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Smad2/3 and IRF4 Play a Cooperative Role in IL-9-Producing T Cell Induction

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IL-9 is a pleiotropic cytokine that can regulate autoimmune and allergic responses. Th9 cells can develop from naive T cells or Th2 cells through stimulation by TGF- β in vitro. In this study, we demonstrated that Smad2 and Smad3 are necessary for IL-9 production from T cells in an OVA-induced asthma model using T cell-specific Smad2- and Smad3-deficient mice. Smad2 and Smad3 were also redundantly essential for TGF- β signaling to induce histone modifications for *Ii9* transcription. Although Smad2/3 was recruited to the *Ii9* promoter by TGF- β stimulation, they are not sufficient to activate the *Ii9* promoter. By the screening the transcription factors, we found that IFN regulatory factor 4 (IRF4) was essential for the Smad2/3-mediated *Ii9* promoter activation. In addition, Smad2/3 physically interacted with IRF4, and Smad2/3 did not bind to the *Ii9* promoter and could not induce Th9 in IRF4-deficient T cells. Similarly, IRF4 could not stimulate *Ii9* transcription in the absence of Smad2/3, and TGF- β enhanced IRF4 recruitment to the *Ii9* promoter in a Smad2/3-dependent manner. We propose that Smad2/3 and IRF4 cooperatively transactivate the *Ii9* promoter and play an important role in regulating allergic immune responses by inducing Th9 cells. *The Journal of Immunology*, 2013, 191: 000–000.

Transforming growth factor- β is predominantly expressed in the immune system and is an important pleiotropic cytokine with potent immunoregulatory properties (1). TGF- β regulates T cells, dendritic cells, and macrophages (2–5), suppresses Th1 and Th2, and induces induced regulatory T cells (iTreg), effector T cells, Th17, and Th9 (4, 6).

Th9 cells are a recently described subset of effector T cells that secrete IL-9. They develop from naive T cells in the presence of TGF- β and IL-4 or from Th2 cells in the presence of TGF- β in vitro (7–9). IL-9 production requires transcription factors in-

cluding STAT6 (10), GATA3 (8), IFN regulatory factor 4 (IRF4) (11), and PU.1 (12). In addition, the Notch (13) and OX40 signals (14) and the CGRP/cAMP/protein kinase A pathway (15) have been shown to promote Th9 development. These results suggest that various Th2-type transcription factors and additional signals are involved in Th9 development. Although many pathophysiological functions have been attributed to Th9 cells, the role of TGF- β in Th9 development in vivo remains to be determined.

The main TGF- β signaling pathway is the Smad pathway. TGF- β first binds to the TGF- β receptor, which then activates Smad transcription factors including three structurally similar proteins: Smad2, Smad3, and Smad4. Smad2 and Smad3 are phosphorylated and activated by TGF- β receptor and heterodimerize with Smad4. The activated Smad complex translocates into the nucleus and regulates target gene transcription. TGF- β has been shown to induce Foxp3, a master transcription factor for Tregs. We have demonstrated in experiments with Smad2 and Smad3 double-deficient T cells that Smad2 and Smad3 are necessary for Foxp3 induction in TGF- β iTreg differentiation (4) and suppression of IL-2 production (16). Smad2 and Smad3 are redundantly essential for the suppression of inducible NO synthase expression in macrophages (2). However, the nuclear orphan receptor retinoic acid–related orphan receptor γ t (ROR γ t) induction by TGF- β is independent of the Smad signaling pathway (5). Although Elyaman et al. (13) demonstrated that cooperative interaction of the Notch pathway and Smad3 was required for *Ii9* promoter activation, the precise mechanism and significance of Smad signaling for IL-9 production remains to be clarified.

In this study, using T cell-specific Smad2- and Smad3-deficient mice, we found that Smad2 and Smad3 play an important role in regulating IL-9 levels in OVA-induced allergic asthma, suggesting that the TGF- β –Smad2/3 pathway is crucial for Th9 development in vivo. We also showed that Smad2 and Smad3 were redundantly essential for IL-9 production in CD4⁺ T cells in cooperation with IRF4.

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Abbreviations used in this article: alum, aluminum hydroxide; BAL, bronchoalveolar lavage; ChIP, chromatin immunoprecipitation; CNS, conserved noncoding sequence; H3K4me3, H3 lysine 4 trimethylation; IRF4, IFN regulatory factor 4; iTreg, induced regulatory T cell; PAS, periodic acid–Schiff; ROR γ t, retinoic acid–related orphan receptor γ t; WT, wild-type.

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Materials and Methods

Mice

T cell-specific *Smad2* conditional knockout (*Lckcre-Smad2^{fl/fl}*) mice and *Smad3^{-/-}* mice have been described previously (4). T cells from *Lckcre-Smad2^{fl/fl}* mice are henceforth described as *Smad2^{del/del}* T cells. The *Irf4* knockout mice from Dr. K. Honma have been described elsewhere (17). Mice were kept in specific pathogen-free facilities at Keio University. All experiments using mice were approved by the Animal Ethics Committee of Keio University and performed according to the Animal Ethics Committee's guidelines.

Abs

The Ab to Flag-tag (M2) was from Sigma-Aldrich. PerCP-Cy5.5-conjugated anti-CD4 mAb (L3T4) and PE-conjugated anti-Thy1.1 mAb (HIS51) were purchased from eBioscience. Allophycocyanin-conjugated anti-IL-9 mAb (RM9A4) was purchased from BioLegend. Anti-IRF4 goat polyclonal Ab (sc-6059) and anti-hemagglutinin-tag rabbit polyclonal Ab (sc-805) were purchased from Santa Cruz Biotechnology. Smad2- and Smad3-specific mAbs were from Cell Signaling Technology (5339 for Smad2 and 9523 for Smad3).

Plasmid construction

Murine Smad2 and Smad3 cDNAs were cloned as described previously (2). Mouse Smad3 was subcloned into eMIGR1 or MThyR vectors. Mouse IRF4 was PCR-amplified from the mouse cDNA library and subcloned into eMIGR1 and pCMV-FLAG vectors. The mouse *I19* promoter was PCR-amplified from mouse DNA and subcloned into a pGL3 luciferase vector (18).

Isolation of naive T cells

Naive CD4⁺ T cells (CD4⁺CD25⁻CD62L^{hi}) were prepared from the spleen and lymph nodes as described (19). Briefly, the spleen and lymph node cells from 6–8-wk-old mice were depleted of non-CD4⁺ T cells through the application of biotin-conjugated Abs against CD8a, B220, CD25, CD11b, CD11c, CD49b, and TER-119 in combination with streptavidin microbeads using large depletion columns (Miltenyi Biotec). Cells were then incubated with CD62L microbeads (Miltenyi Biotec), and the CD62L^{hi} cells were positively selected using large selection columns (Miltenyi Biotec). The purity of CD4⁺CD25⁻CD62L^{hi} cells was consistently >95%.

Culture of primary T cells

CD4⁺ T cell primary culture conditions in this study were as follows: Th1 conditions: IL-12 (PeproTech; 20 ng/ml) and IL-4 neutralizing Ab (11B11, 5 µg/ml); Th2 conditions: IL-4 (PeproTech; 20 ng/ml) and IFN-γ neutralizing Ab (R4-6A2; 5 µg/ml); Th9 conditions: IL-4 (20 ng/ml), recombinant human TGF-β (1 ng/ml; R&D Systems), and IFN-γ neutralizing Ab (5 µg/ml); and iTreg conditions: recombinant human TGF-β (2 ng/ml), IL-2 (PeproTech; 10 ng/ml), IFN-γ neutralizing Ab (5 µg/ml), and IL-4 neutralizing Ab (5 µg/ml). All cultures were performed in RPMI 1640 (Invitrogen) supplemented with anti-CD28 (57.31; 1 µg/ml) Abs, 10% FBS, 1% penicillin/streptomycin, 100 nM nonessential amino acids, 2 mM glutamine, and 0.05 mM 2-ME on plate-coated anti-CD3e (2C11; 2 µg/ml).

Retroviral transduction

Retroviral transduction was performed as described (20). Briefly, naive T cells were plated and subjected to the Th2-differentiation conditions described above starting on day 0. On day 2, fresh retroviral supernatants were added, and the cells were centrifuged at 2500 rpm for 2 h at 35°C. After spin infection, the cells were cultured in the appropriate Th cell differentiation media and harvested on day 4 for intracellular cytokine staining and real-time PCR analysis.

mRNA preparation and quantitative PCR

Total RNA was extracted using RNAiso Plus (Takara Bio) and subjected to reverse transcription using a High Capacity cDNA Synthesis Kit (Applied Biosystems). PCR analysis was performed using an iCycler iQ multicolor real-time PCR detection system (Bio-Rad) and SsoFast EvaGreen Supermix (Bio-Rad). All primer sets yielded a single product of the correct size. Relative expression levels were normalized to *Gapdh*.

Flow cytometry

For IL-9 intracellular cytokine staining, cells were stimulated for 5 h in complete medium with PMA (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 anti-IL-9 Ab with the Fixation and Permeabilization kit (eBioscience) according to the manufacturer's protocol instructions. Data were acquired on a BD FACSCanto II (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Luciferase assay

293T cells were seeded on 24-well plates and transfected with various amounts of expression vector with PEI. After transfection, 293T cells were cultured for 24 h. All cells were harvested with 100 µl lysis buffer. The luciferase activity was assessed using a luciferase substrate kit (Promega), and luciferase activity was determined using a Packard luminometer (Packard Instruments) and normalized to β-galactosidase as an internal control.

Induction of OVA-induced asthma

Mice were immunized with an i.p. injection of OVA (Sigma-Aldrich) adsorbed with aluminum hydroxide (alum; Pierce) at a dose of 20 µg OVA/2 mg alum on days 0 and 7. On day 14, mice received an inhaled challenge of nebulized OVA (0.5% in PBS) using an ultrasonic nebulizer (Omron) for 20 min daily from days 1–6. Mice were sacrificed using an i.p. injection of pentobarbital 48 h after the final challenge. The trachea was cannulated, and lungs were lavaged four times with 0.5 ml PBS. Cells recovered in bronchoalveolar lavage (BAL) fluid were counted using trypan blue dye exclusion. Eosinophils, neutrophils, T cells, B cells, and mononuclear cells in the BAL fluid were distinguished by cell size and expression of CD3, B220, CCR3, CD11b, and Gr-1 and analyzed by flow cytometry. The serum from treated mice was analyzed for IL-4 and IL-9 expression using an ELISA.

H&E and periodic acid–Schiff staining

Lungs were fixed in 3.7% paraformaldehyde. Fixed tissues were embedded in paraffin, cut into 6-µm sections, and stained with H&E for cellular infiltration analysis or periodic acid–Schiff (PAS) for goblet cell hyperplasia analysis as described (21).

Chromatin immunoprecipitation assay

Primary T cells were fixed with 1% formaldehyde at room temperature for 60 min and then suspended in an SDS lysis buffer. After sonication by the S220 Focused-ultrasonicator (Covaris), samples were incubated with 2 µg Abs or control IgG for 4 h at 4°C. After the addition of Dynabeads protein G (Life Technologies), the immunoprecipitates were sequentially washed once each with a low-salt buffer, a high-salt buffer, and a LiCl buffer and twice with a TE buffer. The DNA–protein complex was eluted by heating at 65°C overnight. Proteins were then digested with proteinase K. DNA was recovered using the QIAquick PCR Purification Kit (Qiagen) and then subjected to real-time PCR analysis.

Immunoprecipitation and Western blotting

The 293T cells that had been transfected with the indicated plasmids were washed once with ice-cold PBS and lysed in 0.3 ml TNE lysis buffer containing 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40 supplemented with protease inhibitor mixture (Nacalai Tesque) (22). After sonication, cellular debris was removed by centrifugation at 15,000 × g for 10 min. Protein from cell lysates was precipitated with 2 µg Ab and 20 µl Protein G-Sepharose (GE Healthcare) for 4 h at 4°C. The immune complex was washed three times with TNE buffer. For Western blotting, the immunoprecipitates or whole-cell lysates were resolved using SDS-PAGE and transferred to Immobilon-P membranes (Millipore). The membranes were blotted with the indicated Abs, and the bound Abs were visualized using HRP-conjugated Abs against goat, rabbit, or mouse IgG and Chemi-Lumi One L Western-blotting detection reagents (Nacalai Tesque).

Proximity-ligation assay

The Duolink In Situ PLA Kit (Olink Bioscience) was used for in situ proximity-ligation assays (23). T cells were attached to glass slides with cytospin, fixed with 4% paraformaldehyde, and then permeabilized for 15 min in 0.1% Triton X-100 in TBS (25 mM Tris and 100 mM NaCl [pH 7.4]). The slides were then blocked for 2 h with 0.5% BSA in TBS in a humidified chamber at 37°C and incubated overnight at 4°C with goat polyclonal anti-IRF4 Ab and rabbit anti-Smad2/3 mAb. After washing with TBST, proximity ligation was performed using the Rabbit PLUS and Goat MINUS Duolink In Situ PLA kits according to the manufacturer's protocol (Olink Bioscience). CD4 was stained with FITC-conjugated anti-CD4 Ab to show the cell shape.

Statistics

A paired, two-tailed Student *t* test was used. The *p* values < 0.05 were considered significant. All error bars represent the SD.

Results

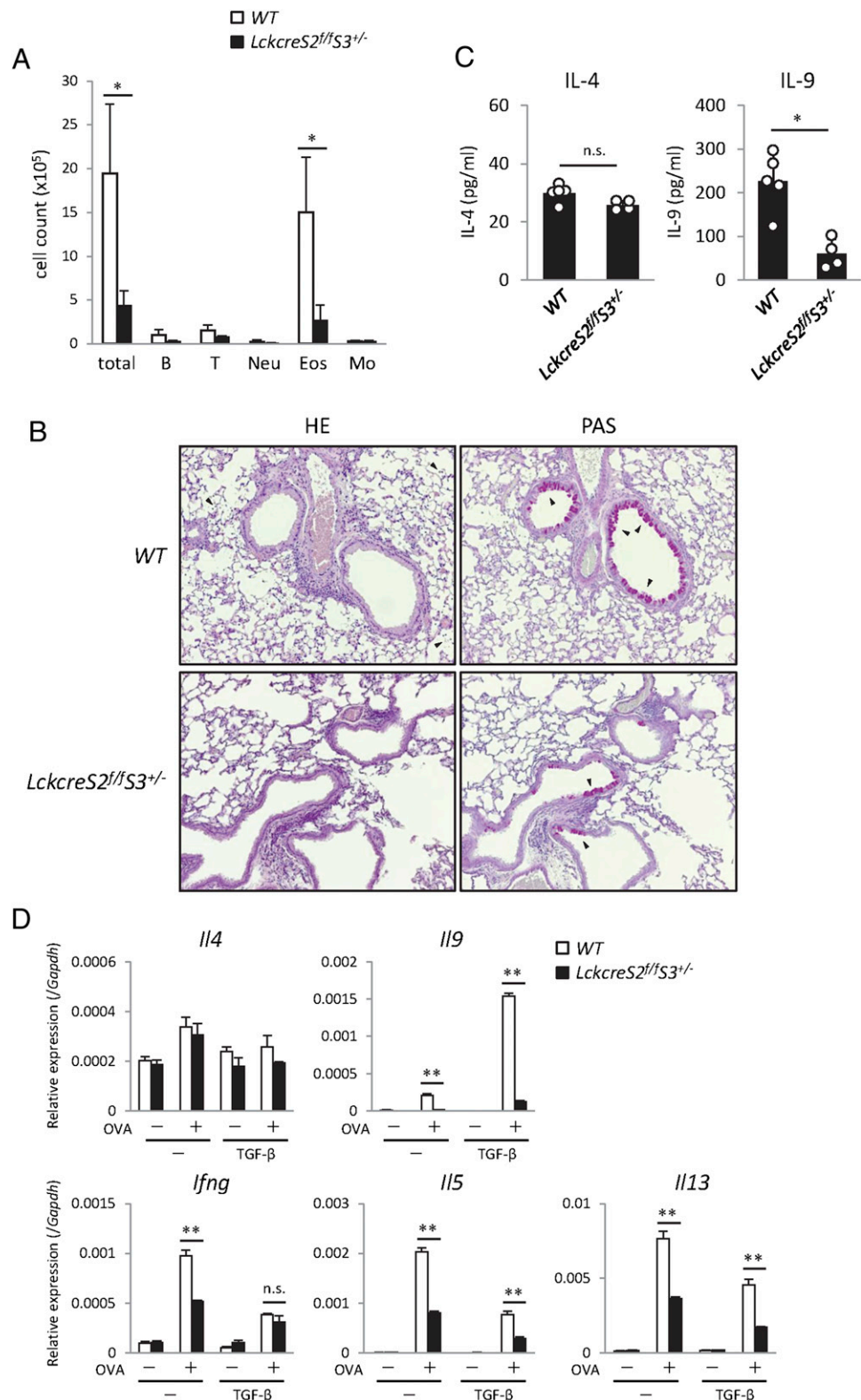
Smad2/3 deficiency in T cells attenuates OVA-induced asthma

Th9 cells have been implicated in allergic inflammation such as asthma. First, we investigated the effect of *Smad2/3* deficiency in

T cells in an OVA-induced asthma model, in which IL-9 has been shown to play an important role. Because T cell-specific *Smad2* and *Smad3* double-knockout mice died within 5 wk after birth and had severe inflammation (4, 24), we used *Lck-cre Smad2^{fllox/fllox} Smad3^{+/-}* (*LckcreS2^{fl}/S3^{+/-}*) mice.

We immunized mice with OVA and alum and then challenged them with nebulized OVA to induce airway inflammation. As shown in Fig. 1A, infiltration of inflammatory cells, especially eosinophils,

FIGURE 1. *Smad2/3*-deficient mice are resistant to OVA-induced airway inflammation. WT (*n* = 5) and *LckcreS2^{fl}/S3^{+/-}* (*n* = 4) mice were immunized with OVA and alum and then challenged with nebulized OVA. BAL fluids were collected 48 h after the final challenge. (A) Specific cell types in the BAL fluid from challenged mice were assessed using flow cytometry. (B) H&E staining (left panel) and PAS staining (right panel) of paraffin-embedded lung sections from challenged mice. Original magnification $\times 100$. (C) ELISA of IL-4 (left panel) and IL-9 (right panel) present in the serum of challenged mice. (D) Splenocytes of challenged mice were stimulated with OVA in the presence or absence of TGF- β . *Il4*, *Il5*, *Il9*, *Il13*, and *Ifng* mRNA levels were assessed using quantitative RT-PCR. Data shown are representative of at least three independent experiments. **p* < 0.05, ***p* < 0.01. B, B cell; Eos, eosinophil; Mo, monocyte; Neu, neutrophil; T, T cell.



in BAL fluid was much lower in *LckcreS2^{fl/fl}S3^{+/-}* mice than in wild-type (WT) mice. This was confirmed by histological examination (Fig. 1B, H&E staining). We observed a marked reduction of mucus-producing cells in the lung of *LckcreS2^{fl/fl}S3^{+/-}* mice compared with WT mice (Fig. 1B, PAS staining). These data indicated that Smad2/3 deficiency in T cells attenuated OVA-induced asthma. In the serum from treated mice, the amount of IL-4 was similar, but IL-9 was lower in *LckcreS2^{fl/fl}S3^{+/-}* mice compared with WT mice (Fig. 1C). We isolated splenocytes from treated mice and stimu-

lated them with OVA in the presence or absence of TGF- β . OVA stimulation induced asthma-related cytokine expression (*Il4*, *Il9*, *Ifng*, *Il5*, and *Il13*). Adding TGF- β inhibited *Il4*, *Ifng*, *Il5*, and *Il13* expression, but stimulated *Il9* expression (Fig. 1D). In *LckcreS2^{fl/fl}S3^{+/-}* mice, all of the cytokines except for *Il4* were lower than in WT mice. In particular, *Il9* levels in *LckcreS2^{fl/fl}S3^{+/-}* splenocytes were much lower than those of WT splenocytes. These data suggest that reduced T cell-derived IL-9 production ameliorated OVA-induced asthma in *LckcreS2^{fl/fl}S3^{+/-}* mice.

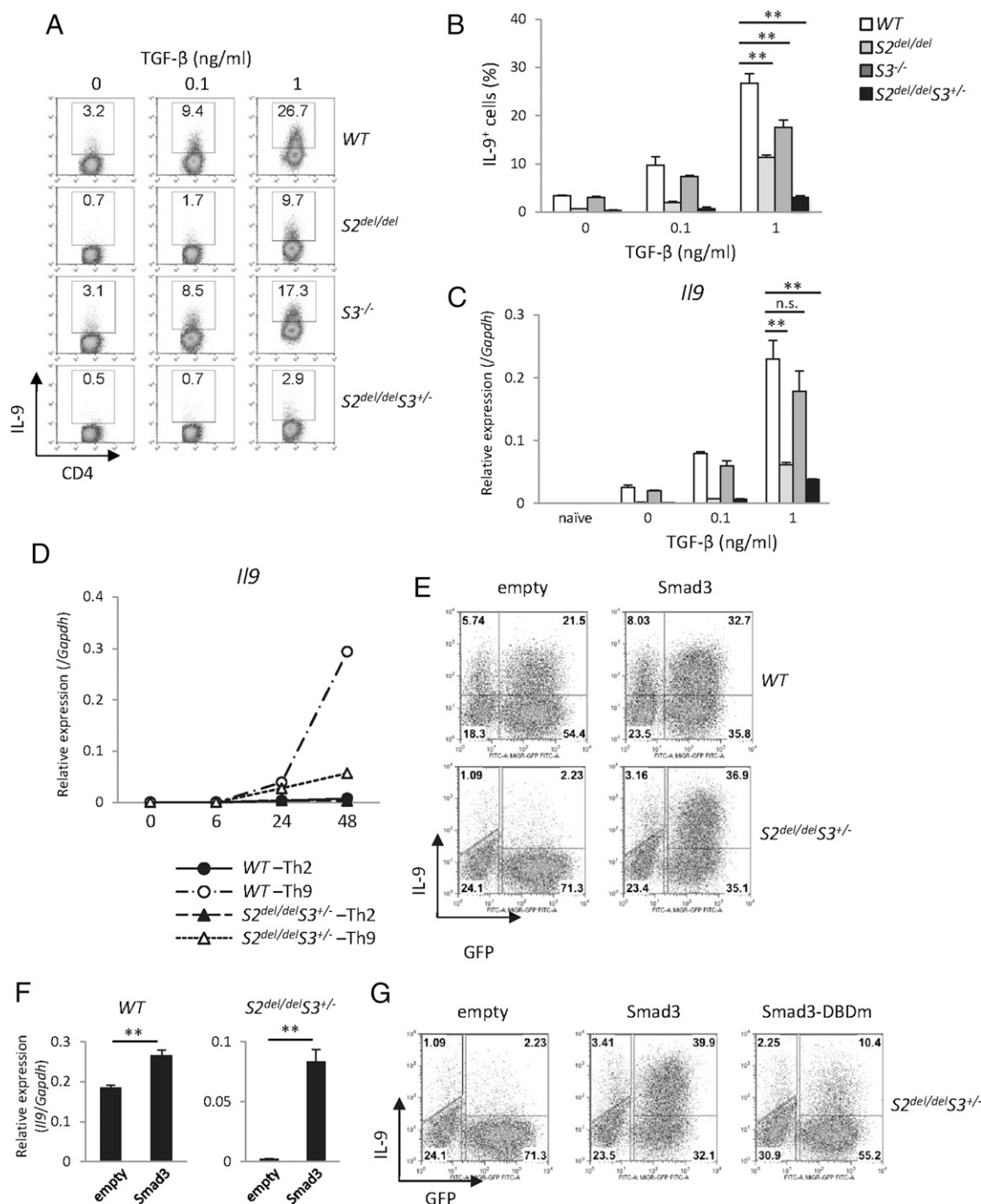


FIGURE 2. Smad2 and Smad3 are redundantly essential for TGF- β -mediated Th9 development in vitro. (A–C) Naïve T cells from WT, *LckcreS2^{fl/fl}*, *S3^{-/-}*, or *LckcreS2^{fl/fl}S3^{+/-}* mice were stimulated for 3 d under Th2-skewing conditions in the presence or absence of TGF- β at the indicated concentrations. IL-9 production was tested in cells using intracellular staining (A, B) and quantitative RT-PCR (C). $n = 3$. (D) Naïve T cells from WT or *LckcreS2^{fl/fl}S3^{+/-}* mice were stimulated under Th2 or Th9 conditions for the indicated times. The amount of *Il9* mRNA was measured using quantitative RT-PCR. $n = 3$. (E–G) Primary T cells from WT or *LckcreS2^{fl/fl}S3^{+/-}* mice were infected with a retrovirus carrying EGFP, Smad3, and DNA binding domain-mutant Smad3 (Smad3-DBDm). Infected T cells were cultured under Th9 conditions for 2 d and then assessed for IL-9 production using intracellular staining (E, G) and quantitative RT-PCR (F). $n = 3$. ** $p < 0.01$.

Smad2 and Smad3 are redundantly essential for TGF- β -mediated Th9 development in vitro

To examine whether Smad2 and Smad3 are involved in Th9 differentiation in vitro, we stimulated naive CD4⁺ T cells in the presence of IL-4 with (Th9 condition) or without TGF- β (Th2 condition). In WT T cells, IL-9 secretion increased dose dependently in response to TGF- β (Fig. 2A–C). In Smad single-deficient, *Lck-cre Smad2^{flox/flox}* (*S2^{del/del}*), or *Smad3^{-/-}* (*S3^{-/-}*) T cells, IL-9 production was partially reduced, and in addition, IL-9 expression in *S2^{del/del}S3^{+/-}* T cells was almost completely suppressed (Fig. 2A–C). These data indicate that Smad2 and Smad3 are redundantly essential for Th9 differentiation.

We then compared the time course of IL-9 expressions under Th2 or Th9 conditions (Fig. 2D). The IL-9 expression was observed from 24 to 48 h in WT T cells. *S2^{del/del}S3^{+/-}* T cells produced less IL-9 than WT T cells under Th9 conditions, and IL-4 level in *S2^{del/del}S3^{+/-}* T cells under Th9 conditions was higher than those under Th2 conditions (data not shown). These data suggested that in the absence of Smad2/3, Th differentiation was skewed toward Th2 under Th9 conditions.

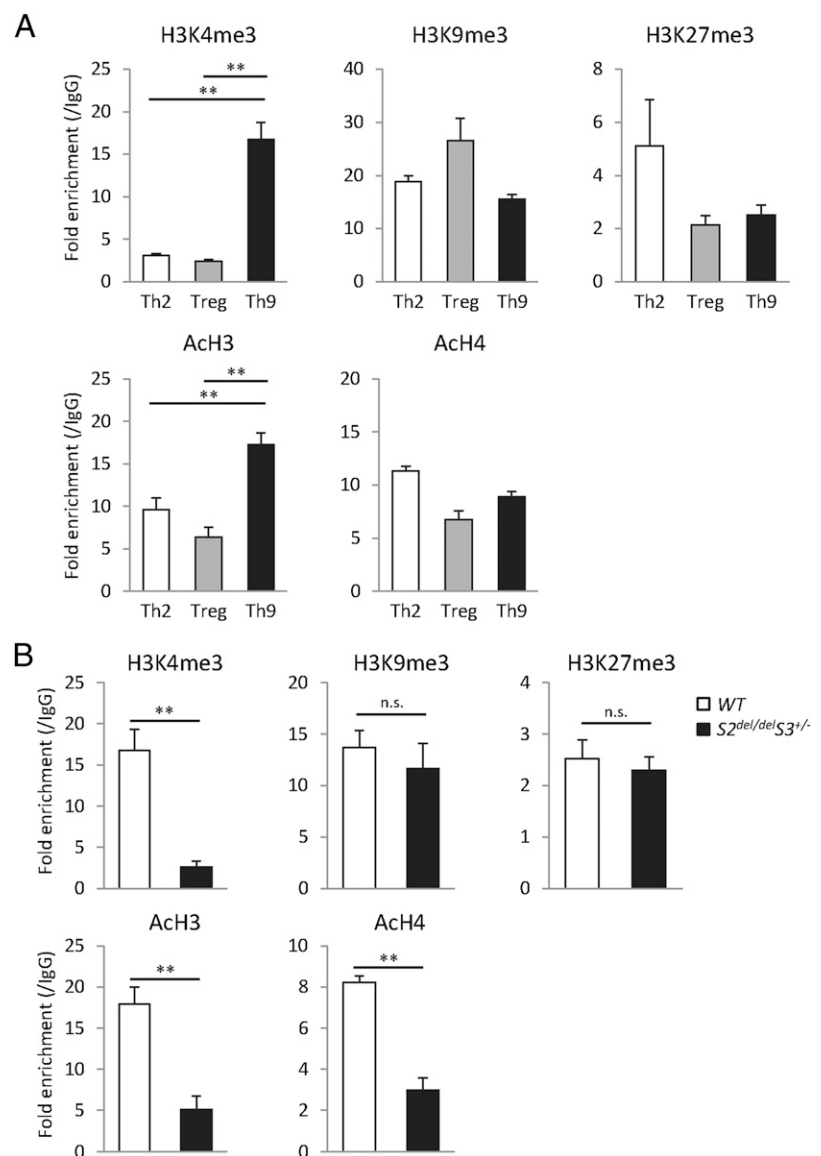
To further confirm the relationship between IL-9 production and Smads, Smad3 was overexpressed in T cells using retroviral gene

transfer. We could not express high levels of Smad2 by retrovirus gene transfer into T cells. Forced expression of Smad3 increased IL-9 protein and mRNA expression in WT T cells (Fig. 2E, 2F). In *S2^{del/del}S3^{+/-}* T cells, IL-9 production was completely restored by Smad3 overexpression (Fig. 2E, 2F); however, induction of IL-9 was much lower by the DNA binding domain-mutant Smad3 than WT Smad3 (Fig. 2G), indicating that IL-9 production requires the Smad3 DNA binding domain in T cells. Thus, Smad2/3 seems to induce IL-9 by directly activating the *Il9* promoter.

Smad2/3 affects gene activation-related histone modifications

To further confirm a direct effect of Smads on the *Il9* promoter, we examined histone modifications in the *Il9* promoter region (conserved noncoding sequence [CNS] 1) using a chromatin immunoprecipitation (ChIP) assay. The increase in transcription is associated with histone H3 and H4 acetylation (AcH3 and AcH4) and/or H3 lysine 4 trimethylation (H3K4me3), whereas silenced genes possess H3K27me3 and/or H3K9me3. In WT T cells, compared with Th2 (+ IL-4) and iTreg (+ TGF- β) conditions, AcH4, H3K9me3, and H3K27me3 were similar under Th9 (+ IL-4 + TGF- β) conditions (Fig. 3A). In contrast, AcH3 and H3K4me3 were higher in the *Il9* promoter region under Th9 conditions than in that under Th2 and iTreg conditions (Fig. 3A). In *S2^{del/del}S3^{+/-}*

FIGURE 3. Smad2/3 affects gene activation-related histone modifications. **(A)** Naive T cells were stimulated under the indicated Th conditions for 3 d, and then a ChIP assay was performed using the indicated Abs. **(B)** Naive T cells from WT or *LckcreS2^{fl}S3^{+/-}* mice were stimulated under Th9 conditions for 3 d, and then a ChIP assay was performed using the indicated Abs. Data shown are representative of at least three independent experiments. ***p* < 0.01.



Th9 cells, gene silencing-related modifications (H3K9me3 and H3K27me3) were not changed, but gene activation-related modifications (AcH3, AcH4, and H3K4me3) were drastically impaired (Fig. 3B). These data indicate that Smad2/3 promoted activation-related histone modifications in the *Il9* promoter rather than affecting gene-silencing histone modification.

Relationship between Th master genes and Smad2/3 for Th9 development

We then investigated the mechanism of how Smad2/3 induces *Il9* promoter activation. First, we examined the effect of Smad2/3 deficiency on the expression of the Th cell master regulators (Fig. 4A). Th2 key transcription factor GATA3 has been impli-

cated in IL-9 expression (8, 10). Our expression profiles for WT T cells were consistent with the previous report (10). However, *Gata3* expression in *S2^{del/del}S3^{+/-}* T cells was similar to that in WT T cells under both Th2 and Th9 conditions, indicating that loss of Th9 induction by Smad2/3 deficiency was not due to reduction in *Gata3* expression.

In contrast, *Tbx21* (T-bet) and *Rorc* (ROR γ t) were higher, whereas *Foxp3* was much lower in *S2^{del/del}S3^{+/-}* T cells compared with WT T cells (Fig. 4A). Modified expression of these master genes could be a mechanism for defective Th9 development that results from Smad2/3 deficiency. To investigate this possibility, we used retroviral gene transfer to test whether overexpression of the other master regulators inhibits IL-9 production. GATA3 increased

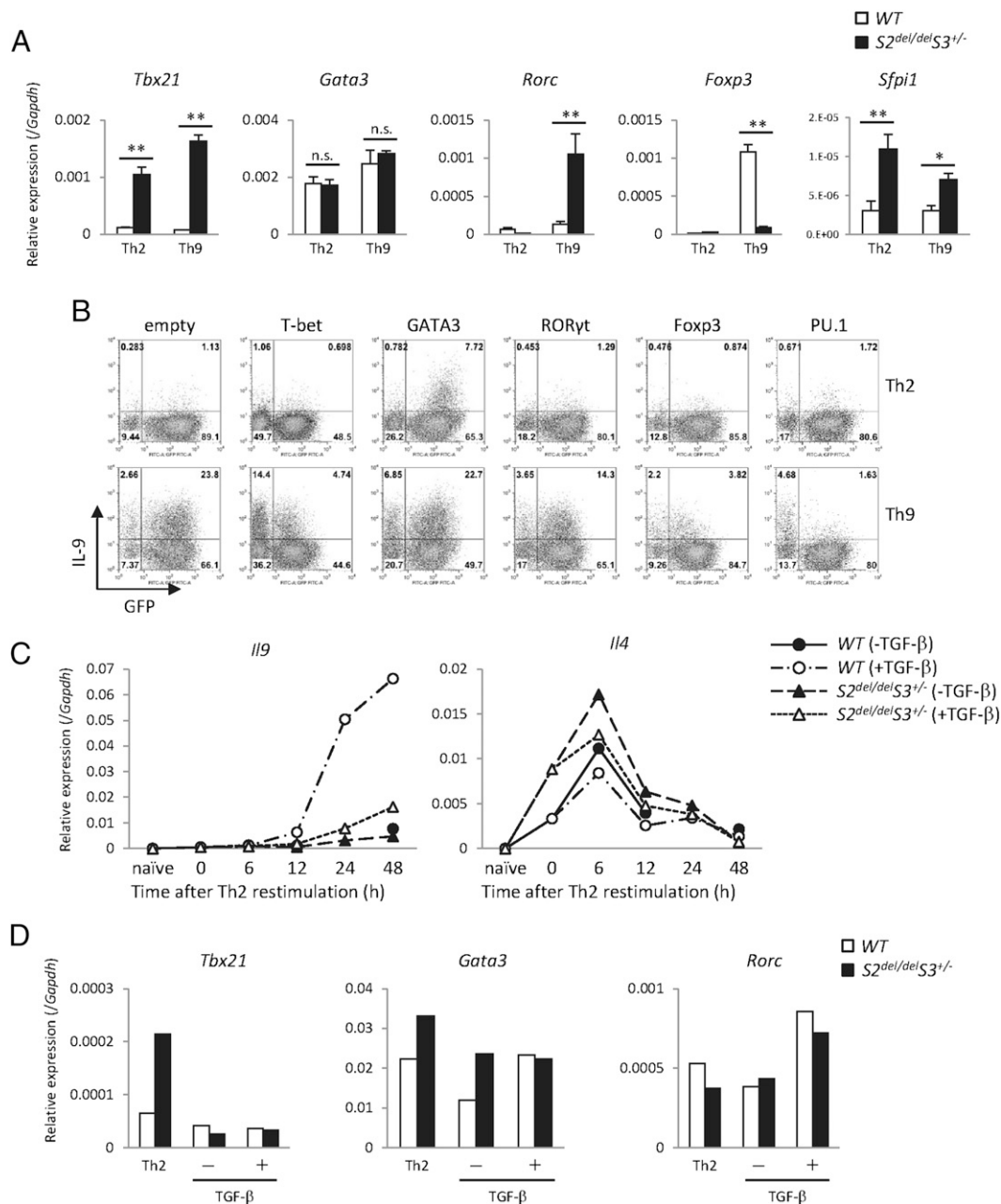


FIGURE 4. Effect of other Th cell subset master regulators on IL-9 production in Th9 cells. **(A)** Naive T cells from WT or *LckcreS2^{del/del}S3^{+/-}* mice were stimulated under Th2 or Th9 conditions. The amount of *Tbx21*, *Gata3*, *Rorc*, *Foxp3*, and *Sfp1* mRNA was measured using quantitative RT-PCR. **(B)** WT primary T cells were infected with retrovirus carrying EGFP, T-bet, GATA3, ROR γ t, Foxp3, and PU.1. Infected T cells were cultured under Th2 or Th9 conditions for 2 d, and then IL-9 production was assessed using intracellular staining. Representative profile from at least three independent experiments. **(C)** Naive T cells from WT or *LckcreS2^{del/del}S3^{+/-}* mice were stimulated under Th2 conditions for 2 d and then were restimulated in the presence or absence of TGF- β for the indicated periods. **(D)** The amount of *Tbx21*, *Gata3*, and *Rorc* mRNA was measured using quantitative RT-PCR. $n = 3$. * $p < 0.05$, ** $p < 0.01$.

IL-9 production, whereas T-bet, ROR γ t, and Foxp3 strongly inhibited IL-9 (Fig. 4B). Thus, Smad2/3 deficiency could be the mechanism that induces high *Tbx21* and *Rorc* expression.

It has been known that transcription factor PU.1 (*Sfp1*) is essential for Th9 differentiation (12), and *Sfp1* was shown to be a downstream target of TGF- β (10). As shown in Fig. 4A, right panel, *Sfp1* expression was not increased in Th9 compared with Th2 in our experimental conditions. Furthermore, we observed higher levels of *Sfp1* in *Smad2^{del/del}Smad3^{+/-}* T cells in both Th2 and Th9 conditions (Fig. 4A). These data indicate that PU.1 is not the downstream of Smad2/3 in our Th9 conditions. In addition, overexpression of PU.1 did not increase IL-9⁺ CD4⁺ T cells under Th9 conditions (Fig. 4B). These data suggest that PU.1 is not involved in Smad2/3-mediated IL-9 expression at least in our Th9 differentiation condition.

To avoid the effect of such abnormal skewing to Th1 or Th17 by Smad2/3 deficiency, naive T cells were first differentiated into Th2 cells, and then Th9 cells were induced by culturing Th2 cells with TGF- β (9). Similar to normal Th9 differentiation

conditions, WT Th2 cells were converted into Th9 cells by TGF- β , whereas *S2^{del/del}S3^{+/-}* Th2 cells expressed little IL-9 using this method (Fig. 4C, left panel). Interestingly, IL-4 expression was suppressed in both WT and *S2^{del/del}S3^{+/-}* T cells (Fig. 4C, right panel). The expressions of *Tbx21*, *Gata3*, and *Rorc* were similar between WT and *S2^{del/del}S3^{+/-}* T cells under this condition (+ TGF- β in Fig. 4D). Therefore, differences in the expression of master regulators between WT and *S2^{del/del}S3^{+/-}* T cells cannot fully explain reduced Th9 differentiation in Smad2/3-deficient T cells.

The transcription factor IRF4 is identified as an IL-9-producing partner of Smad2/3

Because TCR stimulation in the presence of TGF- β alone induces iTregs, but not Th9, additional factors that are induced by IL-4 or TCR signals should cooperatively upregulate the *Il9* promoter with Smad2/3. To identify such factors, we constructed an *Il9* promoter luciferase vector and screened various genes, such as transcription factors, transcriptional coactivators, and modifying enzymes expressed highly in T cells. Among

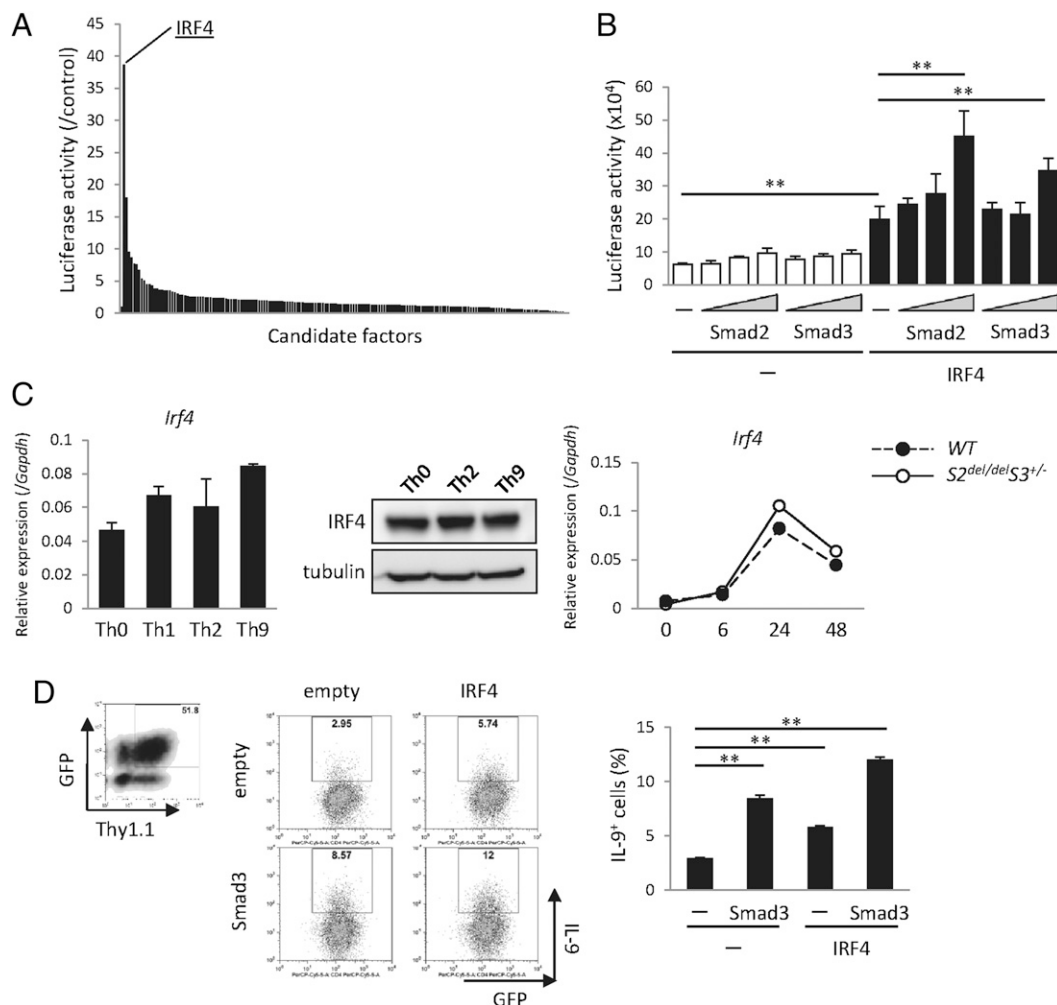


FIGURE 5. Smad2/3 activates the *Il9* promoter in cooperation with IRF4. **(A)** A screening luciferase assay was performed on 300 candidate factors (list of the genes will be provided on request) using the *Il9* promoter luciferase vector for IL-9-promoting factor. The *Il9* promoter luciferase vector was transfected into 293T cells together with the cDNA from candidate factors, and luciferase activity was measured. **(B)** *Il9* promoter activation by IRF4 and Smad2/3. The *Il9* promoter luciferase vector was transfected into 293T cells together with IRF4 and Smad2/3 cDNA, and the luciferase activity was measured. Data shown are representative of at least three independent experiments. **(C)** *Irf4* expression in various Th cells was assessed using quantitative RT-PCR (left panel) and Western blotting (middle panel). The time course of *Irf4* mRNA in primary T cells under Th2 or Th9 conditions was measured using quantitative RT-PCR (right panel). *n* = 3. **(D)** Primary T cells were infected with a retrovirus carrying Thy1.1-marked Smad3 and EGFP-marked IRF4. Infected T cells were cultured under Th2 condition for 2 d and then assessed for IL-9 production using intracellular staining (D). Representative profile from at least three independent experiments. ***p* < 0.01.

>300 genes, the transcription factor IRF4 upregulated *Ii9* promoter activity most efficiently in 293T cells (Fig. 5A). Moreover, Smad2 and Smad3 coexpression enhanced IRF4-mediated *Ii9* promoter activity, although Smad2 or Smad3 alone did not activate *Ii9* promoter activity (Fig. 5B). It has been shown that IRF4-deficient T cells cannot differentiate into Th9 cells (11). Forced expression of IRF4 enhanced IL-9 production under both Th2 and Th9 conditions (data not shown). Thus, we focused on IRF4.

IRF4 expression was similar among Th cell subsets (Fig. 5C). During Th9 differentiation, IRF4 levels were increased 24 h after stimulation in naive T cells, and there was no difference in IRF4 expression between WT and *S2^{del/del}S3^{+/-}* T cells (Fig. 5C, right panel). These data suggest that IRF4 expression is induced by TCR signals and is not affected by cytokines.

To investigate whether IRF4 and Smad2/3 cooperatively function in primary T cells, we introduced Thy1.1-marked Smad3 and

EGFP-marked IRF4 into Th2 cells using retroviral gene transfer. An individual introduction of Smad3 or IRF4 increased Th9 cells, whereas the introduction of both molecules together further increased Th9 cells (Fig. 5D). These data suggest that Smad2/3 and IRF4 cooperate to induce Th9 development.

Smad2 and Smad3 physically interact with IRF4

To examine whether Smad2/3 directly interacts with IRF4, we expressed Smad2/3 and IRF4 in 293T cells. Smad2 and Smad3 were coimmunoprecipitated with IRF4 (Fig. 6A). Smad2 and Smad3 contain two conserved structural domains in the N terminus and C terminus, respectively, and there are linker sequences between the MH1 and MH2 domains. The MH1 and MH2 domains participate in DNA binding and transcriptional activation, respectively. Results of an immunoprecipitation assay using truncated Smad3 indicated that the MH2 domain of Smad3 interacts with IRF4 (Fig. 6B).

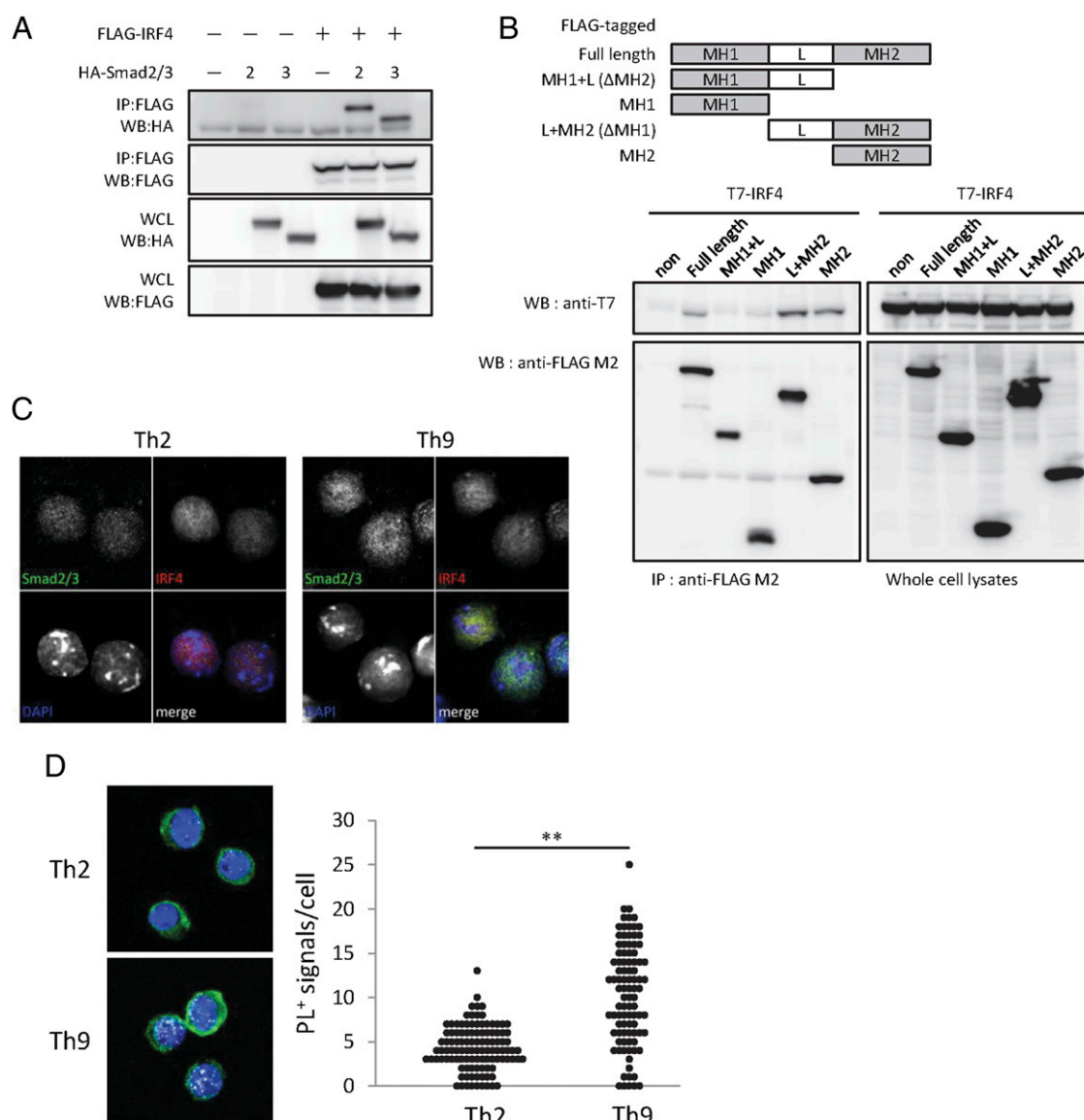


FIGURE 6. Smad2 and Smad3 physically interact with IRF4. (**A** and **B**) Binding of Smad2/3 to IRF4. 293T cells were transfected with the indicated plasmids and then lysed. After immunoprecipitation (IP) with anti-FLAG Ab, coprecipitated proteins were analyzed using Western blotting (WB) with the indicated Abs. (**C**) Immunocytochemistry of Smad2/3 and IRF4 in Th2 or Th9 cells: green, Smad2/3; red, IRF4; and blue, nuclei (DAPI staining). Original magnification $\times 1000$. (**D**) Proximity-ligation assay of IRF4 and Smad2/3 in Th2 or Th9 cells: white, proximity ligation-positive (PL⁺) signals; green, CD4; and blue, nuclei. Punctate staining (white) indicates a Smad2/3–IRF4 interaction as detected by the assay. Original magnification $\times 1000$. Quantification of the white dots is shown in the right panel. ** $p < 0.01$.

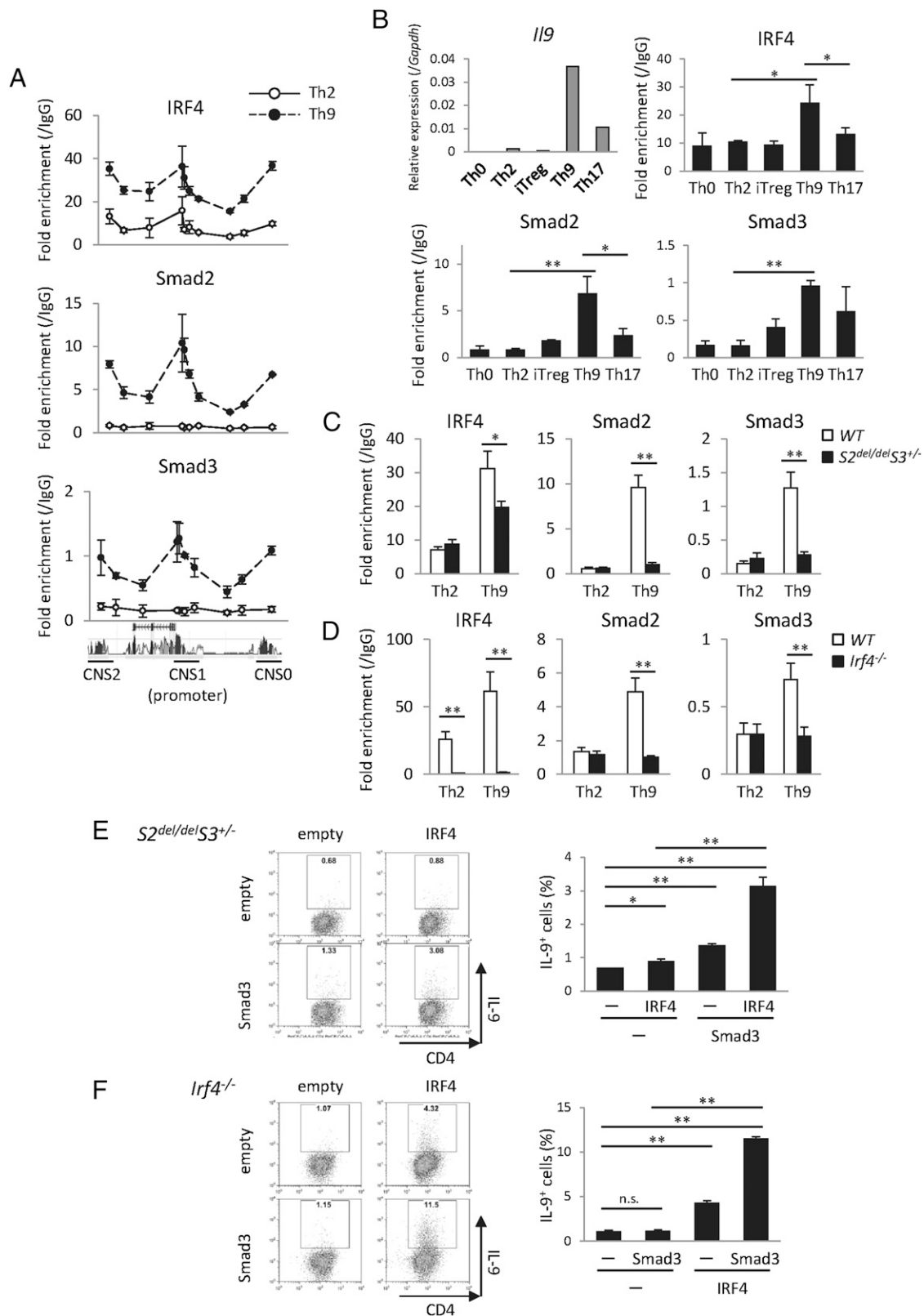


FIGURE 7. Smad2/3-mediated IL-9 production is dependent on IRF4. (**A** and **B**) Naive T cells were stimulated under the indicated Th conditions for 3 d, and then a ChIP assay was performed using anti-Smad2-, anti-Smad3-, and anti-IRF4-specific Abs. The amount of *Il9* promoter DNA precipitated with Abs was measured using quantitative RT-PCR. $n = 3$. The expression levels of *Il9* in each Th condition were quantified by quantitative RT-PCR (**B**, top left panel). ChIP assay using *S2^{del/del}S3^{+/-}* T cells (**C**) or *Irf4^{-/-}* T cells (**D**). Naive T cells from indicated mice were cultured under the indicated Th conditions for 3 d, and then a ChIP assay was performed using anti-Smad2-, anti-Smad3-, and anti-IRF4-specific Abs. $n = 3$. Primary T cells from *LckcreS2^{del/del}S3^{+/-}* (**E**) or *Irf4^{-/-}* (**F**) mice were infected with a retrovirus carrying Thy1.1-marked Smad3 and EGFP-marked IRF4. Infected T cells were cultured under Th2 conditions for 2 d and then assessed for IL-9 production using intracellular staining. Representative data of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$.

We confirmed that Smad2/3 and IRF4 were colocalized in the nucleus of Th9 cells (Fig. 6C). However, it was difficult to conclude colocalization by conventional fluorescence microscopy, because a substantial amount of Smad2/3 was already localized in the nucleus without exogenous TGF- β (Fig. 6C, Th2). To demonstrate Smad-IRF4 interaction in primary T cells, we used a proximity-ligation assay that can detect two proteins within 40 nm (23), because we barely obtained a sufficient amount of Th9 cells for immunoprecipitation. This assay confirmed a close association between IRF4 and Smad2/3 in Th9 cells compared with Th2 cells (Fig. 6D).

Functional interaction between Smad2/3 and IRF4 for IL-9 production

We investigated the functional interaction between IRF4 and Smad2/3 in the *Il9* promoter. There are three CNSs in the *Il9* regulating region: CNS0, CNS1 (which functions as a promoter), and CNS2 (Fig. 7A, bottom panel). Both IRF4 and Smad2/3 bound to the same *Il9* CNS regions in CNS1, CNS0, and CNS2 (Fig. 7A). IRF4 was bound to the *Il9* promoter region even in Th0 and Th2 cells, and IRF4 binding was enhanced by Th9 conditions (Fig. 7A, 7B). Conversely, Smad2 and Smad3 recruitment only occurs in the presence of TGF- β . Therefore, these data suggest that Smad2/3 and IRF4 interaction enhanced recruitment of these two factors into the *Il9* CNS regions.

IL-9 is also produced by Th17 cells (25, 26). We examined the role of Smad2/3 and IRF4 under Th17 conditions. First, we examined the expression of IL-9 in Th17 conditions. IL-9 was apparently expressed under Th17 conditions, although *Il9* levels were much lower than those under Th9 conditions (Fig. 7B). Then we examined recruitment of Smad2, Smad3, and IRF4 to the *Il9* promoter by ChIP assay. We observed a significant recruitment of Smad2/3 and IRF4 to the *Il9* promoter under Th17 conditions (Fig. 7B). However, recruitment of Smad2 and IRF4 under Th17 conditions was significantly lower than that under Th9 conditions. Smad3 recruitment was also lower under Th17 than under Th9, although it was not statistically significant due to low levels of expression of Smad3. Thus, IL-9 levels were well correlated with the recruitment levels of Smad2/3 and IRF4 to the *Il9* promoter, which supports cooperation of Smad2/3 and IRF4 for IL-9 induction.

To confirm this hypothesis, we used *S2^{del/del}S3^{+/-}* and *IRF4^{-/-}* T cells. As shown in Fig. 7C and 7D, IRF4 binding to the *Il9* promoter region was reduced in *S2^{del/del}S3^{+/-}* Th9 cells under Th9 conditions, but not under Th2 conditions (Fig. 7C). Smad2 and Smad3 recruitments to the *Il9* promoter CNS1 region were completely abolished in *IRF4^{-/-}* Th9 cells (Fig. 7D). These data indicate that binding of IRF4 to the *Il9* promoter region was enhanced by the presence of Smad2/3, whereas Smad2/3 binding to the promoter was completely dependent on IRF4.

In addition, we confirmed the functional cooperation between IRF4 and Smad for IL-9 production. By using retroviral infection, we transduced IRF4 and Smad3 in *S2^{del/del}S3^{+/-}* and *IRF4^{-/-}* Th2 cells (Fig. 7E, 7F). In the Smad2/3-deficient condition, forced expression of IRF4 only slightly increased IL-9 production (Fig. 7E, second bar from left). However, IL-9 was highly expressed with Smad3 coexpression (Fig. 7E, rightmost bar). Similarly, Smad3 overexpression did not enhance IL-9 expression in *IRF4^{-/-}* T cells (Fig. 7F, second bar from left), whereas it was enhanced in *IRF4^{+/+}* T cells (see Fig. 2D). Again, coexpression of IRF4 and Smad3 synergistically enhanced IL-9 expression even under Th2 conditions (Fig. 7F, rightmost bar). Taken together with the ChIP assay results, these data indicate that both Smad2/3 recruitment to the *Il9* promoter region and its activation are completely dependent on

IRF4. Conversely, IRF4 alone can bind the *Il9* promoter without Smad2/3, but not activate *Il9* transcription. Interaction with Smad2/3 is required for tight binding of IRF4 to the *Il9* promoter and its activation (Fig. 8).

Discussion

In this study, we demonstrated that Smad2 and Smad3 are redundantly essential for Th9 development and that they induce IL-9 expression in collaboration with IRF4. IRF4 can bind to the *Il9* promoter without TGF- β signals, but it cannot induce *Il9* transcription. After stimulation with TGF- β , Smad2/3 was recruited to the *Il9* promoter by interacting with IRF4, and this complex promoted active chromatin modification to initiate *Il9* transcription. This model may explain why IRF4 alone cannot activate the *Il9* promoter even though IRF4 is essential for Th9 development. PU.1 and GATA3 play essential roles in *Il9* promoter activation (8, 12). In addition, the Notch1 intracellular domain together with RBP-Jk have been shown to be necessary for IL-9 expression (13). However, we found that IRF4 and Smad2/3, but not PU.1 and GATA3, directly interacted and cooperatively induced IL-9 expression. Therefore, we propose that Smad2/3 and IRF4 are essential partners for stimulating IL-9 induction (Fig. 8).

IRF4 is a member of the IFN regulatory transcription factor family, for which expression is primarily restricted to lymphoid and myeloid cells. In CD4⁺ T cells, IRF4 expression is induced by TCR stimulation, and it is essential for Th2 and Th17, as well as Th9, development (27). Shindo et al. (28) demonstrated a specific association of IRF4 with c-Rel, which is involved in IL-2 and IL-4 promoter activation. IRF4 activates the Ig L chain genes by binding to a specific DNA sequence in the 3' enhancer regions, in cooperation with the external transcribed spacer family transcription factor PU.1 (29). IRF4 has also been shown to be important for the induction of IL-17. IRF4 binds to the *Il17a* transcriptional regulatory region, even under Th0 conditions, and IRF4-B cell-activating transcription factor complexes have been shown to precede the access of Th17-related transcription factors, such as ROR γ t (30, 31). Similarly, in this study, we demonstrated that IRF4-Smad2/3 interaction in the *Il9* CNS regions is important to start *Il9* transcription. Thus, IRF4 plays a critical role in the production of various cytokines by cooperating with different interaction partners.

We demonstrated that IRF4 and Smad3 overexpression was sufficient to induce IL-9 under Th2 conditions. However, these two factors did not induce IL-9 under Th0 conditions (without IL-4 and TGF- β), indicating that additional factors are required for full

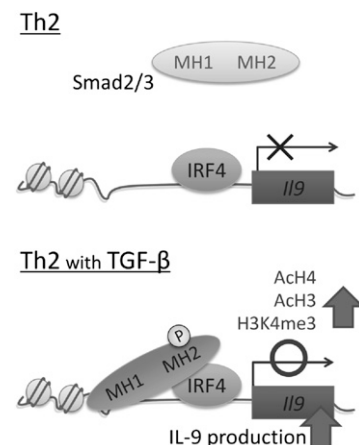


FIGURE 8. Schematic representation of the functional role of Smad2/3 and IRF4 for *Il9* promoter activation.

Th9 development. IL-4 activates STAT6 and induces GATA3, both of which have been shown to be important for the induction of Th9 (8, 10). STAT6 has been shown to directly bind to the *IL9* promoter (10). Thus, IL-4-mediated STAT6 activation and GATA3 expression could be additional events that are required for Th9 development. Further study is necessary to define a complete set of transcription factors for Th9 development. Unlike other Th subsets, a single master transcription factor may not be able to determine Th9.

Elyman et al. (13) discovered RBP-J κ -binding site (GTGGGA) and Smad3-binding site (GTCTG) in the *IL9* promoter. They found that RBP-J κ and Smad3 binding was detected in Th9 cells but not in the other Th cell types. These consensus sequences were located between CNS1 and CNS0 (Fig. 7A). We observed enhanced recruitments of Smad2 and Smad3 to this region under Th9 conditions compared with under Th2 conditions. Thus, our study is not contradictory to that by Elyman et al. (13). However, our scanning search defined previously unidentified Smad2 and Smad3 recruiting region near CNS1. Furthermore, we observed that both Smad2 and Smad3 recruitments to the *IL9* promoter was strictly dependent on IRF4. Thus, Smad-binding sequences on the genomic DNA seem not to be sufficient for Smad2/3 recruitment to the *IL9* promoter. Other factors such as Notch and IRF4 are necessary for a transcriptionally active binding of Smad2 and Smad3 to their binding sites on promoter DNA.

Th9 cells have been implicated in airway hypersensitivity (7–9). In humans, Th9 cells are found in the peripheral blood of allergic patients, and this population is rare in nonallergic individuals (6, 32). However, few reports have clearly demonstrated the involvement of Th9 in allergic inflammation. Xiao et al. (14) reported that transgenic expression of OX40 in T cells resulted in deterioration of airway inflammation and enhanced Th9 induction in vivo. Mikami et al. (15) reported that CGRP promoted allergic asthma and Th9 development through the cAMP/protein kinase A pathway. Using RAMP1-deficient mice that specifically lack CGRP signaling, they also showed partial reduction of IL-9 and amelioration of asthma when these mice were immunized and challenged with OVA. These data supported the hypothesis that Th9 is actually involved in airway inflammation. We also showed that Smad2/3-deficient mice had a lower IL-9 level compared with control mice in a model of asthma. These in vivo and in vitro studies strongly support the involvement of Th9 cells in an allergic asthma; however, further study is necessary to clearly demonstrate a direct link between Th9 and allergic airway inflammation. A recent study identified an innate lymphoid cell (innate lymphoid cell 9) that produces IL-9 predominantly in acute airway responses (33). However, it is still unknown whether the TGF- β –Smad2/3 pathway is involved in innate lymphoid cell 9 development as well as Th9 development.

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Disclosures

The authors have no financial conflicts of interest.

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