The Journal of Immunology

Smad2 and Smad3 Are Redundantly Essential for the TGF- β–Mediated Regulation of Regulatory T Plasticity and Th1 Development

Tomohito Takimoto, Yu Wakabayashi, Takashi Sekiya, Naoko Inoue, Rimpei Morita, Kenji Ichiyama, Reiko Takahashi, Mayako Asakawa, Go Muto, Tomoaki Mori, Eiichi Hasegawa, Saika Shizuya, Toshiro Hara, Masatoshi Nomura and Akihiko Yoshimura

J Immunol 2010; 185:842-855; Prepublished online 14 June 2010;
doi: 10.4049/jimmunol.0904100
http://www.jimmunol.org/content/185/2/842

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Smad2 and Smad3 Are Redundantly Essential for the TGF-β–Mediated Regulation of Regulatory T Plasticity and Th1 Development

Tomohito Takimoto,*,† Yu Wakabayashi,* Takashi Sekiya,* Naoko Inoue,* Rimpei Morita,* Kenji Ichiyama,* Reiko Takahashi,* Mayako Asakawa,* Go Muto,* Tomoaki Mori,* Eiichi Hasegawa,* Saika Shizuya,‡ Toshiro Hara,† Masatoshi Nomura,† and Akihiko Yoshimura*,§

Although it has been well established that TGF-β plays a pivotal role in immune regulation, the roles of its downstream transcription factors, Smad2 and Smad3, have not been fully clarified. Specifically, the function of Smad2 in the immune system has not been investigated because of the embryonic lethality of Smad2-deficient mice. In this study, we generated T cell-specific *Smad2* conditional knockout (KO) mice and unexpectedly found that Smad2 and Smad3 were redundantly essential for TGF-β–mediated induction of Foxp3-expressing regulatory T cells and suppression of IFN-γ production in CD4$^+$ T cells. Consistent with these observations, *Smad2*/*Smad3*-double KO mice, but not single KO mice, developed fatal inflammatory diseases with higher IFN-γ production and reduced Foxp3 expression in CD4$^+$ T cells at the periphery. Although it has been suggested that Foxp3 induction might underlie TGF-β–mediated immunosuppression, TGF-β still can suppress Th1 cell development in Foxp3-deficient T cells, suggesting that the Smad2/3 pathway inhibits Th1 cell development with Foxp3-independent mechanisms. We also found that Th17 cell development was reduced in Smad-deficient CD4$^+$ T cells because of higher production of Th17-inhibotory cytokines from these T cells. However, TGF-β–mediated induction of RORγt, a master regulator of Th17 cell, was independent of both Smad2 and Smad3, suggesting that TGF-β regulates Th17 development through Smad2/3-dependent and -independent mechanisms. ***The Journal of Immunology*****, 2010, 185: 842–855.**

Among the three TGF-β isoforms, TGF-β1 is predominantly expressed in the immune system and is an important pleiotropic cytokine with potent immunoregulatory properties (1, 2). Mice deficient in TGF-β1 develop a multiorgan autoimmune inflammatory disease and die a few weeks after birth (3, 4). Various transgenic mice whose T cells are unable to respond specifically to TGF-β1 have also been shown to develop autoimmunity, indicating that TGF-β1 signaling is essential for T cell homeostasis (5–7).

Various mechanisms have been proposed for the immunoregulatory functions of TGF-β on T cells, such as suppression of cell proliferation, cytokine production, and cytokine signaling, as well as inducing apoptosis (8). TGF-β has been shown to induce Foxp3 (9), a master transcriptional factor of regulatory T cells (Tregs) (10, 11). The overexpression of Foxp3 in CD4$^+$ T cells suppressed the production of proinflammatory cytokines, including IL-2, IFN-γ, IL-4, and IL-17 (12–14). Foxp3 is predominantly expressed in thymus-derived, naturally occurring Tregs, which are critically important for immunoregulation (15). Likewise, in vitro TGF-β–induced Tregs (iTregs) can suppress effector T cell proliferation in vitro and autoimmune diseases in vivo, just as nTregs can (16). Furthermore, TGF-β signaling has been reported to be essential for the maintenance of nTregs at the periphery and for their development in the thymus (17).

The major signaling pathways of the TGF-βRs are relatively simple (18). TGF-β first binds to the TGF-βR, which then mainly activates Smad transcription factors, including three structurally similar proteins, two receptor-associated Smads, Smad2 and Smad3, and one common Smad4. Smad2 or Smad3 are directly phosphorylated and activated by TGF-βR and heterodimerize with Smad4 or TIF1γ (19). The activated Smad-complex translocates into the nucleus and, in a cooperative manner with other nuclear cofactors, regulates the transcription of target genes.

Many previous reports have indicated that TGF-β is required to orchestrate T cell immunity, but it is not clear whether various TGF-β–mediated effects on T cells are equally dependent on Smad signaling. It has been reported that Smad2 or Smad3 regulates a distinctive set of genes in fibroblasts and tumor cells (18). *Smad2*-knockout (KO) mice are embryonic-lethal (20) and *Smad3*-KO mice exhibit inflammatory diseases (21), suggesting that Smad2 is involved in mediating signals during development, whereas Smad3 is important for anti-inflammation. A recent

*Department of Microbiology and Immunology, Keio University School of Medicine, Shinjyuku-ku; §Japan Science and Technology Agency, Core Research for Evolutional Science and Technology, Chiyoda-ku, Tokyo; †Graduate School of Medical Sciences, Kyushu University, Fukuoka; and ‡Department of Ophthalmology, Wakayama Medical University School of Medicine, Wakayama, Japan

Received for publication December 22, 2009. Accepted for publication May 3, 2010.

This work was supported by Grants-in-Aid for Scientific Research and for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation; the Naito Foundation; the Mochida Foundation; and the Astellas Foundation for Research on Metabolic Disorders.

Address correspondence and reprint requests to Dr. Akihiko Yoshimura, Department of Microbiology and Immunology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, 160-8582, Japan. E-mail address: yoshimura@a6.keio.jp

The online version of this article contains supplemental material.

Abbreviations used in this paper: DKO, double knockout; EAE, experimental autoimmune encephalomyelitis; iTreg, in vitro TGF-β–induced Treg cell; KO, knockout; MLN, mesenteric lymph node; MOG, myelin oligodendrocyte glycoprotein; Treg, regulatory T cell.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0904100

report suggested that Smad3, but not Smad2, is critical for the induction of Foxp3 (22). Moreover, the disruption of Smad4 specifically in T cells results in colitis and an increased susceptibility to the spontaneous colo-rectal tumorigenesis (23). These reports might indicate that the Smad3/4 pathway is an important mediator of TGF-β signaling in immune regulation. However, the phenotypes of Smad3- or Smad4-deficient mice were much milder than those of T cell-specific *TGF-βRII* KO mice (7), suggesting that Smad2 may also play a role in immune regulation.

Because *Smad2* completely-null mice were embryonic-lethal, the roles of Smad2 in the immune system have not been clarified; one question that remains open is whether Smad2 and Smad3 have any overlapping and/or specific functions in vivo. To address these questions, we generated T cell-specific *Smad2* conditional KO mice. Our study also revealed unexpected overlapping functions of Smad2 and Smad3 in the regulation of various T cell responses and gene expressions. Our data indicated that Smad3 and Smad2 are important for TGF-β–mediated Foxp3 induction and suppression of Th1 development. We also found that Th17 cell development was indirectly regulated by Smad2/3 signaling. Furthermore, we found that TGF-β suppressed Th1 cell development in a Foxp3-independent manner. These data suggest that the TGF-β/Smad pathway regulates T cell responses via multiple mechanisms.

Materials and Methods

Mice

Construction of the *Smad2-flox* vector and generation of the *Smad2*$^{f/f}$ mice will be described elsewhere (M. Nomura, manuscript in preparation). LckCre mice with a C57BL/6 background (24) expressing Cre recombinase under the control of the mouse proximal *Lck* gene were crossed with *Smad2*$^{f/f}$ mice. Offspring carrying both *LckCre* and floxed *Smad2* genes were used for intercrossing and further analysis. Littermates without the LckCre allele (*Smad2*$^{f/f}$) were used as control mice for analysis purposes. *Smad3* KO mice with a C57BL/6 background were provided by Dr. Flanders (National Institutes of Health, Bethesda, MD) (25). Nomenclature of genotypes of Smad2/Smad3 KO mice and T cells is listed in Table I. *Scurfy* mice (C57BL/6) have been described elsewhere. Age- and sex-matched littermates were used as control mice in all experiments. Genotypes were determined by PCR, using the following primer pairs: 5′-TGAGACTTCTCTGTACCC-GAT-3′ and 5′-CATCAGATTCCATTAGAGATGG-3′. Mice were kept in specific pathogen-free facilities in Keio University. All experiments using mice were approved by and performed according to the guidelines of the Animal Ethics Committee of Keio University.

T cell isolation and differentiation

CD4$^+$CD25$^-$CD44^{low}CD62L^{high} naive T cells or CD4$^+$CD25^{high}CD44^{low} CD62L^{high} nTregs from spleens and lymph nodes and also, in some cultures, from thymi were enriched by negative selection using the magnetic cell sorting system (Miltenyi Biotec, Auburn, CA) with biotin-conjugated anti-CD8.2 (53-6.7), anti-B220/CD44 (RA3-6B2), anti-CD11b (M1/70), anti-CD11c (N418), anti-TER119, and anti-NK1.1 (PK136) Abs (eBioscience, San Diego, CA) as well as streptoavidin-conjugated magnetic beads (Miltenyi Biotec). Cells were then FACS-sorted using a BD FACS aria cell sorter (BD Biosciences, San Jose, CA). The purity of the sorted CD4$^+$ T cell populations was consistently >98%. T cells were activated by anti-CD3e (145-2C11) and anti-CD28 (37.51) and cultured in the presence of recombinant murine IL-12 (10 ng/ml; PeproTech, Rocky Hill, NK) and anti–IL-4 (5 μg/ml; 11B11) for Th1 cell differentiation; recombinant murine IL-4 (10 ng/ml; PeproTech) and anti–IFN-γ (5 μg/ml, R4-6A2) for Th2 cell differentiation; recombinant hTGF-β1 (2 ng/ml; R&D Systems, Minneapolis, MN), anti–IFN-γ (5 μg/ml), and anti–IL-4 (5 μg/ml) for Treg differentiation; and recombinant human TGF-β1 (0.5 ng/ml), recombinant human IL-6 (20 ng/ml; R&D Systems), anti–IFN-γ (5 μg/ml), and anti–IL-4 (5 μg/ml) for Th17 cell differentiation (26, 27). In some cultures, for induction of Th17 cells, anti–IL-2 (10 μg/ml; 1A12, eBioscience) was added. T cells were maintained in a complete medium containing RPMI 1640 supplemented with 10% FCS, 1% penicillin/streptomycin, 100 nM non-essential amino acids, 2mM glutamine, and 0.05 mM 2-ME.

Western blot analysis

Cells were washed, lysed in sample buffer, and boiled. Whole cell lysates were separated by SDS-PAGE under reducing conditions and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) as described elsewhere (28). The membrane was blocked and probed with the following primary Abs: anti–β-actin rabbit polyclonal Ab (A2066; Sigma-Aldrich, St. Louis, MO) and anti-Smad2/3 rabbit mAb (#3102, Cell Signaling Technology, Beverly, MA). The membrane was then probed with appropriate secondary Abs conjugated to HRP and visualized with the ECL Detection System (GE Healthcare, Tokyo, Japan), according to the manufacturer's instructions. β-Actin was used as a loading control.

Flow cytometry

For intracellular cytokine staining, cells were stimulated for 6 h in complete medium with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (500 ng/ml; both from Sigma-Aldrich) in the presence of brefeldin A (eBioscience). Surface staining was then performed in the presence of Fc-blocking Abs (2.4G2), followed by intracellular staining for cytokines with the Fixation and Permiabilization kit (eBioscience) according to the manufacturer's protocol. All Abs were from eBioscience. For intracellular staining of Foxp3, the Foxp3-Staining Buffer Set (fixation/permeabilization and permeabilization buffers, eBioscience) was used according to the manufacturer's protocol. Data were acquired on a BD FACS aria and were analyzed with FlowJo software (Tree Star, Ashland, OR).

ELISA

Supernatants were collected after the indicated periods of cell culture and were analyzed for IL-2, IFN-γ, IL-4, and IL-17A with an ELISA kit (eBioscience) according to the manufacturer's protocol.

Real-time RT-PCR

cDNA was synthesized and analyzed by real-time quantitative PCR as described previously (29). Gene expression was examined with a Bio-Rad iCycler Optical System with the iQ SYBR green real-time PCR kit (Bio-Rad, Hercules, CA). The data were normalized to HPRT. The primers have been described previously (29). The relative expression of the indicated gene to HPRT was calculated as $(2^{-[\text{experimental CT} - \text{HPRT CT}]}) \times 1000$, where *CT* is the cycle threshold of signal detection.

CFSE labeling and suppression assay

For CFSE labeling, sorted naive CD4$^+$ T cells were washed twice with HBSS (Invitrogen), and labeled with 5 mM CFSE (Sigma-Aldrich) in HBSS for 10 min at 20°C. The labeling was then stopped by adding 1/5 volume of FCS. The labeled cells were washed twice with the T cell culture medium before they were seeded and stimulated as described in the text. CD4$^+$CD25^{high}CD44^{low} CD62L^{high} nTregs from *Smad2*$^{f/f}$ mice or LckCre-*Smad2*$^{f/f}$ mice were FACS-sorted (purity >98%), as were naive CD4$^+$ T cells as responder cells. Naive CD4$^+$ T cells (1×10^5 cells) and the indicated number of nTregs were cultured with irradiated splenocytes (3×10^4 cells) with 2 μg/ml anti-CD3e Ab for 3 d. CFSE dilution was analyzed on a BD Biosciences FACS Aria.

Microarrays

RNA was prepared from 3×10^6 cells of stimulated cells as described previously (28). Total RNA was cleaned with RNeasy Micro Kit (QIAGEN). Microarray processing was done by the Central Research Laboratory, School of Medicine, Keio University on a fee-for-service basis according to the recommendations in the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). The cRNA was hybridized to M430 2.0 microarray chips (Affymetrix). Hybridized chips were stained and washed and were scanned with a GeneArray scanner 3000 7G (Affymetrix). GeneSpring software (Tomy, Tokyo, Japan) was used for comparison analyses. Microarray data are deposited in the National Center for Biotechnology Information GEO database. (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=zpotpcgkcyqauhg&acc=GSE19601)

Experimental autoimmune encephalomyelitis induction

Myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 (MEVGWYRSPFSRVVHLYRNGK) (BEX) was used to induce experimental autoimmune encephalomyelitis (EAE) in mice (30, 31). Mice were injected s.c. with 200 μg of MOG peptide in 100 μl of PBS emulsified in 100 μl CFA that was further enriched with 5 mg/ml *Mycobacterium tuberculosis* (H37Ra; Difco BD Biosciences). In addition, 500 ng pertussis toxin (Calbiochem, San Diego, CA) was injected i.p. on days 0 and 2. Paralysis was evaluated according to the following scores: 0, no signs; 1, full tail; 2, hind limbs; 3, complete back; 4, forelimbs; 5, dead.

Statistics

A paired Student *t* test was used to determine statistical significance, and $p < 0.05$ was considered significant. Error bars show standard deviations.

Results

Generation of Smad2 conditional KO mice

To clarify the role of Smad2 in immune regulation, we generated T cell-specific *Smad2*-conditional KO mice. We first generated mice carrying a modified *Smad2* allele in which exon 7 and exon 8 were flanked by *lox*P sites (floxed). There was no difference between C57BL/6 wild-type mice and *Smad2*$^{f/fl}$ control mice in T cell development, nTreg functions in vitro, and CD4$^+$ T cell responses to TGF-β (Supplemental Fig. 1) (data not shown). We then deleted *Smad2* specifically in T cells by crossing *Smad2*$^{f/f}$ mice with LckCre transgenic mice, in which Cre recombinase was driven by the *Lck* promoter (LckCre). Smad2 protein was undetectable in naive CD4$^+$T cells isolated from LckCre-*Smad2*$^{f/f}$ (*Smad2*-cKO) mice (Fig. 1*A*). The resulting *Smad2*-cKO mice were fertile and were born with the expected Mendelian frequency (data not shown). *Smad2*-cKO mice appeared healthy and had no evident signs of autoimmunity, but a small fraction (<5%) of aged *Smad2*-cKO mice spontaneously developed inflammatory bowel diseases (data not shown). Moreover, *Smad2*-cKO mice were much more susceptible to dextran sodium sulfate induced colitis,

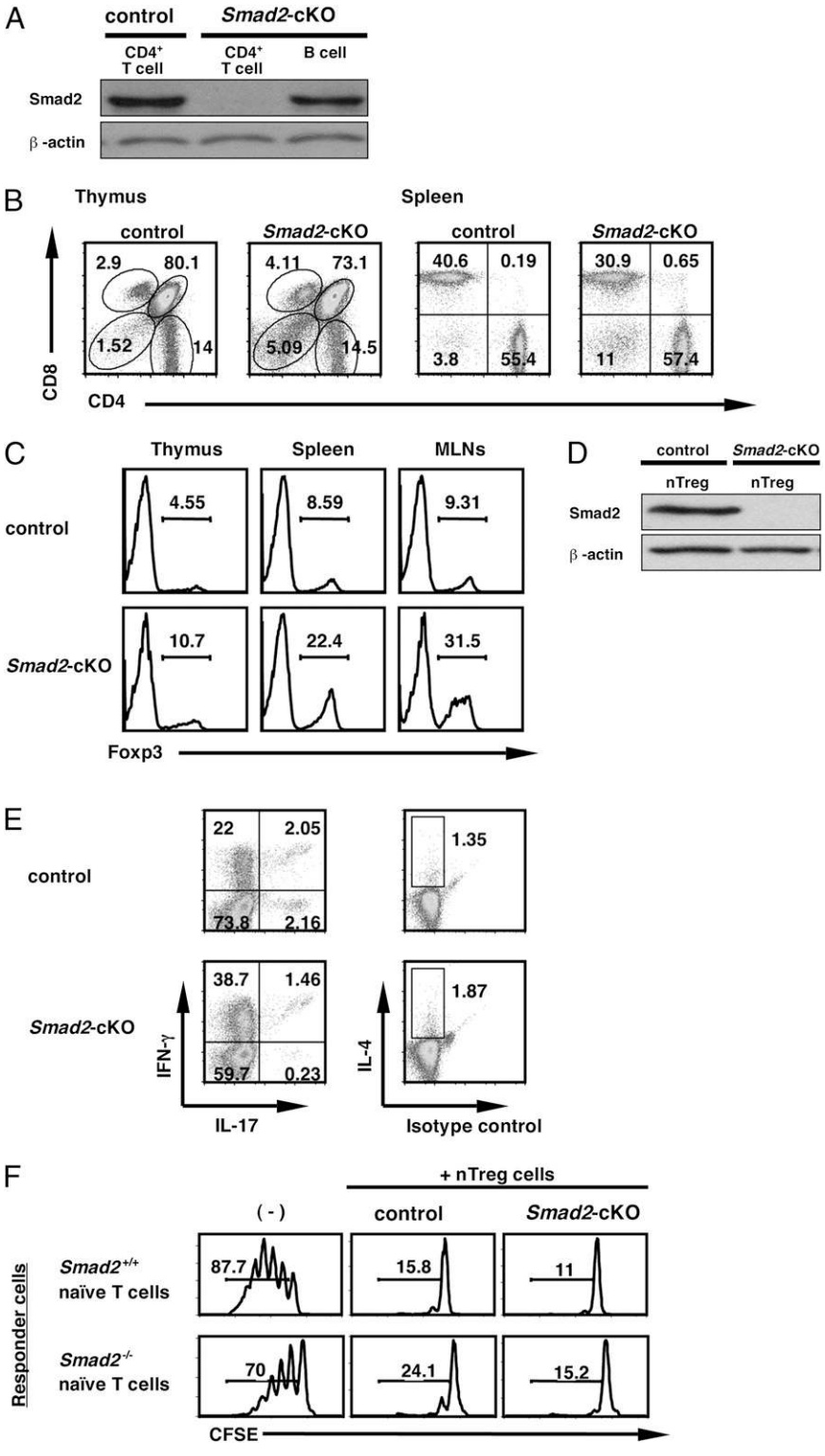

FIGURE 1. T cell-specific deletion of *Smad2* abrogates T cell homeostasis in vivo. *A*, Immunoblot of lysates of FACS-sorted CD4$^+$ CD25$^-$CD44^{low}CD62L^{high} T cells (naive T cells) isolated from *Smad2*$^{f/f}$ (control) or LckCre-*Smad2*$^{f/f}$ (*Smad2*-cKO) mice (loading control: β-actin). Data shown are representative of two independent experiments on different mice. *B*, Flow cytometry of freshly isolated T cells from thymus and spleen in control and *Smad2*-cKO mice at 8 wk old. Five mice per group were analyzed with similar results. *C*, Flow cytometry of freshly isolated CD4$^+$ T cells from thymus, spleen, and MLNs in indicated mice at 8 wk old. Cells were fixed and stained for intracellular Foxp3. Histograms are gated on CD4-single-positive subsets. Five mice per group were analyzed with consistent results. *D*, Immunoblot of lysates of FACS-sorted CD4$^+$CD25^{high}CD44^{low}CD62L^{high} nTregs isolated from control or *Smad2*-cKO mice (loading control: β-actin). Data shown are representative of two independent experiments on different mice with similar results. *E*, Flow cytometry of freshly isolated CD4+ T cells from spleen in indicated mice at 8 mo old. Cells were stimulated for 5 h with PMA and ionomycin. Brefeldin A was added to cultures before 1 h. Cells were assessed for the indicated cytokine expression by intracellular staining. Data shown are representative of five mice per group. *F*, CFSE-labeled naive CD4$^+$ T cells from control or *Smad2*-cKO mice were cultured with or without CD4$^+$ CD25^{high}CD44^{low}CD62L^{high} nTregs from control or *Smad2*-cKO mice in triplicate wells with irradiated splenocytes and stimulated with 2 μg/ml of anti-CD3 Abs for 3 d. Proliferation was estimated by CFSE dilution. Each histogram was gated on a CD4-positive subset. Representative data of two independent experiments are shown. MLNs, mesenteric lymph nodes.

which is deteriorated by IFN-γ (32), than were control littermates (data not shown). These observations suggest that *Smad2*-cKO mice were more sensitive to inflammatory stress than control (*Smad2*$^{f/fl}$) littermate mice were.

Characterization of Smad2-deficient T cells

First, we characterized T cells in *Smad2*-cKO mice. The development of T cells in the thymus and spleen were normal in *Smad2*-cKO mice (Fig. 1*B*). As was found to be true of *Smad3*-KO mice (33), *Smad2*-cKO mice can also generate nTregs, and the fraction of nTregs in the indicated organs was 2- to 3-fold higher in *Smad2*-cKO mice than in control littermate mice (Fig. 1*C*). We confirmed that Smad2 protein was also undetectable in nTregs isolated from *Smad2*-cKO mice (Fig. 1*D*). Peripheral CD4$^+$ T cells from *Smad2*-cKO mice produced more IFN-γ, but less IL-17, after stimulation with PMA and ionomycin (Fig. 1*E*). IL-4 levels were not found to be significantly different. These activated phenotypes of CD4$^+$ T cells in *Smad2*-cKO were not due to nTreg dysfunction, because nTregs from *Smad2*-cKO mice (*Smad2*$^{-/-}$ in Fig. 1*F*) showed a sufficient suppressive function, equivalent to that seen in nTregs from control mice (*Smad2*$^{+/+}$ in Fig. 1*F*). Naive *Smad2*$^{-/-}$ CD4$^+$ T cells also showed similar sensitivity to the suppressive effect of nTregs (Supplemental Fig. 2).

Phenotypes of LckCre-Smad2 $^{f/f}$*Smad3*$^{-/-}$ *mice*

Then we generated *Smad2* and *Smad3* double KO (DKO) mice by crossing *Smad2*-cKO mice and *Smad3*$^{-/-}$ (*Smad3*-KO) mice to compare T cell phenotypes among Smad2-cKO, Smad3-KO and *Smad2/3*-DKO mice. To understand the genotype easier, description of genotypes of T cells and mice is listed in Table I.

Overall, CD4$^+$ T cell phenotypes of *Smad2*-cKO mice resembled those of *Smad3*-KO mice (Fig. 2) (21). Although Smad2 signaling seems to be important in T cells, as we demonstrated above, the phenotypes of *Smad2*-cKO mice and those of *Smad3*-KO mice were much milder than those of *TGF-β1*–KO mice (4) or T cell-specific *TGF-βRII*–cKO mice (7). Whereas *Smad2/3*-DKO mice showed severe inflammation and died within 3–5 wk (Fig. 2*A*–*C*). Severe atrophy of the thymus was observed, and the total number of T cells in the thymus was extremely reduced (Fig. 2*D*). The population of activated–memory phenotype with CD25$^+$CD44^{high} CD62L^{low} was increased in peripheral CD4$^+$ T cells isolated from Smad2-cKO mice but not from Smad3-KO mice (Fig. 2*E*). Most of the peripheral CD4$^+$ T cells in *Smad2/3*-DKO mice showed an activated memory phenotype (Fig. 2*E*). The fraction of Foxp3-expressing cells in the thymus of *Smad2/3*-DKO mice was similar to that of littermate control (*Smad2*$^{fl/fl}$*Smad3*$^{+/+}$) mice, whereas Foxp3-expressing cells in the periphery of these mice were severely reduced (Fig. 2*F*). These results suggested that TGF-β–Smad signaling was not always necessary for the generation of nTregs in vivo. These phenotypes of *Smad2/3*-DKO mice were similar to those of T cell-specific *TGF-βRII*–cKO mice, suggesting that the immunosuppressive functions of Smad2 and Smad3 overlap each other and are redundantly essential for TGF-β signaling in T cells.

The compatible roles of Smad2 and Smad3 in regulating the gene transcription by TGF-β

It has been shown that, unlike Smad3, Smad2 lacks a DNA-binding domain; therefore, Smad2 has been suggested to regulate different genes than does Smad3 (18). However, the functions of Smad2 and Smad3 in gene regulation of T cells have not been investigated. To investigate the requirement of Smad2 or Smad3 for TGF-β–mediated gene regulation, we performed a microarray analysis. We used TGF-β1 in all experiments because TGF-β1 is believed to be the major TGF-β involved in immune regulation. Because *Smad2/3*-DKO mice had too low a number of naive CD4$^+$ T cells as shown in Fig. 2*E*, we used *Smad2*$^{-/-}$*Smad3*$^{+/-}$ CD4$^+$ T cells from LckCre-*Smad2*$^{f/f}$/*Smad3*$^{+/-}$ (2cKO/3hetero) mice for microarray analysis. A similar approach was performed previously to identify Smad2 and Smad3 common target genes in keratinocytes (34). We considered that *Smad2*$^{-/-}$*Smad3*$^{+/-}$ CD4$^+$ T cells were still useful to identify Smad2/3-dependent and -independent genes, because Foxp3 induction by TGF-β was severely impaired in *Smad2*$^{-/-}$ *Smad3*$^{+/-}$ T cells (Supplemental Fig. 3).

Naive CD4$^+$ T cells from control, *Smad2*-cKO, *Smad3*-KO, or 2cKO/3hetero mice were stimulated with anti-CD3e/CD28 with or without TGF-β for 24 h. We compared genes upregulated >4-fold and downregulated <0.25-fold by TGF-β in *Smad2*$^{+/+}$*Smad3*$^{+/+}$ CD4$^+$ T cells. A significant number of genes were regulated by Smad2 and Smad3 specifically (8% was Smad2-specific and 20% was Smad3-specific). However, the role of these genes in T cells has not been clarified. We found that a large proportion of TGF-β–regulated genes were both Smad2 and Smad3-dependent (Smad2/3-dependent); 64% (increase) and 90% (decrease) of genes, including *Foxp3, Ahr, IRF8, Ikzf4 (Eos), c-maf* (up), and *IL-2* (down), were equally affected by either Smad2 or Smad3 deficiency (Fig. 3*A*, 3*B*, Supplemental Fig. 4). These data suggested that not only Smad3 but also Smad2 were required in TGF-β–mediated transcriptional regulation in T cells. Notably, most of the Treg signature genes (35, 36) were similarly affected by Smad2 or Smad3 deficiency (Fig. 3*C*).

Interestingly, we found several TGF-β–regulated genes that which were not affected by Smad2/3-deficiency, including *RORγt*, *CD73*, *Ccr8*, *Socs2* (up), and *eomes* (down). These data collectively indicated that TGF-β organizes the gene transcription in T cells through both a Smad2/3-dependent and -independent manner; however, most TGF-β–inducible genes involved in T cell functions and differentiation are shown to be common targets of Smad2 and Smad3.

Induction and maintenance of Foxp3 by TGF-β were redundantly dependent on Smad2 and Smad3

Next, we confirmed the involvement of Smad signaling in iTreg differentiation by TGF-β. As shown in Fig. 4*A*, when cultured in the presence of TGF-β and IL-2, Foxp3 mRNA expression levels

Table I. *Description of the genotypes used in this study*

T Cell		Mouse	
Control	*Samd2*$^{fl/fl}$*Smad3*$^{+/+}$	Control	*Samd2*$^{fl/fl}$*Smad3*$^{+/+}$
Samd2$^{-/-}$*Smad3*$^{+/+}$	*Samd2*$^{del/del}$*Smad3*$^{+/+}$	Smad2-cKO	LckCre*Smad2*$^{fl/fl}$*Smad3*$^{+/+}$
Samd2$^{-/-}$*Smad3*$^{-/-}$	*Samd2*$^{fl/fl}$*Smad3*$^{-/-}$	Smad3-KO	*Smad2*$^{fl/fl}$*Smad3*$^{-/-}$
Samd2$^{-/-}$*Smad3*$^{+/-}$	*Samd2*$^{del/del}$*Smad3*$^{+/-}$	2cKO/3hetero	LckCre*Smad2*$^{fl/fl}$*Smad3*$^{+/-}$
Samd2$^{-/-}$*Smad3*$^{-/-}$	*Samd2*$^{del/del}$*Smad3*$^{-/-}$	2/3-DKO	LckCre*Smad2*$^{fl/fl}$*Smad3*

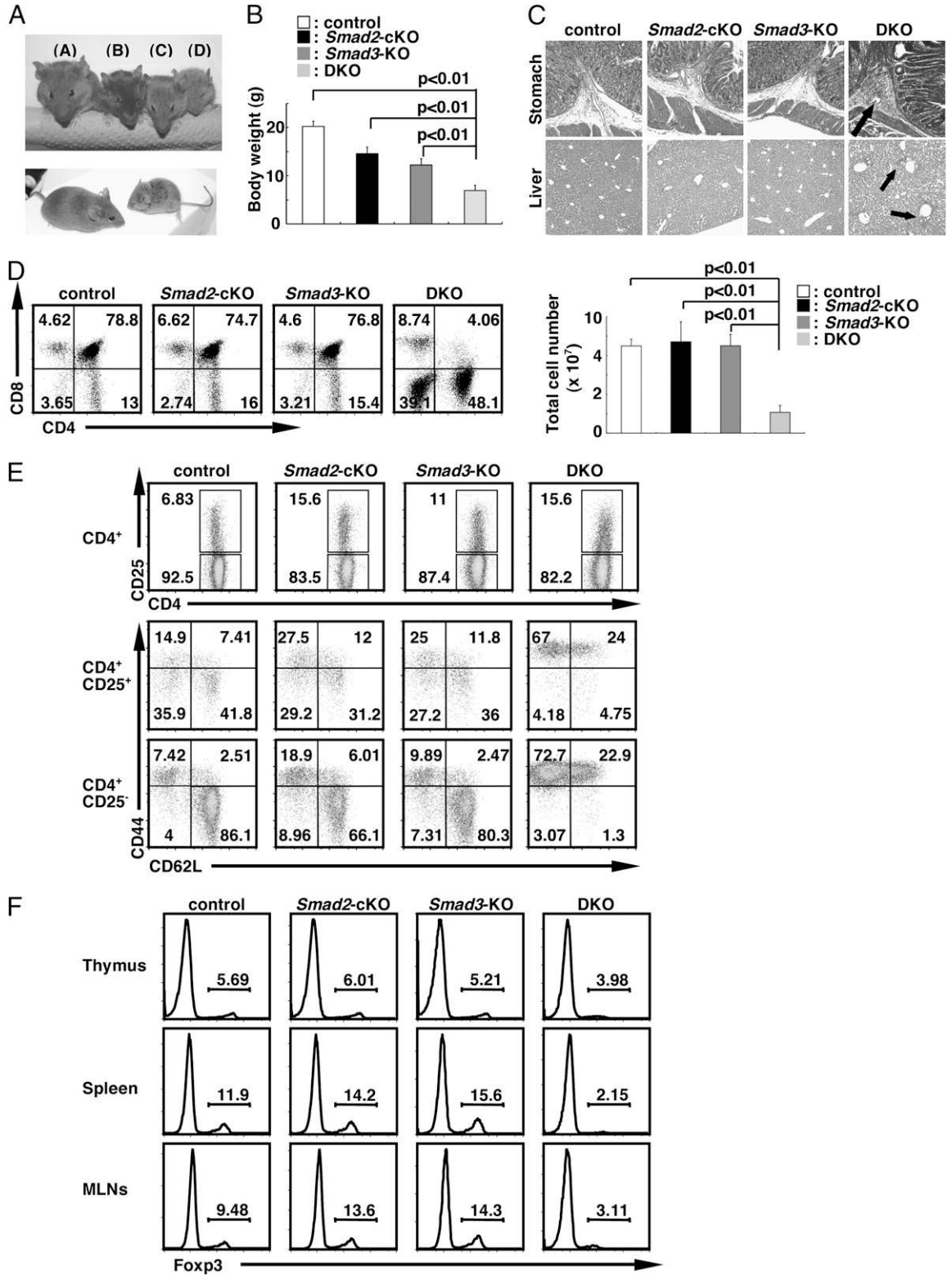

FIGURE 2. The genetic ablation of both Smad2 and Smad3 in T cells causes severe inflammatory diseases. Appearance of LckCre-*Smad2*$^{f/f}$*Smad3*$^{-/-}$ (DKO) mice. The left panel shows, from left to right, the front image of (*A*) control (*Smad2*$^{f/f}$*Smad3*$^{+/+}$), (*B*) *Smad2*-cKO (LckCre-*Smad2*$^{f/f}$*Smad3*$^{+/+}$), (*C*) *Smad3*-KO (*Smad2*$^{f/f}$*Smad3*$^{-/-}$), and (*D*) DKO (LckCre-*Smad2*$^{f/f}$*Smad3*$^{-/-}$) mice, respectively. All mice were 4-wk-old male littermates. The *right panel* shows lateral images of mice; *left*, control (*Smad2*$^{f/f}$*Smad3*$^{+/+}$) and *right*, *Smad2/3*-DKO (LckCre-*Smad2*$^{f/f}$*Smad3*$^{-/-}$). A bar graph shows the mean body weights of *Smad2*$^{f/f}$*Smad3*$^{+/+}$ (control; n = 15), LckCre-*Smad2*$^{f/f}$*Smad3*$^{+/+}$ (*Smad2*-cKO; n = 13), *Smad2*$^{f/f}$*Smad3*$^{-/-}$ (*Smad3*-KO; n = 12) and LckCre-*Smad2*$^{f/f}$*Smad3*$^{-/-}$ (DKO; n = 10) mice at the age of 4 wk. Statistical differences were verified by paired Student t test. Histologic analysis by H&E staining of indicated organs from indicated mice at 4 wk old (original magnification ×20). Black arrows indicate inflammatory cell infiltration. Data shown are representative from one of the five tissue samples with similar results. *D*, CD4/CD8 profile of freshly isolated CD3$^+$ T cells from the thymus of indicated mice at 4 wk old. A bar graph shows the mean total cell number of thymocytes. *E*, Expression of activation–memory markers on freshly isolated CD4$^+$ T cells from spleens of indicated mice at 4 wk old. *Upper*, *middle*, and *lower panels* are gated on CD4–single-positive, CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ subsets, respectively. Ten mice per group were analyzed and representative data are shown. *F*, Foxp3 expression in freshly isolated CD4$^+$ T cells from

were partially decreased in *Smad2*$^{-/-}$*Smad3*$^{+/+}$ CD4$^+$ T cells and *Smad2*$^{+/+}$*Smad3*$^{-/-}$ CD4$^+$T cells. Reduced iTreg differentiation of *Smad2*$^{-/-}$*Smad3*$^{+/+}$ CD4$^+$ T cells was confirmed by Foxp3 immunostaining (Fig. 4*B*). In the absence of Smad2, the iTreg fraction was rapidly decreased after day 4 in culture (Fig. 4*B*).

Foxp3 induction was markedly reduced in *Smad2*$^{-/-}$*Smad3*$^{+/-}$ CD4$^+$T cells. Furthermore, TGF-β never induced Foxp3 mRNA in naive *Smad2*$^{-/-}$*Smad3*$^{-/-}$ CD4$^+$ T cells (Fig. 4*A*), which was consistent with microarray data (Fig. 3*A*). These data indicate that TGF-β–mediated Foxp3 induction is completely dependent on Smad signaling. In addition, one Smad in Foxp3 induction can partly compensate for the loss of another Smad.

Previous reports have suggested that TGF-β could also be involved in the maintenance of Foxp3 expression in nTregs at the periphery (7, 17); however, the role of Smad signaling has not been identified. We isolated CD4$^+$CD25^{high}CD44^{low}CD62L^{high} T cells as nTregs by FACS (>98% Foxp3-positive day 0) (Fig. 4*C*). The fraction of Foxp3-positive cells was decreased to 44% in *Smad2*$^{+/+}$*Smad3*$^{+/+}$ nTregs in the absence of TGF-β, and TGF-β partly maintained their Foxp3 expression to 61% (Fig. 4*C*, *top row*). *Smad2*$^{-/-}$*Smad3*$^{+/+}$ or *Smad2*$^{+/+}$*Smad3*$^{-/-}$ nTregs lost their Foxp3 expression more intensively than did *Smad2*$^{+/+}$*Smad3*$^{+/+}$ nTregs within this in vitro culture period, but TGF-β still increased the levels of Foxp3 in these cells (Fig. 4*C*, *middle two rows*). However, *Smad2*$^{-/-}$*Smad3*$^{+/-}$ nTregs lost Foxp3 expression more profoundly, and TGF-β showed little effect on Foxp3 maintenance in these cells (Fig. 4*C*, *bottom row*). We also observed that Smad2-deficient nTregs more rapidly lost their Foxp3 expression in vivo, when transferred into *Rag2*$^{-/-}$ mice (Supplemental Fig. 5). These data suggest that TGF-β is involved in the maintenance of Foxp3 in nTregs via a Smad2 and Smad3-dependent mechanism.

TGF-β–mediated suppression of Th1 cell differentiation was dependent on both Smad2 and Smad3

It has been shown that TGF-β strongly inhibits Th1 cell differentiation from naive CD4$^+$ T cells (37). Furthermore, Foxp3 in CD4$^+$ T cells has been shown to suppress the production of various cytokines, including IFN-γ. To clarify the role of Smad2 and Smad3 in TGF-β–mediated suppression of Th1 cell differentiation, and its relationship to Foxp3 induction, we compared Th1/iTreg differentiation among *Smad2*$^{+/+}$*Smad3*$^{+/+}$, *Smad2*$^{-/-}$*Smad3*$^{+/+}$, *Smad2*$^{+/+}$*Smad3*$^{-/-}$, or *Smad2*$^{-/-}$*Smad3*$^{+/-}$ CD4$^+$ T cells. Naive CD4$^+$ T cells were cultured in the Th1-skewing condition in the presence of various concentrations of TGF-β. As shown in Fig. 5*A* and 5*B*, in control *Smad2*$^{+/+}$*Smad3*$^{+/+}$ cells, TGF-β efficiently suppressed generation of IFN-γ–producing cells, but strongly induced Foxp3-positive cells. Th1 cell development was partially suppressed in *Smad2*$^{-/-}$*Smad3*$^{+/+}$ or *Smad2*$^{+/+}$*Smad3*$^{-/-}$ CD4$^+$ T cells by TGF-β at the concentration where Foxp3 induction was partially impaired. Moreover, the suppression of Th1 cell development and Foxp3 induction was barely observed in *Smad2*$^{-/-}$*Smad3*$^{+/-}$ CD4$^+$ T cells at any concentrations of TGF-β. These data indicate that TGF-β–mediated Th1 suppression and iTreg induction were strictly dependent on Smad2/3 signaling. The data in Fig. 5*B* suggest an inverse correlation between Th1 suppression and Foxp3 induction.

TGF-β–mediated CD103 induction was Smad2/3-dependent

The $\alpha_E\beta_7$ integrin is a cell surface receptor that interacts with E-cadherin on epithelial cells (38). The α_E subunit, CD103 is expressed on immune cells, including CD8$^+$ T cells, Tregs, and dendritic cells, and CD103$^+$ cells can accumulate in nonlymphoid tissues expressing E-cadherin. In addition, CD103 in Tregs has been implicated in suppressive activity (39). Because TGF-β also has been reported to upregulate CD103 expression in CD4$^+$ T cells (38), we examined the role of Smad signaling in CD103 expression by TGF-β (Fig. 5*C*). Induction of CD103 by TGF-β occurred mainly in Foxp3$^+$ T cells in *Smad2*$^{+/+}$*Smad3*$^{+/+}$ CD4$^+$ T cells. TGF-β–mediated expression of CD103 was dependent on Smad2/3 signaling, because almost no induction of CD103 was observed in *Smad2*$^{-/-}$*Smad3*$^{+/-}$ T cells. Interestingly, *Smad2*$^{+/+}$*Smad3*$^{-/-}$ and *Smad2*$^{-/-}$*Smad3*$^{+/+}$ CD4$^+$ T cells expressed low levels of CD103, even in Foxp3$^+$ cells (compare Fig. 5*A*, 5*C*). These data suggest that CD103 induction by TGF-β was more strictly dependent on Smad2 or Smad3 than Foxp3 induction.

TGF-β suppresses Th1 cell differentiation by Foxp3-independent mechanisms

Because Foxp3$^+$ cells barely produced IFN-γ (12), the above data suggested that Foxp3 induction by TGF-β in naive CD4$^+$ T cells could be the most important mechanism in TGF-β–mediated regulation of Th1 cell differentiation.

To verify this possibility, we used Foxp3-deficient T cells from *scurfy* mice, which have a nonfunctioning mutant *Foxp3* gene allele (40). Unexpectedly, TGF-β sufficiently inhibited Foxp3-deficient CD4$^+$ T cells from differentiating into Th1 cells, just as it did in wild-type CD4$^+$ T cells (Fig. 6*A*, 6*B*). Next, we examined whether TGF-β could also regulate Th1 cell differentiation in a Foxp3-independent manner in vivo. We administered recombinant TGF-β into *scurfy* mice on a daily basis. It is well known that *scurfy* mice develop fatal autoimmune diseases, and that various cytokines secreted by excessively activated CD4$^+$ T cells play an important role in their pathologies (40, 41). Notably, exogenously administered TGF-β efficiently suppressed severe autoimmune phenotypes of *scurfy* mice (Fig. 6*C*, 6*D*). Microscopic studies showed milder tissue destruction and inflammatory cell infiltration in the livers of TGF-β–treated *scurfy* mice than in those of PBS-treated *scurfy* mice (Fig. 6*D*). Consistent with these results, CD4$^+$ T cells isolated from lymph nodes or spleens of TGF-β–treated *scurfy* mice showed lower production levels of IL-2, IFN-γ, and IL-4 than did those from PBS-treated *scurfy* mice (Fig. 6*E*) (data not shown). These observations indicate that TGF-β can suppress the responses of CD4$^+$ T cells without inducing Foxp3.

Smad2/3 signaling is required for effective Th17 cell development, but not for the induction of RORγt

Recent studies have shown that TGF-β acts as an inducer of IL-17–producing Th17 cells in mice and humans, especially when combined with proinflammatory cytokines, such as IL-6 or IL-21 (42–44). Th17 cells are now accepted as one of the main causes of the pathogenesis of various murine autoimmune diseases including EAE (30). First, we examined the effect of Smad2 deficinecy in T cells on EAE. As shown in Fig. 7*A* and 7*B*, the clinical features associated with the EAE model were milder in *Smad2*-cKO mice than in control littermate mice. The level of IL-17 production from CD4$^+$ T cells in diseased *Smad2*-cKO mice was lower than those from the control littermate mice. These data suggest that Smad2 positively regulates Th17 cell differentiation (Fig. 7*C*). However, CD4$^+$ T cells in *Smad2*-cKO mice produced higher levels of IFN-γ or IL-2 than did those in the control

thymi, spleens, and MLNs of indicated mice at 4 wk old. Cells were fixed and stained for intracellular Foxp3. Data are representative of five independent experiments with similar results. MLNs, mesenteric lymph nodes.

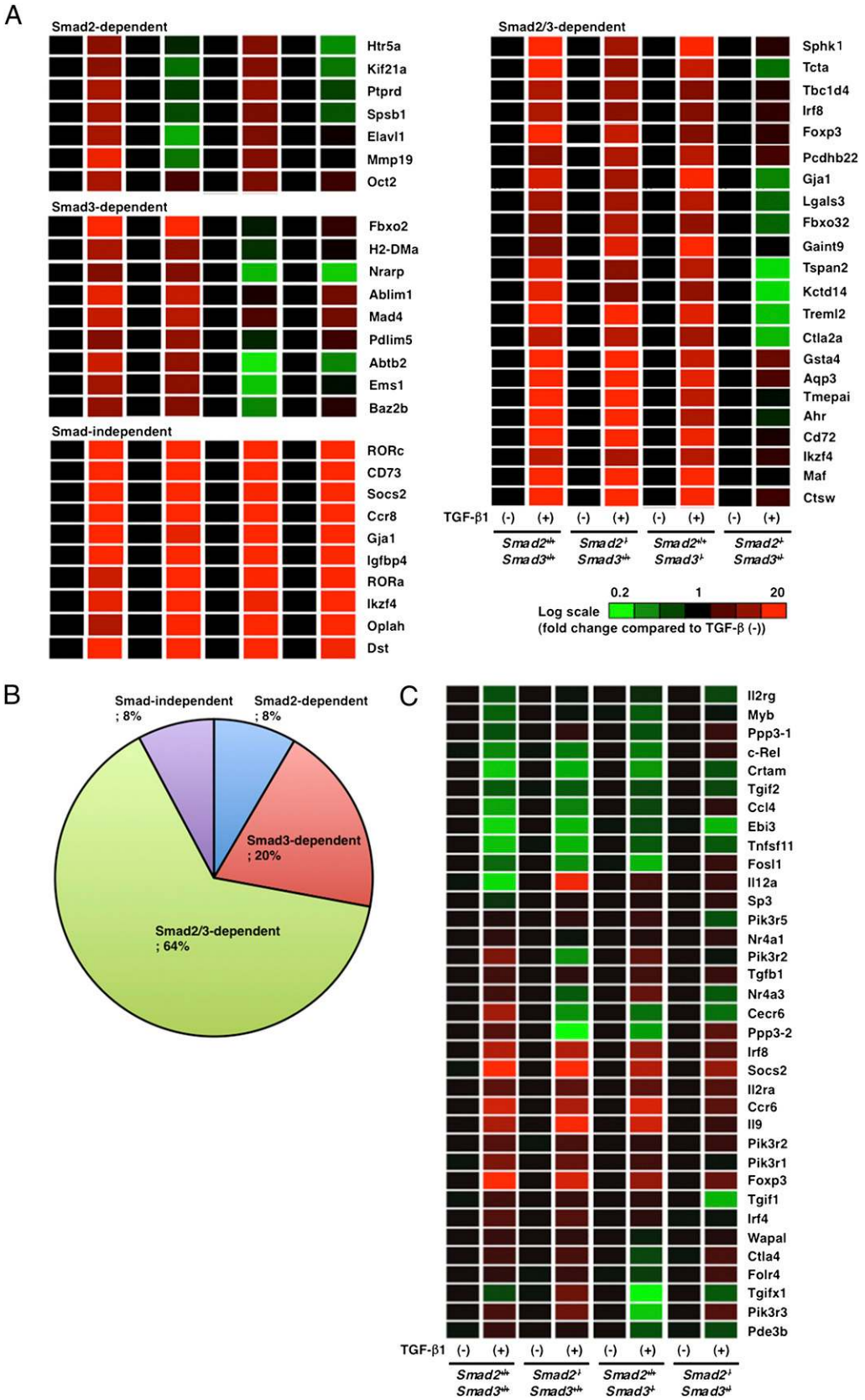

FIGURE 3. Microarray analysis of TGF-β-regulated genes in CD4$^+$ T cells. *A*, Fold change comparing the effect of TGF-β on CD4$^+$ T cells in gene expression. FACS-sorted naive CD4$^+$ T cells cells from control (*Smad2*$^{+/+}$*Smad3*$^{+/+}$), *Smad2*-cKO (*Smad2*$^{-/-}$*Smad3*$^{+/+}$), *Smad3*-KO (*Smad2*$^{+/+}$*Smad3*$^{-/-}$), and 2cKO/3hetero (*Smad2*$^{-/-}$*Smad3*$^{+/-}$) mice were stimulated by anti-CD3e/CD28 and IL-2 with or without 2ng/ml of TGF-β1 in the presence of anti-IFN-γ and anti-IL-4 for 24 h. Genes upregualted >4-fold by TGF-β1 in *Smad2*$^{+/+}$*Smad3*$^{+/+}$ CD4$^+$ T cells were compared. Levels of upregulated genes by TGF-β1 in indicated cells were compared with the same genotype cells stimulated without TGF-β, normalized as 1 of the expression value. Transcriptional profiling of indicated cells was performed using Affymetrix M430 2.0 microarrays. *B*, Relationship between Smad-dependent and Smad-independent genes revealed by expression array analysis in upregulated genes by TGF-β. *C*, Same comparison of fold change as in *A*, with the Treg signature genes.

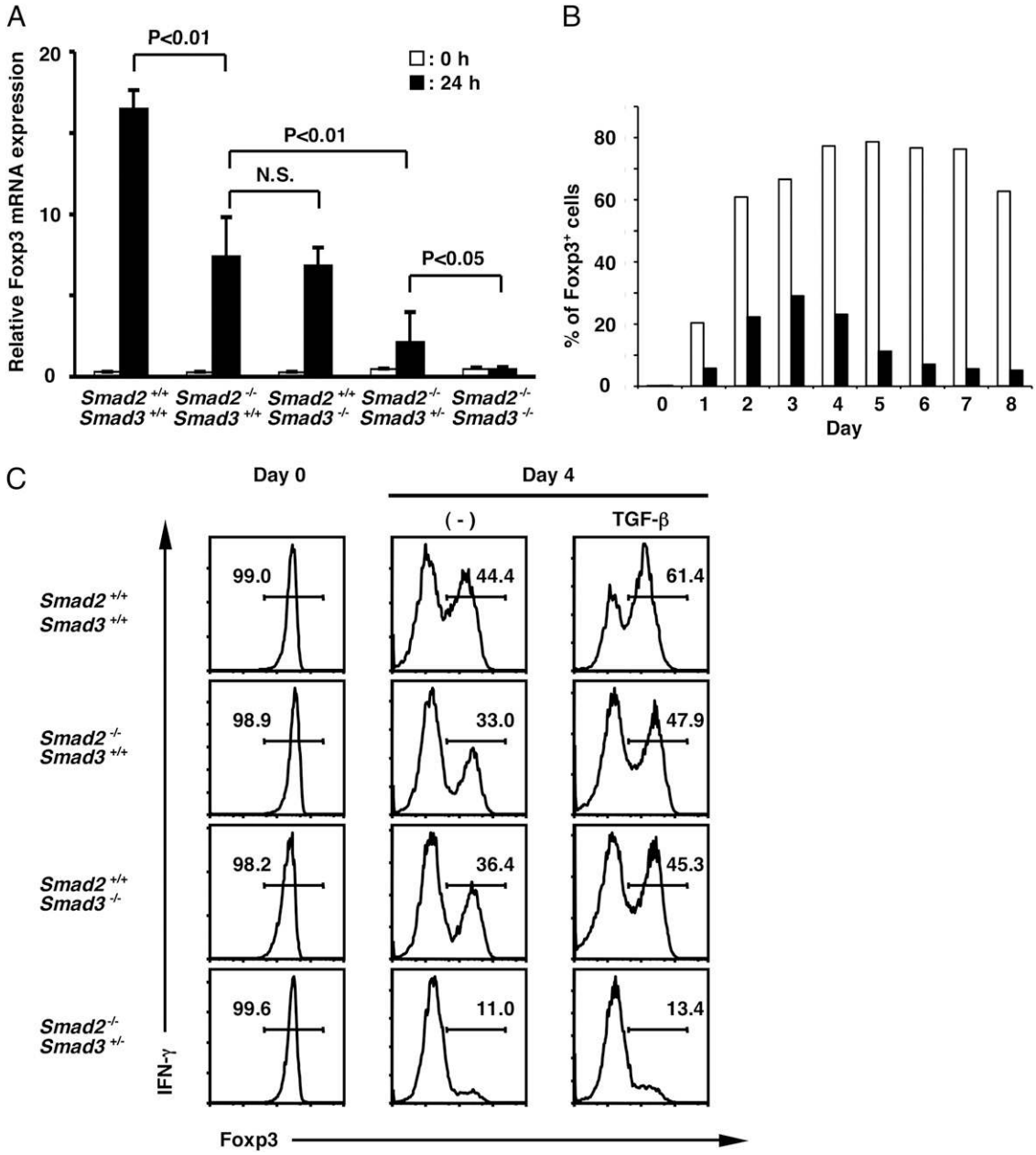

FIGURE 4. TGF-β–mediated induction and maintenance of Foxp3 in Smad2- and Smad3- deficient T cells. *A*, FACS-sorted naive CD4$^+$ T cells from control (*Smad2*$^{+/+}$*Smad3*$^{+/+}$), *Smad2*-cKO (*Smad2*$^{-/-}$*Smad3*$^{+/+}$), *Smad3*-KO (*Smad2*$^{+/+}$*Smad3*$^{-/-}$), 2cKO/3hetero (*Smad2*$^{-/-}$*Smad3*$^{+/-}$), and DKO (*Smad2*$^{-/-}$*Smad3*$^{-/-}$) mice were stimulated under the Treg-skewing condition for 24 h. At 0 and 24 h, mRNA expression of Foxp3 was assessed by real-time RT-PCR. The experiment was performed in duplicate. Data shown were normalized to the expression of a reference gene, *HPRT*. The expression for *Foxp3* gene of *Smad2*$^{+/+}$*Smad3*$^{+/+}$ CD4$^+$ T cells at 0 h was set as 1. Bars show means ± SD. Data are representative of two independent experiments with consistent results. Statistical differences were verified by paired Student *t* test. *B*, FACS-sorted *Smad2*$^{+/+}$*Smad3*$^{+/+}$ (open square) or *Smad2*$^{-/-}$*Smad3*$^{+/+}$ (closed square) naive CD4$^+$ T cells were cultured in the iTreg-skewing condition and collected everyday. Cells were fixed, stained for intracellular Foxp3, and assessed by FACS. A bar graph shows the percentage of Foxp3$^+$ cells. Data shown resulted from experiments that were repeated three times with similar results. *C*, FACS-sorted nTregs from control (*Smad2*$^{+/+}$*Smad3*$^{+/+}$), *Smad2*-cKO (*Smad2*$^{-/-}$*Smad3*$^{+/+}$), *Smad3*-KO (*Smad2*$^{+/+}$*Smad3*$^{-/-}$), and 2cKO/3hetero (*Smad2*$^{-/-}$*Smad3*$^{+/-}$) mice were stimulated by anti-CD3e/CD28 with 20 ng/ml of IL-2 in the absence or presence of TGF-β1 for 4 d. Cells were fixed, stained for intracellular Foxp3, and assessed by flow cytometry. Data are representative of two independent experiments with consistent results.

littermate mice (Fig. 7*C*). Reduced Th17 cells in *Smad2*-cKO mice might be due to upregulation of such anti-Th17 inflammatory cytokines (45, 46).

To investigate this possibility, we examined the levels of cytokines in vitro under Th17-skewing conditions. *Smad2*$^{-/-}$*Smad3*$^{+/+}$ CD4$^+$ T cells produced lower levels of IL-17, but produced higher levels of IL-2, IFN-γ and IL-4 than did *Smad2*$^{+/+}$*Smad3*$^{+/+}$ CD4$^+$ T cells (Fig. 7*D*). Intracellular FACS analysis revealed that production of IL-17 was severely reduced in *Smad2*$^{-/-}$*Smad3*$^{+/+}$ and *Smad2*$^{+/+}$*Smad3*$^{-/-}$ CD4$^+$ T cells (Fig. 7*E*, *upper panels*). *Smad2*$^{-/-}$*Smad3*$^{-/-}$ CD4$^+$ T cells were most severely affected (Fig. 7*E*, *upper panels*). Next, we examined the effect of anti–IL-2 Ab on Th17 differentiation. Anti–IL-2 Ab alone efficiently reduced the levels of both IFN-γ and IL-4 production (data not shown). As shown in Fig. 7*E* (*lower panels*), Smad2- or Smad3-deficient CD4$^+$ T cells effectively developed into Th17 cells when anti–IL-2 Ab was included in the culture. We observed significant TH17 development in *Smad2*$^{-/-}$*Smad3*$^{-/-}$ CD4$^+$ T cells. These

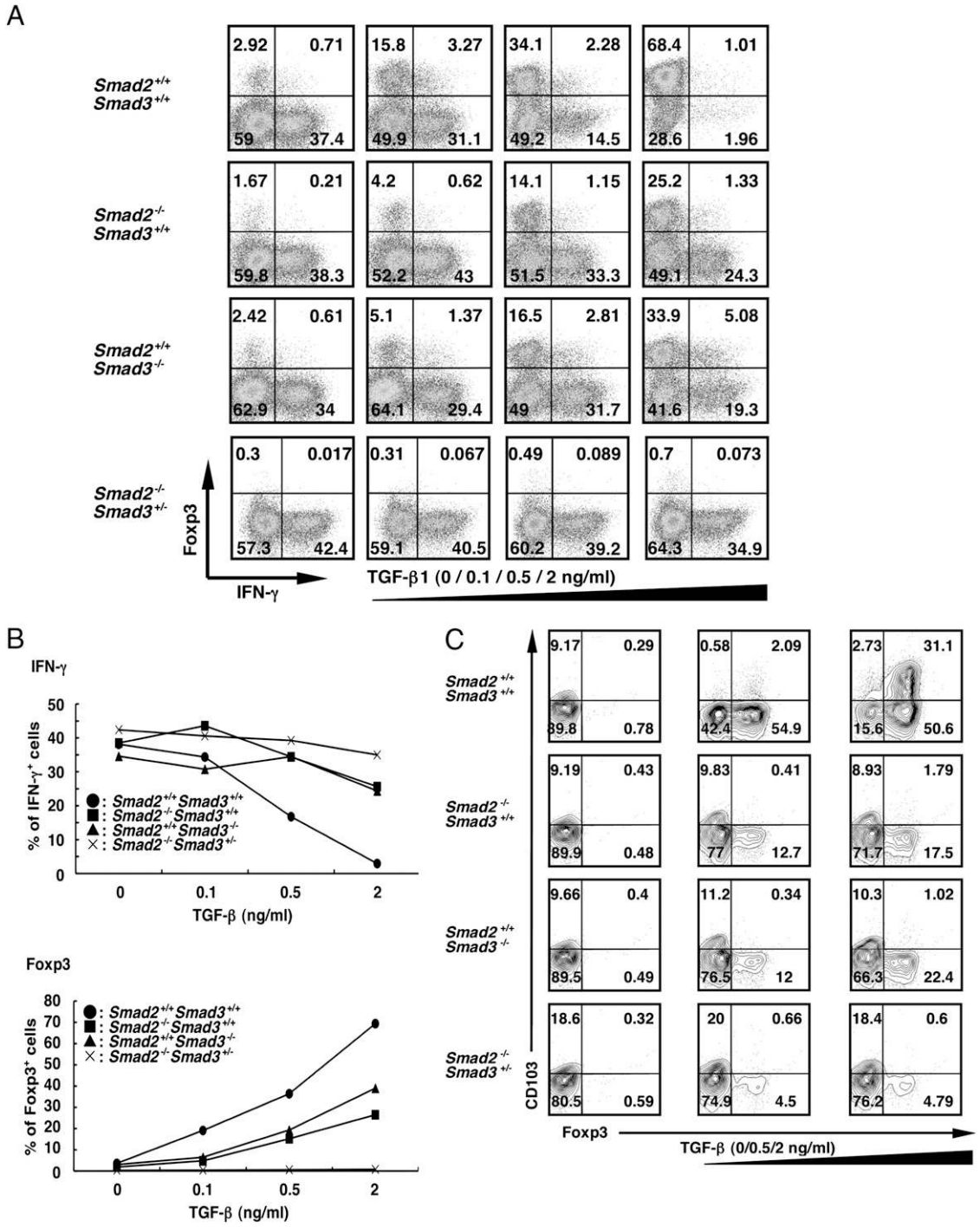

FIGURE 5. Relationship between Th1 cell development and Foxp3 induction in TGF-β-mediated Th1 cell regulation. *A*, FACS-sorted naive CD4$^+$ T cells from control (*Smad2*$^{+/+}$*Smad3*$^{+/+}$), *Smad2*-cKO (*Smad2*$^{-/-}$*Smad3*$^{+/+}$), *Smad3*-KO (*Smad2*$^{+/+}$*Smad3*$^{-/-}$), and 2cKO/3hetero (*Smad2*$^{-/-}$*Smad3*$^{+/-}$) mice were stimulated under the Th1-skewing condition with or without TGF-β1 at various concentrations. On day 4, cells were restimulated for 5 h with PMA and ionomycin. Brefeldin A was added to cultures <1 h after restimulation began. Cells were assessed for IFN-γ production and Foxp3 expression by intracellular staining. Data shown are representative of three independent experiments in triplicate with similar results. The percentage of IFN-γ$^+$ cells (*left panel*) and Foxp3$^+$ cells (*right panel*) of triplicate samples is shown in *B*. Each graph shows means ± SD. Data shown are representative of three independent experiments with similar results. *C*, FACS-sorted *Smad2*$^{+/+}$*Smad3*$^{+/+}$, *Smad2*$^{-/-}$*Smad3*$^{+/+}$, *Smad2*$^{+/+}$*Smad3*$^{-/-}$, and *Smad2*$^{-/-}$*Smad3*$^{+/-}$ naive CD4$^+$ T cells were cultured with anti-CD3e/CD28 and 10 ng/ml of IL-2 in the absence or presence of TGF-β at various concentrations for 4 d. Cells were stained for surface CD103 and intracellular Foxp3 and assessed by flow cytometry. Data shown resulted from experiments that were repeated two times with consistent results.

data suggest that Smad2/3 positively regulates Th17 differentiation by suppressing Th17-inhibitory cytokine production.

Next, to confirm a dispensable effect of Smad2/3 on Th17 differentiation, we examined TGF-β+IL-6–mediated induction of RORγt, a master regulator of Th17 cells (29). As expected from microarray analysis (Fig. 3*A*), the early induction of RORγt normally occurred in *Smad2*$^{-/-}$*Smad3*$^{+/+}$, *Smad2*$^{+/+}$*Smad3*$^{-/-}$, or *Smad2*$^{-/-}$*Smad3*$^{-/-}$ CD4$^+$ T cells in the Th17-skewing condition (Fig. 7*F*). This suggests that both Smad2 and Smad3 were not involved in the induction of RORγt by TGF-β. These results indicate that TGF-β mediates RORγt induction via the Smad-independent pathway. However, the TGF-β–Smad pathway is still required for

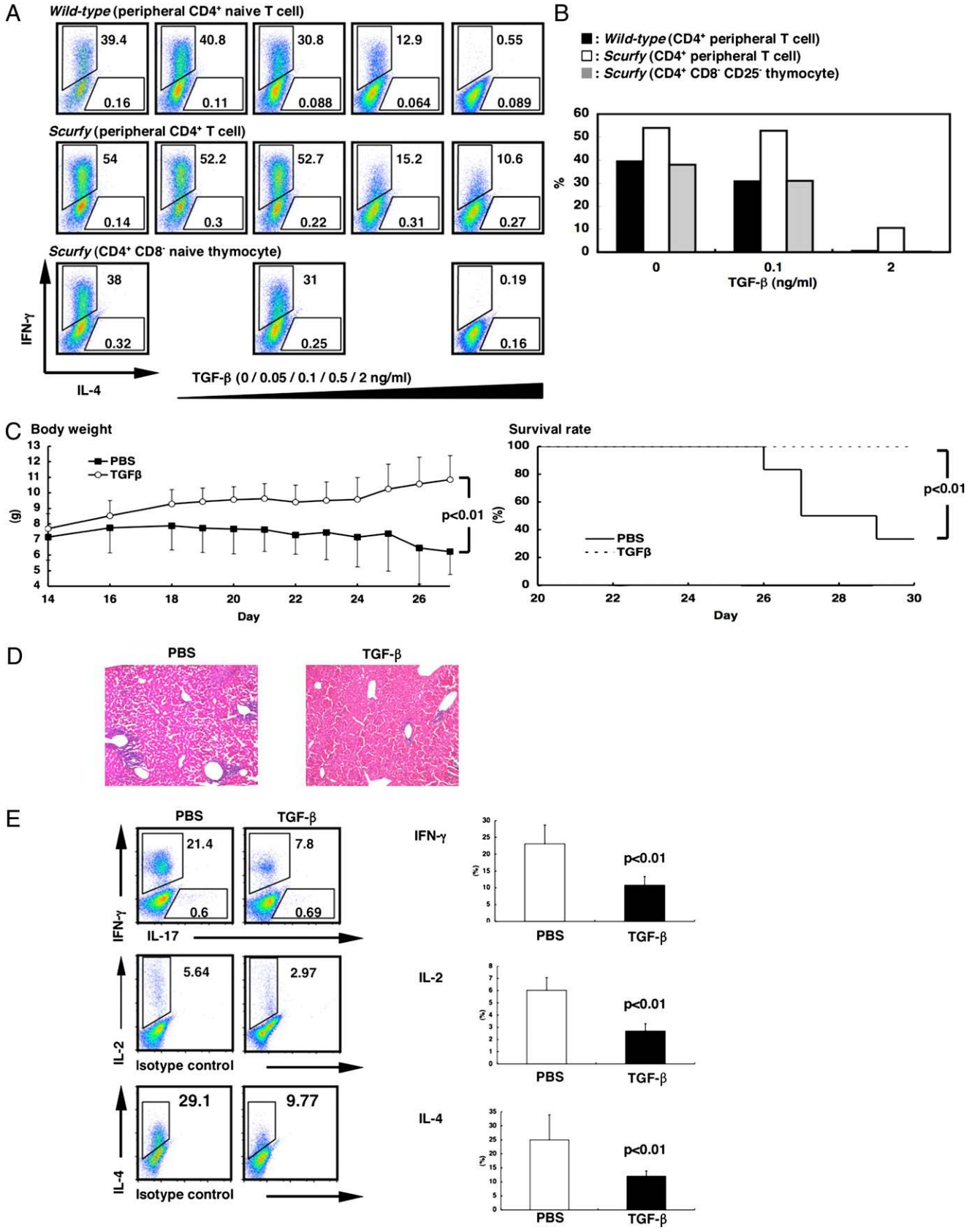

FIGURE 6. Foxp3-independent TGF-β–mediated suppression of Th1 cell development *A*, FACS-sorted peripheral naive CD4$^+$ T cells or thymic naive CD4$^+$ T cells from C57BL/6 (WT) or *scurfy* (*scurfy*) mice were cultured in the Th1-skewing condition with or without TGF-β1 at various concentrations for 4 d. Cells were then restimulated for 5 h with PMA and ionomycin. Brefeldin A was added to cultures <1 h after restimulation began. Cells were assessed for IFN-γ and IL-4 expression by intracellular staining. Data shown are representative of two independent experiments in triplicate with similar results. *B*, A bar graph shows the mean percentage of IFN-γ–producing cells in total CD4$^+$ T cells cultured as described in *A*. *C*, Male *scurfy* mice at 2 wk of age were daily injected i.p. and s.c. with PBS (closed square, solid line, $n = 10$) or 100 ng/ml recombinant hTGF-β1 (open circle, dotted line, $n = 12$) twice per day

efficient Th17 cell development by suppressing Th1 and Th2 cytokines. This finding is consistent with the recent finding that Smad4 is not required for the induction of RORγt (47).

Discussion

In this study, we demonstrated for the first time that Smad2 and Smad3 are essential for TGF-β–mediated regulation of T cell immunity. *Smad2*-cKO mice exhibited some immunologic disturbance, but not at lethal levels. When certain inflammatory events occurred, such as dextran sodium sulfate-induced colitis, *Smad2*-cKO mice failed to maintain the immunologic homeostasis. Furthermore, we showed that Smad2 and Smad3 have functional redundancy, sharing common roles in T cells. One Smad partially compensated for the deficiency of another in particularly important functions, such as Foxp3 induction or maintenance or Th1 cell suppression. Consistent with this idea, Smad2–three-dimensional–KO mice because of severe inflammation, as did *TGF-β1* KO mice. We demonstrated in this study that TGF-β–mediated induction of Foxp3 is also dependent on both Smad2 and Smad3 signaling. Furthermore, we demonstrated that both Smad2 and Smad3 play an important role in the maintenance of Foxp3 expression in nTregs. Like *TGF-β1* KO mice, Smad2–three-dimensional–KO mice possess nTregs, but their number was decreased especially at the periphery. We showed that nTregs lost the expression of Foxp3 in vitro, but TGF-β partially suppressed the loss of Foxp3. Conversion of Foxp3+ cells to Foxp3− cells was much more drastic in $Smad2^{-/-}$ $Smad3^{+/-}$ nTregs, and TGF-β–mediated Foxp3 maintenance was severely impaired in $Smad2^{-/-}Smad3^{+/-}$ nTregs. Molecular basis for such conversion remains to be investigated.

Currently, it is not known whether similar mechanisms were used for Foxp3 maintenance by TGF-β in nTregs and Foxp3 induction by TGF-β in naive $CD4^+$ T cells. Recently, it has been shown that Foxp3 expression is not stable and can be extinguished in Tregs that divert from their original phenotype and become Th17 cells (48, 49) or follicular helper T (Tfh) cells (50) both in vitro and in vivo. Zhou et al. (51) revealed that the loss of Foxp3 in Tregs under autoimmune conditions can result in the conversion of suppressor T cells into highly autoaggressive lymphocytes called *exFoxp3 cells*, which do not currently express Foxp3 but expressed it previously. In addition to the loss of the phenotypic characteristics of Tregs, exFoxp3 cells acquire some new features, including the ability to produce effector cytokines, such as IFN-γ and IL-17. In this study, both Smad2 and Smad3 were essential for the TGF-β–mediated maintenance of Foxp3 expression in Tregs. Our preliminary data also suggest that TGF-β prevents such conversion of Tregs into IFN-γ–producing cells in vitro, which could be one of the mechanisms of immune tolerance induced by TGF-β. It is possible that Smad2/3 interacts with the Foxp3 promoter, thereby maintaining Foxp3 expression and inhibiting pathogenic conversion of nTregs.

It is intriguing that Smad2 and Smad3 shared similar functions in T cells, because Smad3 binds to DNA, whereas Smad2 generally lacks any DNA-binding activity (18). Because Smad2 and Smad3 function redundantly for the regulation of >60% of genes in $CD4^+$ T cells, it is likely that Smad2 and Smad3 have a common partner and bind to a similar region. Our data suggest that Smad3 and Smad2 are involved in the TGF-β–mediated Foxp3 induction. However, it remains unclear how Smad2 regulates the gene expression of Foxp3. The cellular protein content of Smad2 is much higher than that of Smad3. Therefore, Smad2 may activate Foxp3 promoter by binding to the same Smad-binding site as Smad3, even though Smad2 has much lower affinity than Smad3. Another possibility is that Smad2 interacts with other transcription factors, thereby regulating Foxp3 expression by binding to the region of the Foxp3 enhancer different from the Smad3-binding element. We could not rule out the possibility that uncharacterized transcription factors induced by Smad2 contribute to the Foxp3 induction. Further study is necessary to determine the molecular mechanism of Smad2-mediated Foxp3 induction.

Smad4 is the most universal coregulator of canonical TGF-β signaling. However, there are significant differences between T cell-specific *Smad4*-KO mice and *Smad2/3*-DKO mice. For example, Smad4 deficeincy in T cells partially abrogated TGF-β–mediated Foxp3 induction (47), whereas it was completely diminished in $Smad2^{-/-}Smad3^{-/-}$ $CD4^+$ T cells. Thus, there may be a common partner for Smad2 and Smad3 in addition to Smad4. Tob, a member of an anti-proliferative gene family, was shown to bind to Smad2, thereby inhibiting IL-2 production (52). However, the interaction between Tob and Smad3 was not observed. Runx1/3, NF-AT, AP-1, and NF-kB also play essential roles in cytokine production from $CD4^+$ T cells, and they can be potential interaction partners of Smad2 and/or Smad3 (53). It is notable that an essential NF-AT binding site is present, adjacent to the Smad binding elements in the Foxp3 promoter (22). However, the interaction of these transcription factors for both Smad2 and Smad3 has not been reported. Finding a common interaction partner of Smad2 and Smad3 could be the next step in understanding the molecular basis for the immunoregulatory effects of TGF-β.

Smad2 exists in two isoforms, one of which is an alternatively spliced variant of Smad2 with a deletion of exon 3 and has a functional DNA binding domain (54). Because this spliced variant is thought to be important in certain aspects of mouse embryo development (55), we measured the expression of this variant in T cells. We did not observe expression of this spliced form of *Smad2* in naive $CD4^+$ T cells (data not shown), which makes it unlikely that this spliced form functions in $CD4^+$ T cells.

Recent studies demonstrated that TGF-β–induced Foxp3 antagonizes RORγt, which is also induced by TGF-β, to inhibit Th17 cell differentiation (13, 14). However, it has not yet been determined how TGF-β induces the distinct transcription factor Foxp3 or RORγt. We show that the induction of Foxp3 was completely dependent on Smad signaling, but the induction of RORγt was independent of it. However, we have found that Smad signaling indirectly promotes the inducing of Th17 cell differentiation by suppressing its inhibitory cytokine production. These data indicate that TGF-β plays an important role in Th17 cell development in a Smad-dependent and Smad-independent

for 2 wk. Body weight change and survival rate were monitored every day. Each graph shows means ± SD. Data shown are representative of two independent experiments with consistent results. Statistical differences were verified by paired Student *t* test. *D*, Histologic analysis by H&E staining of livers from PBS-treated ($n = 5$) and TGF-β–treated *scurfy* mice ($n = 5$) 10 d after each treatment began (original magnification ×20). Data shown are representative from one of the three tissue samples with similar results. *E*, Cytokine profiles of freshly isolated $CD4^+$ T cells from cervical lymph nodes of PBS- ($n = 5$) or TGF-β–treated ($n = 5$) *scurfy* mice 10 d after treatment began. A bar graph shows the mean percentage of indicated cytokine-producing cells in total $CD4^+$ T cells. Freshly isolated cells were restimulated for 5 h with PMA and ionomycin, and cytokines were detected with intracellular immunostaining (*right*) or ELISA (*left*). Statistical differences were verified by paired Student *t* test. Data shown are representative of two independent experiments. WT, wild-type.

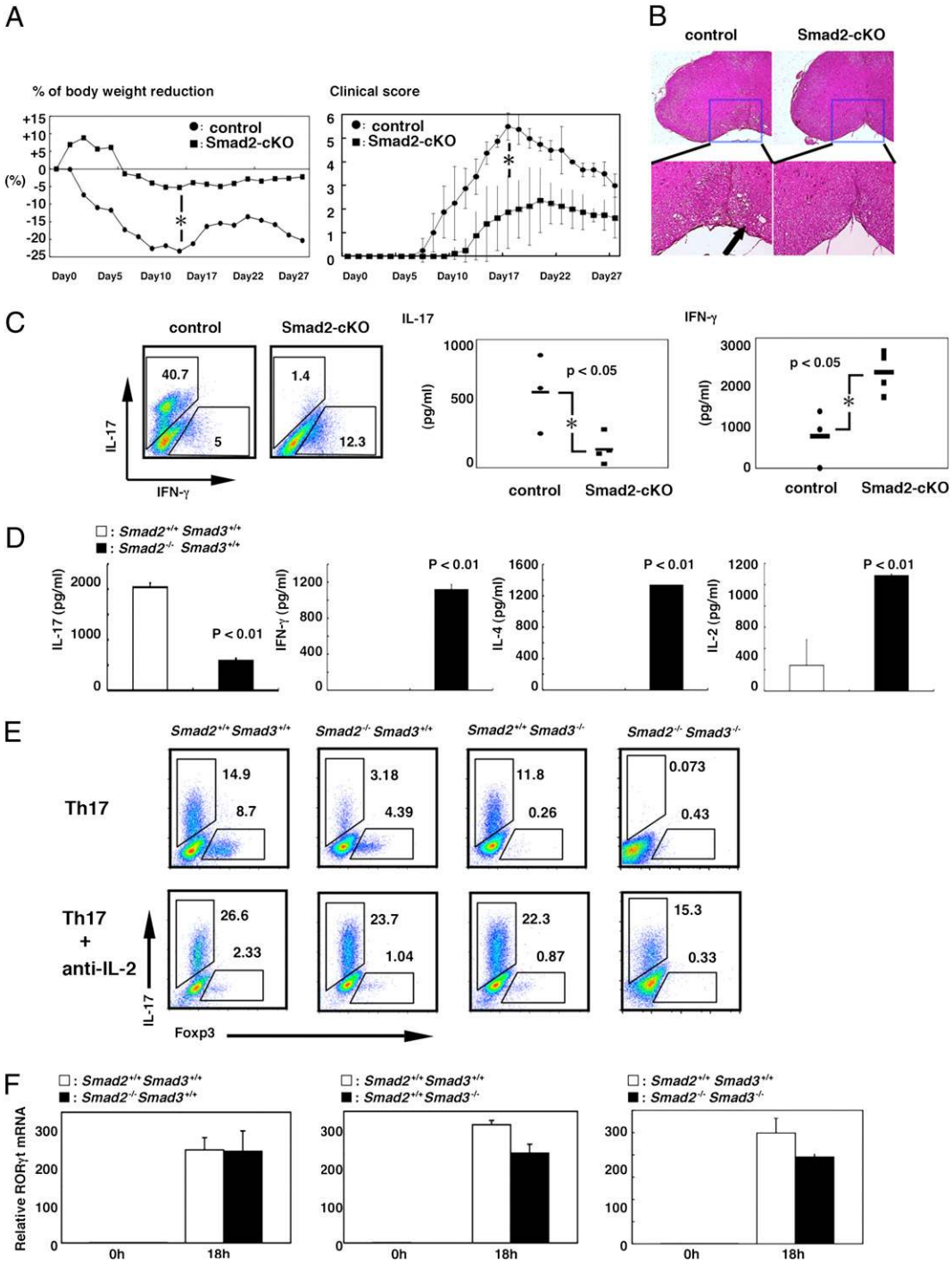

FIGURE 7. TGF-β–Smad signaling is essential for effective Th17 cell development, but not for the induction of RORγt. *A*, The body weight loss and clinical scores of neurologic impairment were recorded daily for control ($n = 8$) and *Smad2*-cKO ($n = 8$) mice after the induction of EAE. The experiments were repeated twice, with five to eight mice in each group, and similar results were obtained each time. The graph shows means ± SD. Statistical differences were verified by paired Student *t* test. $*p < 0.01$ between groups. *B*, Histologic analysis by H&E staining of spinal cords ($n = 5$) of mice 30 d after the induction of EAE (original magnification ×20 and ×200). Black arrows indicate demyelination in the subpial regions. Data shown are representative of two independent experiments with similar results. *C*, Ten days after the immunization, $CD4^+$ T cells were isolated from the draining lymph nodes in control ($n = 3$) and *Smad2*-cKO ($n = 4$) mice. Cells were restimulated for 5 h with PMA and ionomycin for intracellular immunostaining (*left panels*). ELISA of cytokines in supernatants of Ag-specific restimulated $CD4^+$ T cells of draining lymph nodes (*right panels*). Cells were cocultured for 24 h with irradiated splenocytes in the presence of 20 μg/ml MOG. These experiments were repeated twice with similar results. *D*, $Smad2^{+/+}Smad3^{+/+}$ or $Smad2^{-/-}Smad3^{+/+}$ $CD4^+$ T cells were cultured under the Th17-skewing condition for 5 d and then harvested and restimulated with 5 μg/ml plate-bound anti-CD3e Abs for 12 h. The levels of indicated cytokines in the supernatants were determined by ELISA. Experiments were repeated twice with similar results. The graph shows means ± SD. Statistical differences were verified by paired Student *t* test. *E*, FACS-sorted naive $CD4^+$ T cells from control ($Smad2^{+/+}Smad3^{+/+}$), *Smad2*-cKO ($Smad2^{-/-}Smad3^{+/+}$), *Smad3*-KO ($Smad2^{+/+}Smad3^{-/-}$), and *Smad2/3*-DKO ($Smad2^{-/-}Smad3^{-/-}$) mice were cultured in the Th17-skewing condition in the absence or presence of 10 μg/ml of anti-IL-2 Abs for 5 d. Cells were restimulated for 5 h with PMA and ionomycin. Cells were fixed, stained for intracellular Foxp3 and IL-17, and assessed by flow cytometry. Experiments were repeated three times with similar results. *F*, FACS-sorted naive $CD4^+$ T cells with indicated genotypes were stimulated under the Th17-skewing condition for 18 h, and mRNA expression of RORγt was assessed by real-time RT-PCR. The experiment was performed in duplicate. Data shown were normalized to the expression of a reference gene, HPRT. The expression for RORγt gene of $Smad2^{+/+}Smad3^{+/+}$ $CD4^+$ T cells at 0 h was set as 1. The graph shows means ± SD. Data represent two independent experiments with similar results.

manner. Furthermore, $CD4^+$ T cells deficient in Smad2/3 could be useful objects of study for identifying the TGF-β signaling pathway for RORγt induction.

Surprisingly, LckCre-*Smad2*$^{f/f}$*Smad3*$^{+/-}$ mice in which T cells express only one fourth of Smad2/3 transcription factors can survive without any severe inflammatory diseases. In these mice, most of the $CD4^+CD25^-$ T cells showed memory phenotypes in vivo, and TGF-β hardly induced Foxp3 and suppressed IFN-γ production in naive $CD4^+$ T cells in vitro. It would be interesting to know how the immunologic tolerance was maintained in LckCre-*Smad2*$^{f/f}$*Smad3*$^{+/-}$ mice. This may be due to more nTregs in these mice (T. Takimoto, unpublished data).

$CD103^+$ $Foxp3^+$ Tregs have been shown to possess potent immunosuppressive activity (39); therefore, we examined the induction of CD103 in iTregs. We demonstrated that CD103 expression was strongly induced by TGF-β in a Smad-dependent manner in iTregs. We could not detect CD103 in Smad2$^{-/-}$ *Smad3*$^{+/-}$ iTregs. However, we noticed that the number of CD103-expressing $CD4^+$ T cells in 2cKO/3hetero mice was similar to that in control *Smad2*$^{+/+}$*Smad3*$^{+/+}$ mice (data not shown). Thus, CD103 expression in vivo may be regulated by a mechanism other than TGF-β. One candidate is IL-2, because a previous report has shown that IL-2, rather than TGF-β, upregulates CD103 expression in vivo (38). Expression of CD103 in nTregs in a TGF-β–independent mechanism might explain the lack of inflammatory phenotypes in 2cKO/3hetero mice.

In this study, we discovered several Smad2/3-independent genes that were upregulated or downregulated by TGF-β. We found that *eomes* is downregulated by TGF-β in a Smad2/3-independent mechanism. It has been reported that *eomes*, a paralogue of T-bet, plays an important role for IFN-γ production (56). TGF-β might suppress IFN-γ production in $CD4^+$ T cells by inhibiting the expression of *eomes* as well as T-bet. We observed that CD73 and CCR8 was induced by TGF-β in a Smad-independent manner. CD73, an ectonucleotidase, has been shown to be highly expressed in nTregs and could be an immunosuppressive factor (57). CCR8 may be involved in migration of iTregs or Th17 cells into inflammatory cites in Smad2/three-dimensional KO mice. However, further investigation is necessary to clarify the role of these Smad-independent gens in TGF-β–mediated immune tolerance.

Acknowledgments

We thank T. Yoshioka, K. Fukuse, and N. Shiino for technical assistance; Y. Nishi and N. Soma for manuscript preparation; Dr. H. Nishinakamura and Dr. K. Hamada for technical advice; and Dr. S. Hori (Riken, Yokohama, Japan) for providing the *scurfy* mice.

Disclosures

The authors have no financial conflicts of interest.

References

1. Letterio, J. J., and A. B. Roberts. 1998. Regulation of immune responses by TGF-beta. *Annu. Rev. Immunol.* 16: 137–161.
2. Gorelik, L., and R. A. Flavell. 2002. Transforming growth factor-beta in T-cell biology. *Nat. Rev. Immunol.* 2: 46–53.
3. Shull, M. M., I. Ormsby, A. B. Kier, S. Pawlowski, R. J. Diebold, M. Yin, R. Allen, C. Sidman, G. Proetzel, D. Calvin, et al. 1992. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 359: 693–699.
4. Kulkarni, A. B., C. G. Huh, D. Becker, A. Geiser, M. Lyght, K. C. Flanders, A. B. Roberts, M. B. Sporn, J. M. Ward, and S. Karlsson. 1993. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc. Natl. Acad. Sci. USA* 90: 770–774.
5. Gorelik, L., and R. A. Flavell. 2000. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 12: 171–181.
6. Marie, J. C., D. Liggitt, and A. Y. Rudensky. 2006. Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. *Immunity* 25: 441–454.
7. Li, M. O., S. Sanjabi, and R. A. Flavell. 2006. Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. *Immunity* 25: 455–471.
8. Li, M. O., and R. A. Flavell. 2008. TGF-β: a master of all T cell trades. *Cell* 134: 392–404.
9. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+ CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J. Exp. Med.* 198: 1875–1886.
10. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057–1061.
11. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat. Immunol.* 4: 330–336.
12. Bettelli, E., M. Dastrange, and M. Oukka. 2005. Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. *Proc. Natl. Acad. Sci. USA* 102: 5138–5143.
13. Zhou, L., J. E. Lopes, M. M. Chong, I. I. Ivanov, R. Min, G. D. Victora, Y. Shen, J. Du, Y. P. Rubtsov, A. Y. Rudensky, et al. 2008. TGF-β-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature* 453: 236–240.
14. Ichiyama, K., H. Yoshida, Y. Wakabayashi, T. Chinen, K. Saeki, M. Nakaya, G. Takaesu, S. Hori, A. Yoshimura, and T. Kobayashi. 2008. Foxp3 inhibits RORgammat-mediated IL-17A mRNA transcription through direct interaction with RORgammat. *J. Biol. Chem.* 283: 17003–17008.
15. Sakaguchi, S. 2004. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu. Rev. Immunol.* 22: 531–562.
16. You, S., B. Leforban, C. Garcia, J. F. Bach, J. A. Bluestone, and L. Chatenoud. 2007. Adaptive TGF-β-dependent regulatory T cells control autoimmune diabetes and are a privileged target of anti-CD3 antibody treatment. *Proc. Natl. Acad. Sci. USA* 104: 6335–6340.
17. Liu, Y., P. Zhang, J. Li, A. B. Kulkarni, S. Perruche, and W. Chen. 2008. A critical function for TGF-β signaling in the development of natural CD4+CD25+ Foxp3+ regulatory T cells. *Nat. Immunol.* 9: 632–640.
18. Massagué, J. 1998. TGF-beta signal transduction. *Annu. Rev. Biochem.* 67: 753–791.
19. He, W., D. C. Dorn, H. Erdjument-Bromage, P. Tempst, M. A. S. Moore, and J. Massagué. 2006. Hematopoiesis controlled by distinct TIF1gamma and Smad4 branches of the TGFbeta pathway. *Cell* 125: 929–941.
20. Nomura, M., and E. Li. 1998. Smad2 role in mesoderm formation, left-right patterning and craniofacial development. *Nature* 396: 786–790.
21. Yang, X. O., J. J. Letterio, R. J. Lechleider, L. Chen, R. Hayman, H. Gu, A. B. Roberts, and C. Deng. 1999. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-β. *EMBO J.* 18: 1280–1291.
22. Tone, Y., K. Furuuchi, Y. Kojima, M. L. Tykocinski, M. I. Greene, and M. Tone. 2008. Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nat. Immunol.* 9: 194–202.
23. Kim, B. G., C. Li, W. Qiao, M. Mamura, B. Kasprzak, B. Kasperczak, M. Anver, L. Wolfraim, S. Hong, E. Mushinski, et al. 2006. Smad4 signalling in T cells is required for suppression of gastrointestinal cancer. *Nature* 441: 1015–1019.
24. Kinjyo, I., H. Inoue, S. Hamano, S. Fukuyama, T. Yoshimura, K. Koga, H. Takaki, K. Himeno, G. Takaesu, T. Kobayashi, and A. Yoshimura. 2006. Loss of SOCS3 in T helper cells resulted in reduced immune responses and hyperproduction of interleukin 10 and transforming growth factor-beta 1. *J. Exp. Med.* 203: 1021–1031.
25. Flanders, K. C., C. D. Sullivan, M. Fujii, A. Sowers, M. A. Anzano, A. Arabshahi, C. Major, C. Deng, A. Russo, J. B. Mitchell, and A. B. Roberts. 2002. Mice lacking Smad3 are protected against cutaneous injury induced by ionizing radiation. *Am. J. Pathol.* 160: 1057–1068.
26. Takaki, H., K. Ichiyama, K. Koga, T. Chinen, G. Takaesu, Y. Sugiyama, S. Kato, A. Yoshimura, and T. Kobayashi. 2008. STAT6 Inhibits TGF-beta1-mediated Foxp3 induction through direct binding to the Foxp3 promoter, which is reverted by retinoic acid receptor. *J. Biol. Chem.* 283: 14955–14962.
27. Tanaka, K., K. Ichiyama, M. Hashimoto, H. Yoshida, T. Takimoto, G. Takaesu, T. Torisu, T. Hanada, H. Yasukawa, S. Fukuyama, et al. 2008. Loss of suppressor of cytokine signaling 1 in helper T cells leads to defective Th17 differentiation by enhancing antagonistic effects of IFN-gamma on STAT3 and Smads. *J. Immunol.* 180: 3746–3756.
28. Koga, K., G. Takaesu, R. Yoshida, M. Nakaya, T. Kobayashi, I. Kinjyo, and A. Yoshimura. 2009. Cyclic adenosine monophosphate suppresses the transcription of proinflammatory cytokines via the c-Fos protein. *Immunity* 30: 372–383.
29. Ivanov, I. I., B. S. McKenzie, L. Zhou, C. E. Tadokoro, A. Lepelley, J. J. Lafaille, D. J. Cua, and D. R. Littman. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126: 1121–1133.
30. Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* 201: 233–240.

31. Matsumura, Y., T. Kobayashi, K. Ichiyama, R. Yoshida, M. Hashimoto, T. Takimoto, K. Tanaka, T. Chinen, T. Shichita, T. Wyss-Coray, et al. 2007. Selective expansion of foxp3-positive regulatory T cells and immunosuppression by suppressors of cytokine signaling 3-deficient dendritic cells. *J. Immunol.* 179: 2170–2179.
32. Ito, R., M. Shin-Ya, T. Kishida, A. Urano, R. Takada, J. Sakagami, J. Imanishi, M. Kita, Y. Ueda, Y. Iwakura, et al. 2006. Interferon-gamma is causatively involved in experimental inflammatory bowel disease in mice. *Clin. Exp. Immunol.* 146: 330–338.
33. Wang, Z. B., Y. F. Cui, Y. Q. Liu, W. Jin, H. Xu, Z. J. Jiang, Y. X. Lu, Y. Zhang, X. L. Liu, and B. Dong. 2006. Increase of $CD4(^{+})CD25(^{+})$ T cells in $Smad3^{(-/-)}$ mice. *World J. Gastroenterol.* 12: 2455–2458.
34. Descargues, P., A. K. Sil, Y. Sano, O. Korchynskyi, G. Han, P. Owens, X. J. Wang, and M. Karin. 2008. IKKalpha is a critical coregulator of a Smad4-independent TGFbeta-Smad2/3 signaling pathway that controls keratinocyte differentiation. *Proc. Natl. Acad. Sci. USA* 105: 2487–2492.
35. Hill, J. A., M. Feuerer, K. Tash, S. Haxhinasto, J. Perez, R. Melamed, D. Mathis, and C. Benoist. 2007. Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity* 27: 786–800.
36. Wei, G., L. Wei, J. Zhu, C. Zang, J. Hu-Li, Z. Yao, K. Cui, Y. Kanno, T. Y. Roh, W. T. Watford, et al. 2009. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. *Immunity* 30: 155–167.
37. Lúdvíksson, B. R., D. Seegers, A. S. Resnick, and W. Strober. 2000. The effect of TGF-β1 on immune responses of naïve versus memory $CD4^{+}$ Th1/Th2 T cells. *Eur. J. Immunol.* 30: 2101–2111.
38. Sharma, R., S. S. Sung, C. E. Abaya, A. C. Ju, S. M. Fu, and S. T. Ju. 2009. IL-2 regulates CD103 expression on $CD4^{+}$ T cells in Scurfy mice that display both CD103-dependent and independent inflammation. *J. Immunol.* 183: 1065–1073.
39. Banz, A., A. Peixoto, C. Pontoux, C. Cordier, B. Rocha, and M. Papiernik. 2003. A unique subpopulation of CD4+ regulatory T cells controls wasting disease, IL-10 secretion and T cell homeostasis. *Eur. J. Immunol.* 33: 2419–2428.
40. Brunkow, M. E., E. W. Jeffery, K. A. Hjerrild, B. Paeper, L. B. Clark, S. A. Yasayko, J. E. Wilkinson, D. Galas, S. F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* 27: 68–73.
41. Zhang, W., R. Sharma, S. T. Ju, X. S. He, Y. Tao, K. Tsuneyama, Z. Tian, Z. X. Lian, S. M. Fu, and M. E. Gershwin. 2009. Deficiency in regulatory T cells results in development of antimitochondrial antibodies and autoimmune cholangitis. *Hepatology* 49: 545–552.
42. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, and V. K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441: 235–238.
43. Mangan, P. R., L. E. Harrington, D. B. O'Quinn, W. S. Helms, D. C. Bullard, C. O. Elson, R. D. Hatton, S. M. Wahl, T. R. Schoeb, and C. T. Weaver. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* 441: 231–234.
44. Yang, L., D. E. Anderson, C. Baecher-Allan, W. D. Hastings, E. Bettelli, M. Oukka, V. K. Kuchroo, and D. A. Hafler. 2008. IL-21 and TGF-β are required for differentiation of human T(H)17 cells. *Nature* 454: 350–352.
45. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 6: 1133–1141.
46. Laurence, A. C. M., C. M. Tato, T. S. Davidson, Y. Kanno, Z. Chen, Z. Yao, R. B. Blank, F. Meylan, R. Siegel, L. Hennighausen, et al. 2007. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* 26: 371–381.
47. Yang, X. O., R. Nurieva, G. J. Martinez, H. S. Kang, Y. Chung, B. P. Pappu, B. Shah, S. H. Chang, K. S. Schluns, S. S. Watowich, et al. 2008. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity* 29: 44–56.
48. Zhou, L., M. M. Chong, and D. R. Littman. 2009. Plasticity of CD4+ T cell lineage differentiation. *Immunity* 30: 646–655.
49. Feuerer, M., J. A. Hill, D. Mathis, and C. Benoist. 2009. Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nat. Immunol.* 10: 689–695.
50. Tsuji, M., N. Komatsu, S. Kawamoto, K. Suzuki, O. Kanagawa, T. Honjo, S. Hori, and S. Fagarasan. 2009. Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. *Science* 323: 1488–1492.
51. Zhou, X., S. L. Bailey-Bucktrout, L. T. Jeker, C. Penaranda, M. Martínez-Llordella, M. Ashby, M. Nakayama, W. Rosenthal, and J. A. Bluestone. 2009. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat. Immunol.* 10: 1000–1007.
52. Tzachanis, D., G. J. Freeman, N. Hirano, A. A. van Puijenbroek, M. W. Delfs, A. Berezovskaya, L. M. Nadler, and V. A. Boussiotis. 2001. Tob is a negative regulator of activation that is expressed in anergic and quiescent T cells. *Nat. Immunol.* 2: 1174–1182.
53. Miyazono, K., S. Maeda, and T. Imamura. 2004. Coordinate regulation of cell growth and differentiation by TGF-β superfamily and Runx proteins. *Oncogene* 23: 4232–4237.
54. Dunn, N. R., C. H. Koonce, D. C. Anderson, A. Islam, E. K. Bikoff, and E. J. Robertson. 2005. Mice exclusively expressing the short isoform of Smad2 develop normally and are viable and fertile. *Genes Dev.* 19: 152–163.
55. Takenoshita, S., A. Mogi, M. Nagashima, K. Yang, K. Yagi, A. Hanyu, Y. Nagamachi, K. Miyazono, and K. Hagiwara. 1998. Characterization of the MADH2/Smad2 gene, a human Mad homolog responsible for the transforming growth factor-beta and activin signal transduction pathway. *Genomics* 48: 1–11.
56. Pearce, E. L., A. C. Mullen, G. A. Martins, C. M. Krawczyk, A. S. Hutchins, V. P. Zediak, M. Banica, C. B. DiCioccio, D. A. Gross, C. A. Mao, et al. 2003. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. *Science* 302: 1041–1043.
57. Sitkovsky, M., D. Lukashev, S. Deaglio, K. Dwyer, S. C. Robson, and A. Ohta. 2008. Adenosine A2A receptor antagonists: blockade of adenosinergic effects and T regulatory cells. *Br. J. Pharmacol.* 153(Suppl 1): S457–S464.

Corrections

Takimoto, T., Y. Wakabayashi, T. Sekiya, N. Inoue, R. Morita, K. Ichiyama, R. Takahashi, M. Asakawa, G. Muto, T. Mori, E. Hasegawa, S. Shizuya, T. Hara, M. Nomura, and A. Yoshimura. 2010. Smad2 and Smad3 are redundantly essential for the TGF-β–mediated regulation of regulatory T plasticity and Th1 development. *J. Immunol.* 185: 842–855.

The twelfth author's name was published incorrectly. The correct name is Shizuya Saika.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1090121