Histone 3 Lysine 9 (H3K9) Methyltransferase Recruitment to the Interleukin-2 (IL-2) Promoter Is a Mechanism of Suppression of IL-2 Transcription by the Transforming Growth Factor-β-Smad Pathway*

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Background: Suppression of IL-2 production from T cells is an important process for the immune regulation by transforming growth factor-beta (TGF-β).

Results: Smad2 and Smad3 were redundantly essential for IL-2 suppression and recruited histone H3K9 methyltransferase, Suv39h1 to the proximal region of the IL-2 promoter, thereby suppressing IL-2 transcription.

Conclusion: Smad2/3 mediated histone H3K9 trimethylation of the IL-2 promoter is an important mechanism for the suppression of IL-2 transcription.

Significance: Our finding will be useful to establish a novel therapy for autoimmune diseases and inflammatory diseases.

Suppression of IL-2 β production from T cells is an important process for the immune regulation by TGF-β. However, the mechanism by which this suppression occurs remains to be established. Here, we demonstrate that Smad2 and Smad3, two major TGF-β-downstream transcription factors, are redundantly essential for TGF-β-mediated suppression of IL-2 production in CD4+ T cells using Smad2- and Smad3-deficient T cells. Both Smad2 and Smad3 were recruited into the proximal region of the IL-2 promoter in response to TGF-β. We then investigated the histone methylation status of the IL-2 promoter. Although both histone H3 lysine 9 (H3K9) and H3K27 trimethylation have been implicated in gene silencing, only H3K9 trimethylation was increased in the proximal region of the IL-2 promoter in wild type primary T cells; however, this was not observed in Smad2−/−Smad3+/− T cells. Thus, we propose that Smads recruit H3K9 methyltransferases Suv39h1 to the IL-2 promoter, thereby inducing suppressive histone methylation and inhibiting T cell receptor-mediated IL-2 transcription.

The pleiotropic cytokine TGF-β plays several critical roles in suppressing the immune response (1–5). Although multiple types of immune cells can be regulated by TGF-β, helper T cells actually play a central role in the immunosuppressive effect of TGF-β; this is deduced from the observation that the neonatal lethality of TGF-β1-deficient mice is eliminated by deletion of CD4+ T cells (6) and that the crossing of TGF-β1-deficient mice onto a MHC class II null background prevents the inflammation that would otherwise develop (7). One of the most important effects of TGF-β on T cells is the suppression of IL-2 production (8), which leads to the anti-proliferative effect of TGF-β on activated T cells. Other important functions of TGF-β for T cells are the induction of Foxp3, a master regulator of regulatory T cells (Treg cells) and the suppression of the production of effector cytokines such as IFN-γ and IL-4.

The major signaling pathways of the TGF-β receptors are relatively simple (9). TGF-β first binds to a TGF-β receptor, which then primarily activates Smad transcription factors, including three structurally similar proteins: two receptor-associated Smads known as Smad2 and Smad3 and one common

3 The abbreviations used are: Treg cells, regulatory T cells; TCR, T cell receptor; HMT, histone methyltransferase; SBE, Smad-binding element; CREB, cAMP-responsive element-binding protein; CNS, conserved non-coding sequence; NF-AT, nuclear factor of activated T-cells.
Smad known as Smad4. Smad2 and Smad3 are directly phosphorylated and activated by TGF-β receptor; they also heterodimerize with Smad4. The activated Smad complex translocates into the nucleus and, in cooperation with other nuclear cofactors, regulates the transcription of target genes. Apparently, however, Smad-independent pathways also exist (10, 11).

The downstream mechanism for the regulation of T cells by TGF-β remains unclear. It has been reported that either Smad2 or Smad3 can regulate a distinct set of genes in fibroblasts and tumor cells (9). Smad2–knock-out (KO) mice are embryonic-lethal (12), and Smad3–KO mice exhibit inflammatory diseases (13), suggesting that Smad2 is involved in mediating signals during development, whereas Smad3 is important in suppressing inflammation. However, our experiments with T cell-specific Smad2 conditional knock-out mice have revealed that Smad2 and Smad3 unexpectedly share some overlapping functions in TGF-β-induced Foxp3 induction as well as in IFN-γ suppression (14). Smad2/Smad3–double knock-out mice, but not single knock-out mice, develop fatal inflammatory diseases and exhibit elevated IFN-γ production and reduced Foxp3 expression in CD4⁺ T cells at the periphery (14). TGF-β-mediated induction of Foxp3 and suppression of IFN-γ were completely eliminated in Smad2/3–double KO T cells, but only partially impaired in Smad2 or Smad3 single KO T cells (14). Thus, it appears that Smad2 and Smad3 are redundantly essential for Foxp3 induction and IFN-γ suppression. However, the roles of Smad2/3 in IL-2 suppression by TGF-β remain to be completely elucidated.

The signals involved in IL-2 suppression by TGF-β also remain to be elucidated. TGF-β suppresses IL-2 production in T cells potentially through direct inhibition of IL-2 promoter activity. A cis-acting enhancer DNA element has been identified as critical in suppressing IL-2 production via TGF-β (8). Tob, a member of an anti-proliferative gene family, was shown to bind to Smad2, but not to Smad3, thereby inhibiting IL-2 production (16). Runx1/3 also play essential roles in cytokine production from CD4⁺ T cells and may be potential interaction partners of Smad2 and/or Smad3 (17). It is notable that an essential Nuclear factor of activated T cells binding site is present in the Foxp3 promoter, adjacent to the Smad-binding elements (18, 19). The mechanisms by which Smad2 and Smad3 enhance or suppress these transcription factor activities have not been identified.

Recent studies have revealed epigenetic regulation of cytokines during helper T cell differentiation (20). In particular, it has been revealed that gene silencing and suppression of transcription are often associated with certain histone modifications, namely, the di- and trimethylation of histone 3 (H3) lysine (K) 27 or H3K9 (21). Here, using Smad2- and Smad3-deficient T cells, we demonstrate for the first time that Smad2 and Smad3 are redundantly essential for TGF-β-mediated suppression of IL-2 production in CD4⁺ T cells. Smads suppressed IL-2 proximal promoter activity and promoted H3K9 trimethylation in the IL-2 proximal promoter region. We propose that Smads recruit H3K9 methyltransferases to the IL-2 promoter, thereby suppressing IL-2 transcription.

**EXPERIMENTAL PROCEDURES**

**Mice**—T cell-specific Smad2 conditional knock-out (cKO) mice and Smad3⁻/⁻ mice have been described previously (14). T cells from Smad2-cKO mice are henceforth described as Smad2⁻/⁻ T cells. Mice were kept in specific pathogen-free facilities at 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⁻CD4⁰CD62Lhigh naive T cells from spleens and lymph nodes were enriched through negative selection using the magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany) 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) antibodies (all from eBioscience, San Diego, CA) as well as streptavidin-conjugated magnetic beads (Miltenyi Biotec). Cells were then FACSorted using a BD FACSAria™ cell sorter (BD Biosciences). The purity of the sorted CD4⁺ T cell populations was consistently >98%. T cells were activated by anti-CD3ε (145–2C11) and anti-CD28 (37.51) and cultured in the presence of recombinant hTGF-β1 (0.1–2 ng/ml; R&D Systems, Minneapolis, MN) to induce Foxp3 (22). T cells were maintained in a complete medium containing RPMI 1640 supplemented with 10% FCS, 1% penicillin/streptomycin, 100 μM non-essential amino acids, 2 μM glutamine, and 0.05 μM 2-mercaptoethanol.

Cells from the T cell line 68-41 were provided by Dr. Masato Kubo (Research Center for Allergy and Immunology, Yokohama, Japan) and cultured as described previously (22). 68-41 cells were stimulated with anti-CD3ε antibody in the presence or absence of recombinant hTGF-β1 as described above.

**Immunoprecipitation and Western Blot Analysis**—Anti-FLAG (M2) antibody was purchased from Sigma-Aldrich, and T7 antibody was from Novagen (EMD Chemicals, Gibbstown, NJ). 293 cells transfected with indicated plasmids were washed once with ice-cold PBS and lysed in 0.3 ml of 0.5% Triton X-100 lysis buffer containing 20 mM Hepes (pH 7.4), 150 mM NaCl, 1.5 mM MgCl₂, 12.5 mM β-glycerophosphate, 10 mM NaF, and 1 mM sodium orthovanadate supplemented with protease inhibitor mixture (Nacalai Tesque, Tokyo, Japan). Cellular debris was removed by centrifugation at 15,000 × g for 5 min. Proteins from cell lysates were precipitated with 1 μg of an antibody and 20 μl of protein G-Sepharose (GE Healthcare) for 2 h at 4 ºC (23). The immune complex was washed three times with washing buffer containing 20 mM Hepes (pH 7.4), 500 mM NaCl, and 10 mM MgCl₂ and was suspended in 40 μl of rinse buffer containing 20 mM Hepes (pH 7.4), 150 mM NaCl, and 10 mM MgCl₂. For Western blotting, the immunoprecipitates or whole cell lysates were resolved through SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Billerica, MA). The membranes were blotted with the indicated antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG using Chemi-Lumi One L Western-blotting detection reagents (Nacalai Tesque) as described previously (24).

**Flow Cytometry**—For IL-2 intracellular cytokine staining, cells were stimulated for 6 h in complete medium with phorbol ester.
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12-myristate 13-acetate (PMA) (50 ng/ml) and ionomycin (500 ng/ml; both from Sigma-Aldrich) in the presence of brefeldin A (eBioscience) (25). Surface staining was then performed in the presence of Fc-blocking antibodies (2.4G2), followed by intracellular staining for anti-IL-2 antibody (1A12, eBioscience) with the Fixation and Permeabilization kit (eBioscience) according to the manufacturer’s instructions (25). Data were acquired on a BD FACSARia™ and were analyzed with FlowJo software (Treestar, Ashland, OR).

ChIP Assay—The ChIP assay was performed as described previously (18, 22) using a ChIP assay kit (Upstate Biotechnology, Charlottesville, VA). Smad2- and Smad3-specific monoclonal antibodies were purchased from Cell Signaling (5339 for Smad2 and 9523 for Smad3). Anti-Suv39h1 and anti-trimethylated histone antibodies were from Abcam.plc (ab12405 for H3K27me3, ab8898 for H3K9me3, and ab1012 for H3K4me3). Briefly, primary T cells or 69–41 T cells (7 ± 10^6) were fixed with 1% formaldehde at 37 °C for 10 min and then suspended in an SDS lysis buffer. After sonication consisting of 15 30-s pulses from a Bioruptor sonicator (Cosmo Bio Co., Ltd., Tokyo, Japan), samples were incubated with 2.5 μg of antibodies or control IgG overnight at 4 °C. After the addition of salmon sperm DNA and protein A-agarose slurry, the immunoprecipitates were sequentially washed once with a low salt buffer, once with a high salt buffer, once with an LiCl buffer, and twice with a TE buffer. The DNA-protein complex was eluted by heating at 65 °C for 6 h. Proteins were then digested with proteinase K, and RNA was removed by the addition of 10 μg of RNase A. DNA was recovered using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and then subjected to real-time PCR analysis with attention to the IL-2 promoter regions. Real-time PCR was performed using the following primers: proximal (−0.4 to −0.3), 5′-TTCTATCTATCTCTCTTGCCGTGTTT-3′ and 5′-ATTCTCTCTTTGGGGTGAG-3′; IL-2 promoter (−1.6 to −1.4, 5′-ACCCACAGTGTTAGTACG-3′ and 5′-AGGCCCTACACACACCACC-ACC-3′; distal (−8.3 to −8.1), 5′-TGGACATGTGCCGCTTCTTGAC-3′ and 5′-CAGGAAACAGCATCCACAGAG-3′.

Plasmid Construction and Retroviral Infection—cDNA sequences for Suv39h1 and Setdb1 were amplified by RT-PCR and subcloned into pCMV7T vector for T7 tag and pCMV14 vector (Sigma-Aldrich) for FLAG tag (26). Suv39h1 and Setdb1 cDNAs were also subcloned into pMX retroviral vector with internal ribosome entry site (IRES)-GFP.

shRNA target sequences were selected based on the BLOCKiT™ RNAi Designer (Invitrogen). The following oligonucleotides were amplified with the XhoI and EcoRI amplification primers and subcloned into the MSCV-LMP vector (Open Biosystems) as described (27): shSuv39h1−1, 5′-TGCTGTGAGA-GTGAGGAGCCGCTTTTTAGTAGATGTGAGTAGA-GCAAAGATGTAACACATCTCATAAACAAAGGCTG-CTACTGCTGCGGA-3′; and shSuv39h1−2, 5′-TGCTGTTT-GACAGTGACCTGAGATGTGGGAGAGATTATTGAT-GAAGCCACAGATGTAATAATCTCTCCCCACATACAGATGCTCTTGTGCTCGGA-3′.

Retroviral vectors were transfected into HEK293 cells with helper virus plasmids (28) using the FuGENE 6 (Roche Applied Science) to obtain the viruses. Fresh retrovirus supernatant was added and the cells were spun at 2500 rpm for 2 h at 35 °C. After spin infection, the cells were cultured for an additional 2 days, and GFP-positive cells were then sorted with FACS (29). GFP-positive cells were stimulated with anti-CD3 antibody and TGF-β for 24 h and harvested for real-time PCR analysis.

Luciferase Assay—Both the process of transfection into HEK293T cells and the luciferase assay have been described previously (22). Briefly, 68–41 cells were seeded on 12-well plates (7 × 10^5 cells/well) and transfected with various amounts of expression vector using FuGENE HD reagent. After transfection, HEK293T cells were cultured for 18 h and then restimulated with or without PMA for 10 h. All cells were harvested using 50 μl of lysis buffer. The luciferase assay was performed using a luciferase substrate kit (Promega, Madison, WI), and luciferase activity was read with a Packard luminometer and normalized to an internal control, namely, β-galactosidase.

Statistical Analysis—The Student’s paired two-tailed t test was used. Values of p < 0.05 were considered significant. All error bars shown in this article represent S.D.

RESULTS

IL-2 Is Down-regulated by TGF-β in Smad2/3-dependent Manner—First, we compared the time course of IL-2 and Foxp3 induction in primary T cells in the presence or absence of TGF-β (Fig. 1A). TGF-β suppressed IL-2 mRNA induced by TCR stimulation and up-regulated Foxp3 mRNA in cooperation with TCR stimulation. The effect of TGF-β was clearly observed for both genes from 8 to 24 h (Fig. 1A), suggesting that not only Foxp3 but also IL-2 was directly regulated by the TGF-β signaling.

We then compared mRNA gene profiles regulated by TGF-β in a microarray analysis, using WT, Smad2−/−, Smad3−/−, and Smad2−/−Smad3+/− T cells at 24 h stimulation (14). Among the various Smad2/3-redundant genes, Foxp3 was clearly up-regulated by TGF-β in a Smad2- and Smad3-dependent manner (14). Foxp3 induction was severely impaired in Smad2−/−Smad3+/− T cells, though only partially reduced in Smad2 or Smad3 single KO T cells (Fig. 1B). Among the Smad2/3-dependently suppressed genes, IL-2 was the most severely reduced by TGF-β clearly showed Smad2/3 dependence (Fig. 1B).

To confirm the Smad2/3-dependent suppression of IL-2 production, we compared IL-2 protein levels by intracellular cytokine staining and FACS analysis. After T cell receptor (TCR) stimulation with anti-CD3 antibody, primary T cells produced large amounts of IL-2, but this process was strongly suppressed by the presence of a relatively small amount of TGF-β (Fig. 1C, WT). In Smad2−/− T cells, this suppression of IL-2 production was not observed in the presence of 0.1 ng/ml TGF-β, but slight suppression was observed in the presence of 0.5 ng/ml TGF-β. TGF-β also suppressed IL-2 production less efficiently in Smad3−/− T cells than in WT T cells (Fig. 1C). In Smad2−/− Smad3+/− T cells, however, the fraction of IL-2-positive cells was not reduced even in the presence of 0.5 ng/ml TGF-β, in a finding consistent with microarray data. These data clearly demonstrate that TCR-mediated IL-2 production is suppressed by TGF-β through Smad2/3-dependent mechanisms. Smad2 and Smad3 seemed to function redundantly and additively for IL-2 suppression.
Suppression of IL-2 Promoter Activation Does Not Require Direct Binding of Smads to DNA—Then, we investigated the mechanism of Smad2/3-mediated IL-2 promoter suppression. It has been shown that the proximal region of the IL-2 promoter contains Smad-responsive elements to which Smad2/Smad4 binds, but Smad3 does not (16). However, this previously reported Smad-binding element was not present in the mouse IL-2 promoter. Moreover, our data indicate that both Smad2 and Smad3 are involved in IL-2 suppression. Therefore, other Smad2/3-dependent mechanisms must be involved in the IL-2 promoter suppression by TGF-β.

To confirm the recruitment of both Smad2 and Smad3 to the IL-2 promoter, we performed a ChIP assay using Smad2 and Smad3-specific monoclonal antibodies. As shown in Fig. 2A, both Smad2 and Smad3 bound to the proximal region but not to the distal region of the IL-2 promoter. The level of Smad3 recruitment to the IL-2 promoter was much higher than that of Smad2, which may reflect higher DNA-binding ability of Smad3 than that of Smad2 (9).

To determine the part of the proximal region to which Smad3 was bound, we performed a luciferase-reporter assay. The proximal region of the promoter was fused to luciferase, then transfected with NF-AT and Smad3 cDNA into 293 cells and stimulated with PMA (Fig. 2B). Overexpression of Smad3 inhibited NF-AT-mediated activation of the −577 and −194 IL-2 promoters. The suppression activity of Smad3 was reduced in the −145 promoter, however, suggesting that Smad-responsive elements exist between −194 and −145. As shown in Fig. 2C, this region contains several NF-AT and AP-1 binding sites and four potential Smad3/Smad4 binding sequences, termed CAGA boxes (30). We mutated these CAGA boxes, but none of them affected Smad3-mediated suppression of IL-2 promoter activity (Fig. 2D). This raised the possibility that the direct binding of Smads may not be necessary for the suppression of the IL-2 promoter.

This possibility was supported by the data showing that Smad2, which lacks a functional DNA-binding domain (9), still suppressed IL-2 promoter activity like Smad3 (Fig. 3A). To abolish DNA binding ability of Smad3, we mutated several amino acid residues, including Arg-74 and Gln-76, which are necessary for DNA binding in Smad3 (31). As reported, these Smad3 mutants lost the enhancing activity of the reporter containing the Smad-binding elements (SBEs) (Fig. 3B, left; data for Q76A are not shown). Nevertheless, these Smad3 mutants still suppressed NF-AT-mediated IL-2 promoter activity as efficiently as WT Smad3 did (Fig. 3B, right). Taken together, our results suggest that Smad2 and Smad3 suppress IL-2 promoter activity without binding directly to the DNA. Given that our ChIP assay revealed the recruitment of Smad3 to the proximal region of the IL-2 promoter (Fig. 2A), it is likely that Smad2/3 may bind to the DNA indirectly, through association with other transcription factors.

Inhibitory Histone Methylation Is an Important Step in Suppression of IL-2 Transcription by TGF-β—The recognition that specific post-translational modifications of histones are associated with the activation and silencing of specific genes has given rise to the histone code hypothesis (21). Specifically, the post-transcriptional modifications of histones are associated with the activation and silencing of specific genes has given rise to the histone code hypothesis (21).

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promoter of primary naïve T cells in response to TCR stimulation in the presence or absence of TGF-β (Fig. 4A).

H3K4 trimethylation in the proximal region of the IL-2 promoter did not occur in response to TCR stimulation irrespective of TGF-β, and H3K27 trimethylation was rather suppressed by TGF-β. H3K9 trimethylation, on the other hand, was strongly enhanced by TGF-β. This enhancement was partly impaired in Smad2-deficient T cells and almost completely abrogated in Smad3-deficient T cells; these findings are consistent with the possibility of Smad2/3-dependent suppression of IL-2 transcription. In addition, TGF-β-mediated H3K9 trimethylation occurred only in the proximal region but not in distal region of the IL-2 promoter (Fig. 4B). Thus, H3K9 methylation was considered the most likely mechanism for the suppression of the proximal IL-2 promoter activity by the TGF-β-Smad pathway.

Smad3 Interacts with HMTs and Cooperatively Suppresses IL-2 Transcription—Next, we investigated whether Smads recruit histone methyltransferases (HMTs). The K9 HMTs Suv39h1 and Setdb1 have been well characterized (32). First, we expressed Smad3 in 293 cells at levels that did not strongly suppress IL-2 promoter activity. As shown in Fig. 5A, both Suv39h1 and Setdb1 suppressed NF-AT-mediated IL-2 reporter activation in collaboration with Smad3. Suv39h1 exerted stronger suppressive activity against the IL-2 promoter than Setdb1 did (Fig. 5A). Furthermore, Setdb1 and Suv39h1 were co-immunoprecipitated with Smad3 when they were co-expressed in the 293 cells, indicating that Smad3 interacts with Setdb1 and Suv39h1 and therefore inhibits IL-2 promoter activity (Fig. 5B).

To examine whether the recruitment of HMTs promotes the suppression of IL-2 transcription in T cells, Setdb1 and
Suv39h1 were expressed in the 68-41 T cell line by means of retroviral gene transfer. Since the retrovirus vector contains an IRES-GFP cassette, transduced cells were collected via FACS as GFP-positive cells. As shown in Fig. 5C, forced expression of Smad3 or Suv39h1 strongly suppressed IL-2 mRNA transcription in response to TCR, suggesting that overexpression of these two factors can mimic the effect of TGF-β. However, forced expression of Setdb1 did not strongly affect TCR-mediated IL-2 transcription, although it slightly enhanced TGF-β-mediated suppression of IL-2 transcription (Fig. 5C). These data are consistent with the IL-2 reporter assay presented in Fig. 5A, which revealed a strong transcriptional suppression activity of Suv39h1 even in the absence of Smad3.

Then, we examined whether Suv39h1 recruited into the IL-2 promoter proximal region in a Smad-dependent manner. As shown in Fig. 5D, Suv39h1 bound to the proximal region of the IL-2 promoter constitutively without stimulation. TCR stimulation released Suv39h1 from the promoter, and TGF-β re-recruited Suv39h1 to the promoter. The TGF-β-mediated recruitment of Suv39h1 to the IL-2 promoter was not observed in Smad2+/− Smad3−/- T cells (Fig. 5D, right panel). These data indicate that Smads are involved in the TGF-β-mediated recruitment of Suv39h1 to the IL-2 promoter, which may induce H3K9 trimethylation (Fig. 4, A and B).

Knockdown of Suv39h1 Partly Abolished TGF-β-mediated IL-2 Suppression—Then, we confirmed an important role of Suv39h1 in TGF-β-mediated IL-2 suppression. We used two independent shRNA (shSuv39h1-1 and shSuv39h1-2) to reduce the expression of Suv39h1 in 68–41 T cells (Fig. 6A) or primary T cells (Fig. 6, B and C). We transduced shRNA into T cells using a retroviral vector (MSCV-LMP) and infected cells were sorted as GFP-positive cells by FACS (26).

As shown in Fig. 6A (left panel), shSuv39h1-2 strongly, whereas shSuv39h1 partially, suppressed Suv39h1 expression in 68–41 T cells. GFP-positive 68–41 cells were stimulated with TCR and TGF-β for 24 h then IL-2 mRNA levels were measured by real-time RT-PCR (Fig. 6A) or primary T cells (Fig. 6, B and C). We transduced shRNA into T cells using a retroviral vector (MSCV-LMP) and infected cells were sorted as GFP-positive cells by FACS (26).

Next, we examined the effect of shRNAs for Suv39h1 in primary T cells (Fig. 6, B and C). Because retroviral introduction of
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FIGURE 4. Histone modification in the IL-2 promoter region in response to TCR and TGF-β stimulation in primary T cells. A, trimethylation of H3K9, H3K27, and H3K4 in stimulated primary T cells from WT and Smad2- or Smad3-deficient mice as assessed through a ChIP assay. Primary CD4+ T cells from indicated mice were stimulated with anti-CD3 and anti-CD28 antibodies (TCR) in the presence or absence of TGF-β for 24 h. Histone trimethylation (H3K9me3, H3K27me3, and H3K4me2) on the proximal region of the IL-2 promoter (−0.3 to −0.4 kb upstream of the first ATG codon) was detected with ChIP assay using the indicated antibodies. ND, not determined. Values are means ± S.D. (n = 3). *, p < 0.05;**, p < 0.01. B, K9 trimethylation of H3 (H3K9me3) at the IL-2 promoter was analyzed through a ChIP assay. Primary T cells were stimulated with anti-CD3 antibody in the presence or absence of TGF-β for 24 h. The upper panel shows the region amplified after immunoprecipitation with anti-H3K9me3 antibody. **, p < 0.01.
shRNA required cell cycle progression, we stimulated T cells for 24 h, and viral infection was performed for another 24 h. GFP-positive cells were sorted and then restimulated with anti-

CD3 antibody in the presence or absence of TGF-β for 12 h. IL-2 mRNA levels were determined by real-time RT-PCR (Fig. 6B, right), and IL-2 protein levels in the culture were determined by ELISA (Fig. 6C). Reduction of Suv39h1 by shSuv39h1-1 was marginal, and we did not see strong effect by this shRNA in primary T cells. However, similar to 68-41 cells, shSuv39h1-2 significantly reduced the levels of Suv39h1 (Fig. 6A, middle panel) and enhanced IL-2 mRNA and protein levels in response to TCR stimulation. Suppression effect of TGF-β was also partly abrogated by shSuv39h1-2 at both mRNA and protein levels in response to TCR stimulation. These data suggest that Suv39h1 is deeply involved in the suppression of TCR-mediated IL-2 transcription by the TGF-β-Smad pathway.

DISCUSSION

In the current study, we have shown that both Smad2 and Smad3 are redundantly essential for the suppression of IL-2 transcription induced by TCR signals. Similar redundancy has already been proposed (16, 33, 34) have
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reported that TGF-β-mediated suppression of IL-2 production from activated T cells is severely impaired by Smad3 deficiency; another report, however, suggests only a partial impairment of TGF-β-mediated suppression of IL-2 production in Smad3−/− T cells (33). Here, we show that Smad2 and Smad3 can both potentially suppress IL-2 promoter activation in response to TCR stimulation.

One possible reason for discrepancies among the previous reports is the difference of the concentrations of TGF-β used in each experiment. We have shown that at low concentrations of TGF-β, suppression of IL-2 production by TGF-β was almost completely abrogated in both Smad2−/− and Smad3−/− T cells, whereas higher concentrations of TGF-β almost completely suppressed IL-2 production in Smad2−/− or Smad3−/− T cells. Thus, importance of each Smad could be underestimated at high concentration of TGF-β. Because almost no IL-2 suppression was observed in Smad2−/−/Smad3−/− T cells by TGF-β (Fig. 1C), we propose that Smad2 and Smad3 can compensate each other but are additively necessary for full IL-2 suppression by TGF-β.

We propose that the recruitment of HMT by Smads is one of the important mechanisms involved in the suppression of IL-2 promoter activity. We observed that a strong augmentation of Smad3-mediated IL-2 promoter suppression resulted from the co-expression of HMTs such as Suv39h1 and Sedb1. Of these, Suv39h1 exerts stronger suppression activity than Setdb1 does; furthermore, the overexpression of Suv39h1 alone was sufficient to suppress TCR-mediated IL-2 transcription in a T cell line. Suv39h1 was recruited to the IL-2 promoter proximal region by the TGF-β-Smad pathway (Fig. 5D), which strongly correlated with H3K9 trimethylation in this region (Fig. 4, A and B). Furthermore, Suv39h1 knockdown abrogated the suppression effect of TGF-β (Fig. 6). These data strongly support our hypothesis that Smads recruit Suv39h1 to the IL-2 promoter and then induce H3K9 trimethylation to suppress IL-2 transcription.

Suv39h1 did not affect Foxp3 promoter activation by Smad3. Therefore, Suv39h1 seems to have promoter specificity. It is still unclear, however, how such promoter specificity is determined. Moreover, Suv39h1 was constitutively bound to the IL-2 promoter in the absence of TCR stimulation, but H3K9 trimethylation was not observed without TCR stimulation (Fig. 4). Thus, recruitment of Suv39h1 to the promoter is not sufficient for H3K9 trimethylation. In addition, we found that, among the repressive forms of H3 methylation, H3K9 methylation was dependent on Smad2/3, whereas H3K27 methylation was not. The mechanism underlying these phenomena is not clear at present.

Smads have been shown to interact with various transcription factors. Because DNA binding seems not to be required for IL-2 suppression, Smad2/3 may recruit HMT to the IL-2 promoter by binding to such transcription factors. The transcription factors AP-1, NF-AT, and NF-kB bind at distinct sites within the IL-2 promoter where Smads may interact (−196 to −145) and are necessary for the activation of the IL-2 gene (35, 36). Multiple CAGA motifs lie juxtaposed to or overlapping with these critical transcription factor-regulatory response elements (i.e. AP-1, NF-AT, NF-kB, and CREB) in the proximal promoter/enhancer region (+1 to −300) of the IL-2 promoter (GenBank accession no. NM008366). Although we found no evidence that these CAGA motifs were required for the Smad3-mediated suppression of IL-2 promoter activity in our 293 cells, the proximity of the CAGA motifs to AP-1, NF-AT, NF-kB, and cAMP-responsive element modulator/CREB cis-regulatory response elements promotes efficient cooperation between Smads and TCR-regulated transcription factors in T cells. Indeed, Smads have been shown to interact with AP-1, NF-kB, and CREB (37, 38). Therefore, Smad2/3 may bridge HMTs, and these transcription factors in the process of IL-2 suppression. However, we could not rule out the possibility that a direct DNA binding of Smad2 and Smad3 to the IL-2 promoter and Foxp3 promoter is involved in IL-2 promoter suppression.

It has not yet been established how Smad2/3 up-regulate and down-regulate various genes. Smad2/3 are apparently necessary for the induction of Foxp3, a typical up-regulated gene, in response to TCR and TGF-β. We and others have shown that two SBEs and one NF-AT binding site were present in the evolutionarily conserved non-coding sequence 1 (CNS1) region of the Foxp3 enhancer where the TGF-β responsive region is localized (18, 19). CNS1 has been shown to be superfluous in thymus-derived Treg cell development, although it plays a prominent role in induced Treg cell generation in gut-associated lymphoid tissues (39). Using Smad2/3-deficient T cells, we have previously demonstrated that both Smad2 and Smad3 are essential for TGF-β-mediated induction and for the maintenance of Foxp3 expression (14). The mechanisms involved in Foxp3 induction by Smad2/3 remained to be elucidated.

Subsequently, we found that TGF-β induced histone acetylation near the CNS1 region (18). Wei et al. (15) have reported that H3K4me3 was highly induced in the Foxp3 enhancer region in the induced Treg (TCR+TGF-β+IL2) condition; similarly, we have found that H3K9me3 and H3K27me3 were strongly reduced in the CNS1 region in the induced Treg condition. Therefore, it appears that, in contrast to the IL-2 promoter, Smad2/3 may recruit histone acetylase and H3K4 HMT to the Foxp3 promoter while inhibiting the recruitment of H3K9 and H3K27 HMT. One simple possible explanation is that Smads recruit HAT or H3K4 HMT in collaboration with NF-AT when they bind to DNA but that they recruit H3K9 HMTs when they do not bind to DNA; this results in the suppression of transcription. However, it is not clear whether this pattern of histone modification of CNS1 is a direct effect of Smad2/3 binding to the SBE or an indirect effect resulting from a modification of the chromatin structure. Moreover, SBEs in the Foxp3 promoter seem to be necessary for TGF-β mediated induced Treg generation (39). As we mentioned in the text, however, Smad2 has a very low affinity to SBE, unlike Smad3, but it is still necessary for a full induction of Foxp3 (14). Therefore, additional study will be necessary to define the mechanism responsible for the redundancy of Smad2 and Smad3 in TGF-β-mediated Foxp3 induction.

In summary, this study reveals that TGF-β signals through Smad2/3-dependent pathways to suppress IL-2 transcription.

4 Y. Wakabayashi, T. Takimoto, and A. Yoshimura, unpublished data.
5 Y. Wakabayashi, unpublished data.
We have shown that Smads recruit H3K9 HMT to the proximal region of the IL-2 promoter, probably via a direct interaction. This ability of Smads to enhance and suppress the transcription of immunoregulatory genes suggests a mechanism by which we could selectively inhibit immunological disorders such as autoimmune diseases and allergies.

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