. Galle,* and Markus F. Neurath[1]**

$CD4^+CD25^+$ regulatory cells are a subpopulation of T lymphocytes of thymic origin. However, recent data suggest an alternative commitment of regulatory T cells in the periphery, although the precise mechanism is unknown. In the present work, we demonstrate that TGF-β is able to induce Foxp3 expression and subsequently a regulatory phenotype in $CD4^+CD25^-$ peripheral murine T cells. Similarly, TGF-β induced Foxp3 in human $CD4^+CD25^-$ T cells. Moreover, we show that the inhibitory Smad7 protein that is normally induced by TGF-β and limits TGF-β signaling, is strongly down-regulated by Foxp3 at the transcriptional level. Foxp3-mediated down-regulation of Smad7 subsequently rendered $CD4^+CD25^-$ T cells highly susceptible to the morphogenic and regulatory effects of TGF-β signaling via Smad3/4. In summary, we demonstrate that TGF-β induces a regulatory phenotype in $CD4^+CD25^-$ T cells through the induction of Foxp3 and a positive autoregulatory loop of TGF-β signaling due to the absence of Smad7. The Journal of Immunology,* 2004, 172: 5149–5153.

Immunological tolerance is a key feature of the immune system and allows the organism to discriminate between self and nonself. Such tolerance is achieved by processes occurring both during the development of the immune system in the thymus (central tolerance) and the lifespan of lymphocytes in the periphery (peripheral tolerance) (1–2). Peripheral tolerance is required to inhibit reactive T cell clones escaped from thymic selection or originated de novo against self Ags. In this context, a group of specialized T regulatory cells expressing CD25 plays a key functional role (3–5). Recent evidence suggests that naturally occurring $CD4^+CD25^+$ regulatory T cells are characterized by the exclusive expression of the winged-helix/forkhead transcription factor Foxp3 (4–6). Although the thymic origin of $CD4^+CD25^+$ T cells has been demonstrated, several findings suggest that such cells may also be generated in the periphery by an unknown mechanism. In the present report, we provide evidence that TGF-β is able to induce Foxp3 expression in $CD4^+CD25^-$ T cells and promotes the acquisition of regulatory properties in these cells.

Materials and Methods

Reagents and media

Anti-mouse CD3ε (145-2C11), anti-mouse CD28 (37.51), anti-human CD3ε, anti-human CD28 (CD28.2; BD Biosciences, Mountain View, CA), human rTGF-β1 (Strathmann Biotech, Hamburg, Germany), human purified TGF-β (R&D Systems, Minneapolis, MN), X-Vivo15 (BioWhittaker, Heidelberg, Germany), neutralizing anti-TGF-β Abs (I. Fuss, Mucosal Immunology Section, National Institutes of Health, Bethesda, MD), and HepG2 cells (American Type Culture Collection, Manassas, VA) were cultured in complete RPMI 1640 (7).

Cell lines and plasmid

Smad7 promoter reporter pS7-5 was a gift of E. P. Böttinger (Department of Medicine and Molecular Genetics, Albert Einstein College of Medicine, Bronx, NY), Foxp3 expression vector was kindly provided by M. Stassen (Institute of Immunology, Mainz, Germany). pMACS K^k.II vector was purchased from Miltenyi Biotec (Bergisch-Gladbach, Germany); β-galactosidase (β-gal)[2] expression vector was purchased from Invitrogen (Heidelberg, Germany).

Isolation of primary T cells

Murine spleen T cells were isolated by MACS (Miltenyi Biotec) (7). $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were sorted from $CD4^+$ splenocytes using the $CD4^+CD25^+$ Regulatory T cell Isolation kit (Miltenyi Biotec). Human T cells were isolated from buffy coats using CD4-MACS Multisort beads or CD4-MACS negative selection followed by CD25-MACS positive selection (Miltenyi Biotec). Cells were >93% pure, as determined by FACS analysis.

Flow cytometric analysis

Cells were resuspended in PBS 0.5% BSA. For staining, anti-mouse CD25 PE (Caltag Laboratories, Hamburg, Germany) and anti-mouse CD4 FITC (BD PharMingen, San Diego, CA) were used. Stained cells were analyzed by FACScan (BD Biosciences).

T cell proliferation

T cell proliferation was determined using the 5-bromo-2-deoxyuridin (BrdU)-based Cell Proliferation ELISA (Roche, Heidelberg, Germany).

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[2] Abbreviations used in this paper: β-gal, β-galactosidase; BrdU, 5-bromo-2-deoxyuridin.

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RT-PCR

Reverse transcription into complementary DNA was performed using the Moloney murine leukemia virus reverse transcriptase (Invitrogen, San Diego, CA). PCR was performed using the RedTaq PCR reagents (Sigma-Aldrich, St. Louis, MO) and the following primers: human Foxp3, 5′-ATG CCT CCT CTT CTT CCT TGA-3′ and 5′-ATT GTG CCC TGC CCT TCT CA-3′; murine Foxp3, 5′-GGC GAA AGT GGC AGA GAG GTA T-3′ and 5′-AAG ACC CCA GTG GCA GCA GAA-3′; murine IL-2, 5′-ATT GAC ACT TGT GCT CCT TGT C-3′ and 5′-TTG ACA GAA AGG CTA TCC ATC-3′; murine TGF-β, 5′-TGC TGC TTT CTC CCT CAA CCT-3′ and 5′-CAC TGC TTC CCG AAT GTC TGA-3′; human Smad7, 5′-TCA CCT TAG CCG ACT CTG C-3′ and 5′-ACA CCC ACA CAC CAT CCA C-3′; β-actin, 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′ and 5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′. PCR products were analyzed on 1% agarose gels.

Transient transfections and reporter gene analysis

Foxp3 expression vector (2.5 μg) along with 2.5 μg of the pMACS K^k.II vector were transfected into 5×10^6 cells using the AMAXA Human T Cell Nucleofector kit (Cologne, Germany). In addition, 0.1 μg of Foxp3 expression vector or pCDNA3.1 empty plasmid, 0.1 μg of β-gal reporter gene, and 1 μg of pS7-5 Smad7 promoter reporter gene were transfected in 80–90% confluent HepG2 cells in a 24-well plate using Lipofectamine Plus Reagent (Invitrogen,). Luciferase activity was measured with a standard luminometer (Sirius, Berthold, Pforzheim, Germany). Luciferase activity was normalized to the β-gal expression and to the protein content of the sample.

ELISA

IL-10, IL-4, and IFN-γ were measured by commercially available ELISA (BD PharMingen for IL-10 and IL-4, R&D Systems for IFN-γ). In some experiments, serum-free medium in the presence or absence of TGF-β was used.

Western blotting

For human and murine Foxp3 analysis, T cell extracts were analyzed by Western blotting (7). Rabbit anti-mouse Foxp3 (1 μg/ml; E. Schmitt, Institute of Immunology, Mainz, Germany) or goat anti-human Foxp3 (Abcam, Cambridge, U.K.) and 1:1000 HRP-labeled anti-rabbit or anti-goat IgG (DAKO, Hamburg, Germany) and the ECL system (Amersham, Arlington Heights, IL) were used.

Results

TGF-β induces Foxp3 expression in CD4$^+$ T cells

Previous studies have demonstrated a pivotal role of TGF-β in mediating immunological tolerance (8–9). Therefore, we tested the possibility that TGF-β1 might regulate Foxp3 expression in murine and human CD4$^+$ T cells. Accordingly, we stimulated purified splenic CD4$^+$ T cells from FVB mice in the presence or absence of TGF-β. CD4$^+$ T cells were activated with anti-CD3 and anti-CD28 Abs in serum-free medium for 5 days and Foxp3 expression was analyzed by RT-PCR (Fig. 1*A*). It was found that TGF-β induced Foxp3 expression, whereas cells cultured in the absence of TGF-β showed reduced or no expression of Foxp3. Thus, TGF-β emerges as an important factor for the induction of Foxp3 expression in murine CD4$^+$ T cells. Similarly, TGF-β stimulation regulated Foxp3 expression in anti-CD3/CD28-stimulated human CD4$^+$ T lymphocytes at the mRNA and protein level, suggesting that TGF-β controls expression of this key regulatory protein both in human and murine T cells (Fig. 1, *B* and *C*).

TGF-β induces Foxp3 in CD4$^+$CD25$^-$ but not CD4$^+$CD25$^+$ T cells

To determine the subpopulation of CD4$^+$ T cells in which Foxp3 expression is regulated by TGF-β, murine and human CD4$^+$ T lymphocytes were separated into CD25$^+$ and CD25$^-$ fractions. As shown in Fig. 2, *A* and *B*, TGF-β stimulation had little or no effects on Foxp3 expression in the murine CD4$^+$CD25$^+$ T cell subset. In contrast, a strong induction of Foxp3 expression was observed in CD4$^+$CD25$^-$ T cells after 24–120 h of culture in the presence of TGF-β, although TGF-β suppressed the proliferation of these cells (Fig. 2*C*). These data were confirmed by Western blot analysis (Fig. 2*D*) and extended by the observation that TGF-β-dependent Foxp3 induction required stimulation with anti-CD3/28 Abs (Fig. 2*E*). Similarly to murine T cells, TGF-β stimulation induced the expression of Foxp3 in human CD4$^+$CD25$^-$ T cells after 5 days of cell culture (Fig. 2*F*). Taken together, these data suggest that TGF-β controls the generation of Foxp3$^+$ T cells from CD4$^+$CD25$^-$ precursor cells in mice and humans.

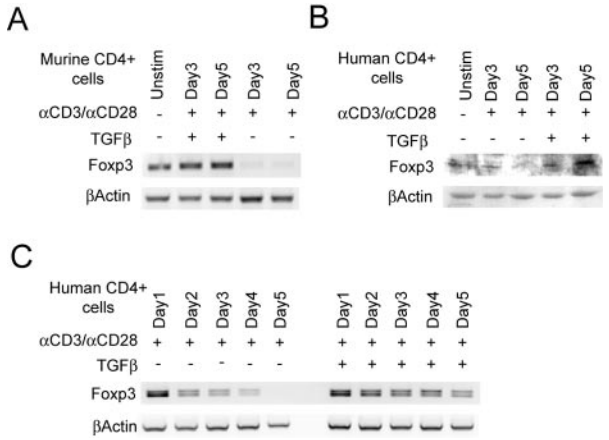

FIGURE 1. TGF-β1 regulates Foxp3 expression. *A*, Murine CD4$^+$ T cells were cultured with anti-CD3/CD28 Abs in serum-free medium for 5 days in presence or absence of 1 ng/ml TGF-β1. Foxp3 levels were determined by RT-PCR at indicated time points. *B* and *C*, Western blotting (*B*) and PCR analysis (*C*) for Foxp3 expression in human CD4$^+$ T cells stimulated in the presence or absence of TGF-β1.

TGF-β-induced Foxp3$^+$ T cells express TGF-β

We next investigated the production of regulatory cytokines by TGF-β-stimulated CD4$^+$CD25$^-$ T cells. CD4$^+$CD25$^-$ T cells cultured in the presence of TGF-β showed lower expression of IL-10 than cells activated in the absence of TGF-β (Fig. 3*A*). In addition, the former cells produced little or no proinflammatory cytokines such as IL-4 and IFN-γ in cell culture. Finally, we observed that CD4$^+$CD25$^-$ T cells stimulated with TGF-β contained higher levels of TGF-β mRNA than cells cultured in the absence of TGF-β (Fig. 3*A*). Taken together, these findings indicate that TGF-β stimulated CD4$^+$CD25$^-$ T cells acquire a cytokine profile consistent with that described for regulatory CD4$^+$CD25$^+$ T lymphocytes (10). Furthermore, CD4$^+$CD25$^-$ T cells cultured in the presence of TGF-β expressed CD25 at day 5 but not day 0, as demonstrated by FACS analysis (Fig. 3*B*).

To test whether CD4$^+$CD25$^-$ T cells activated in presence of TGF-β show regulatory potential, we cultured CD4$^+$CD25$^-$ T cells for 5 days in the presence or absence of TGF-β. Next, cells were extensively washed to eliminate potential traces of TGF-β in the culture medium and mixed with freshly isolated CD4$^+$ T cells in the presence of plate-bound anti-CD3 and soluble anti-CD28 Abs in serum-free medium (Fig. 4). Interestingly, TGF-β-stimulated CD4$^+$CD25$^-$ T cells showed a marked antiproliferative effect on freshly isolated CD4$^+$ T cells as compared with CD4$^+$CD25$^-$ T cells cultured in the absence of TGF-β. In our experimental system, this

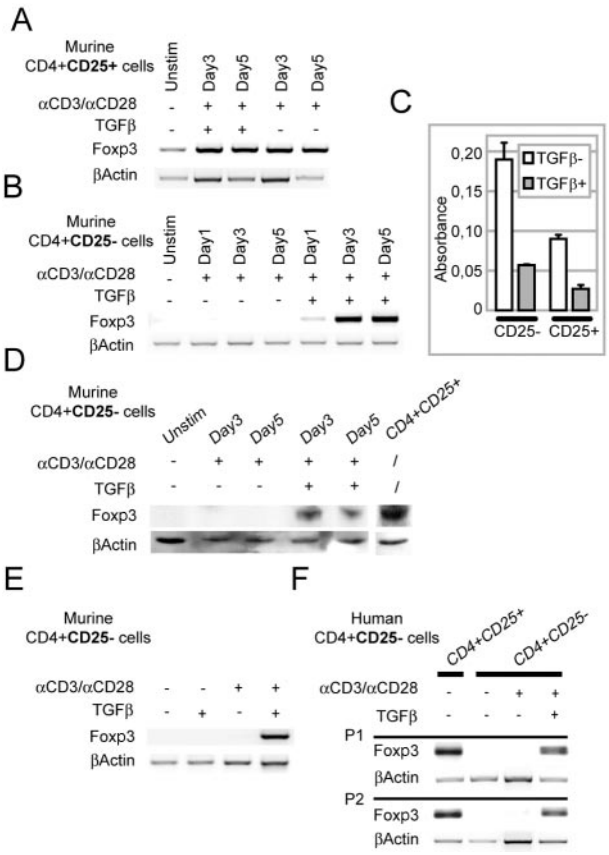

FIGURE 2. TGF-β induces Foxp3 expression in murine $CD4^+CD25^-$ but not $CD4^+CD25^+$ T cells. T cells were cultured for 5 days in presence or absence of TGF-β1 as above. Cells were collected at the indicated points and Foxp3 levels were analyzed by RT-PCR (*A* and *B*) or Western blots (*D*). One representative experiment of three is shown. Stimulation of murine T cell subsets with TGF-β suppressed anti-CD3/CD28-induced T cell proliferation, as shown by a BrdU-based assay. T cells were cultured in the presence (TGF-β^+) or absence (TGF-β^-) of rTGF-β for 5 days as above (*C*). TGF-β-dependent induction of Foxp3 expression required stimulation with anti-CD3/CD28 Abs, as shown by RT-PCR (*E*). In addition, stimulation of human $CD4^+CD25^-$ T cells with TGF-β resulted in the induction of Foxp3 (*F*). Human T cells were isolated (14) and cultured for 5 days in the presence or absence of TGF-β and anti-CD3/CD28 Abs, as indicated. Foxp3 levels were determined by RT-PCR. P1 = patient 1; P2 = patient 2.

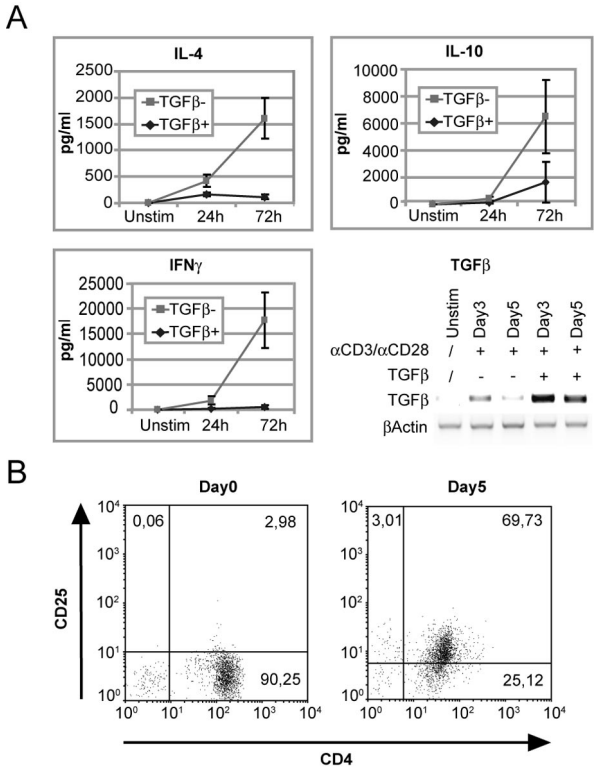

FIGURE 3. Supernatants from the above murine experiments (Fig. 2) were assayed for cytokine content by ELISA (*A*). TGF-β1 expression was assayed by RT-PCR to avoid any potential contamination by rTGF-β. TGF-β-stimulated $CD4^+CD25^-$ cells contained TGF-β mRNA but produced low levels of IL-10 and little or no IFN-γ and IL-4. Mean values $\pm$ SD from three independent experiments are shown. *B*, Analysis of CD25 expression by FACS analysis. $CD4^+CD25^-$ T cells were cultured for indicated periods of time in the presence of TGF-β before FACS analysis. Thirty percent of T cells cultured in the absence of TGF-β for 5 days expressed CD25 probably due to T cell activation by anti-CD3 plus anti-CD28 Abs (data not shown).

antiproliferative effect could only be partially inhibited by the addition of neutralizing anti-TGF-β Abs (25% inhibition: absorbance difference 0.177 vs 0.131 at 10 μg/ml Ab concentration).

Foxp3 expression allows TGF-β signaling through down-regulation of Smad7

Finally, we analyzed TGF-β signaling via Smad proteins in Foxp3-expressing $CD4^+CD25^-$ T lymphocytes. First, we determined potential effects of Foxp3 on expression of the inhibitory Smad7 protein. Accordingly, primary $CD4^+$ T cells were transfected with a Foxp3 expression vector in the presence or absence of TGF-β. Positively transfected cells were then selected using cotransfection of the pMACS K^k.II vector resulting in a sorted cell population with 95% transfection efficacy. Analysis of Smad7 mRNA levels showed Smad7 induction upon TGF-β stimulation in control-transfected cells while overexpression of Foxp3 completely abrogated TGF-β-induced Smad7 expression in $CD4^+$ T cells (Fig. 5*A*).

Next, we investigated whether Foxp3-induced down-regulation of Smad7 was mediated at the transcriptional level. Accordingly, cells were cotransfected with the Foxp3 expression vector or an empty control vector together with a luciferase reporter construct containing the Smad7 promoter region (−303 to +672). Foxp3 expression led to an ~40% down-regulation of basal Smad7 promoter activity but very strongly suppressed TGF-β induced Smad7 promoter activity (Fig. 5*B*). Finally, we analyzed the effect of Smad7 down-regulation on TGF-β signaling by transfecting $CD4^+$ T cells with a construct containing multiple Smad3/4 responsive elements in front of a luciferase gene, along with Foxp3 expression vector or a control vector. It was found that Foxp3 enhances Smad3/4-mediated TGF-β signal transduction in TGF-β-stimulated cells (Fig. 5*C*) suggesting that Foxp3 induces a positive autoregulatory loop of TGF-β signaling in the absence of Smad7.

Discussion

Although the thymic origin of $CD4^+CD25^+$ T cells has been demonstrated, several findings suggest that such cells may also be generated from $CD25^-$ precursor cells in the periphery (11, 12). In the present study, we have found that TGF-β-mediated induction of Foxp3 in $CD4^+$ $CD25^-$ T cells results in the development of T cells with regulatory functions.

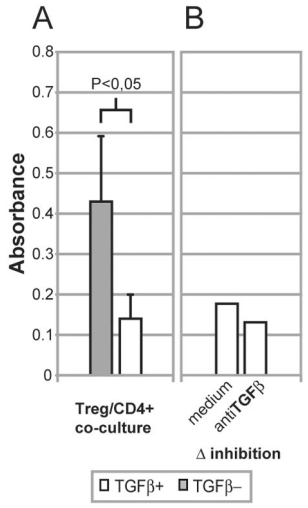

FIGURE 4. *A*, Foxp3$^+$ cells generated from TGF-β-stimulated CD4$^+$CD25$^-$ cells suppress T cell proliferation. Murine CD4$^+$CD25$^-$ T cells were activated in the presence or absence of TGF-β1 for 5 days as above, extensively washed, and cocultured with freshly isolated CD4$^+$ T cells. After 5 days, cells were pulsed with BrdU and 16 h later were assayed for BrdU incorporation. TGF-β-stimulated CD4$^+$CD25$^-$ cells significantly suppressed CD4$^+$ T cell proliferation as compared with T cells cultured in the absence of TGF-β ($p < 0.05$). Data represent mean values ± SD from three independent experiments. *B*, Partial inhibition of the suppressive effect of TGF-β stimulated CD4$^+$ T cells by neutralizing anti-TGF-β-Abs. T cells were cultured as above in the presence or absence of anti-TGF-β-Abs followed by analysis of BrdU incorporation.

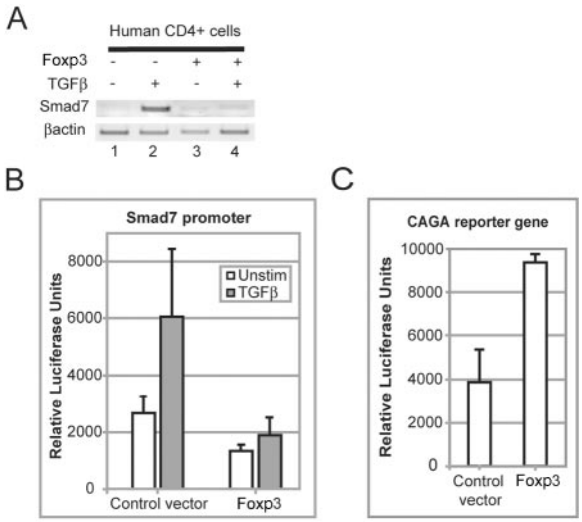

FIGURE 5. *A*, Human CD4$^+$ T cells were cotransfected with a Foxp3 expression vector or an empty control vector and the pMACS K^k.II expression vector. Twenty-four hours later, transfection cells were sorted for the expression of H-2K^k and starved for 16 h in serum-free medium. TGF-β1 (4 ng/ml) was added for 60 min, as indicated, before Smad7 expression was analyzed by RT-PCR. TGF-β stimulation led to induction of Smad7 levels in T cells, while forced Foxp3 expression suppressed Smad7 levels. *B*, HepG2 cells were transiently cotransfected with a Foxp3 expression vector or an empty control vector, along with the pS7-5 Smad7 promoter reporter gene and a β-gal expression vector. After transfection, cells were starved for 12 h before stimulation with TGF-β for an additional 10 h. Cell lysates were assayed for luciferase activity. Data represent mean values ± SD from four independent experiments. *C*, CD4$^+$ T cells were cotransfected with a Foxp3 expression vector or an empty control vector and a vector containing a Smad3/4 response element upstream of the luciferase gene. Foxp3 expression induced up-regulation of the Smad3/4 response element. Data represent mean values ± SD from three independent experiments.

TGF-β regulated Foxp3 expression in both murine and human CD25$^-$ CD4$^+$ T cells, while it had little or no effects on Foxp3 expression in CD25$^+$CD4$^+$ T cells. TGF-β-induced Foxp3$^+$ T cells, generated from CD4$^+$CD25$^-$ precursor T cells, showed increased expression of TGF-β and low IL-10 production but failed to produce proinflammatory cytokines such as IFN-γ and IL-4, consistent with the cytokine profile of naturally occurring CD4$^+$CD25$^+$ cells (10). Moreover, TGF-β-stimulated CD25$^-$ CD4$^+$ T cells showed regulatory potential in a coculture system with activated naive T cells, suggesting that TGF-β induces regulatory T cells from a CD25$^-$ T cell population. Importantly, the induction of Foxp3 by TGF-β required stimulation via the TCR suggesting that Ag-specific activation is required to induce such regulatory T cell responses.

Subsequent studies showed that TGF-β-induced Foxp3 down-regulates Smad7 expression in CD4$^+$ T cells and thereby suppresses the expression of the key negative regulator of TGF-β signaling. This effect of Foxp3 on Smad7 expression was mediated by a direct effect of Foxp3 on Smad7 promoter activity. Taken together, these data suggest a model in which TGF-β plays a pivotal role in the generation of regulatory T cells from a population of peripheral CD4$^+$CD25$^-$ T cells through the induction of the key transcription factor Foxp3. Foxp3 enhances the TGF-β effects in these cells by down-regulation of Smad7 thereby creating a positive autoregulatory loop of TGF-β signaling in CD4$^+$CD25$^-$ T cells that potentially stabilizes their regulatory phenotype. However, Piccirillo et al. (13) showed that CD4$^+$CD25$^+$ T cells isolated from TGF-$\beta^{-/-}$ mice during the first 3–7 days after birth show normal regulatory function. Taken together, these data suggest a dispensable role of TGF-β in mediating CD4$^+$CD25$^+$ regulatory function, but underline a new role for TGF-β in the maintenance of the regulatory compartment in the periphery via its effects on CD4$^+$CD25$^-$ T cells. Consistent with such role in peripheral tolerance, TGF-$\beta^{-/-}$ mice have been shown to develop an autoimmune response with loss of tolerance and multiorgan inflammation within several weeks after birth (8).

The data from the present study suggest a pivotal role for TGF-β in the generation of Foxp3$^+$ regulatory T cells in the periphery via TGF-β mediated induction of Foxp3 in CD4$^+$CD25$^-$ T cells. Foxp3 in turn down-regulates Smad7 and renders T cells highly susceptible to the morphogenic and regulatory effects of TGF-β signaling via Smad3/4. The concept that regulatory T cells can be generated in vitro from the large pool of CD4$^+$CD25$^-$ naive T cells via TGF-β could open the way toward new therapeutic approaches for autoimmune and allergic diseases based on modulation of TGF-β and Foxp3 expression.

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