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Smad2 and Smad4 regulate TGF-β-mediated *Il9* gene expression *via* EZH2 displacement*

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Abstract

IL-9 is a pro-allergic cytokine produced by a newly proposed T helper cell subset T_H9. T_H9 cells can be generated by treatment of naïve T cells with TGF-β and IL-4 *in vitro*. But how TGF-β signaling regulates T_H9 differentiation is still not clear. Here we demonstrate that Smad2 and Smad4, two transcriptional factors activated by TGF-β signaling, are required for T_H9 differentiation *in vitro*. Deficiency of *Smad2* or *Smad4* in T cells resulted in impaired IL-9 expression, which was coincident with enrichment of repressive chromatin modification H3K27Me3 and enhanced EZH2 binding to the *Il9* locus. Pharmacologic inhibition of EZH2 partially rescued IL-9 production in Smad deficient T_H9 cells. Smad proteins may displace EZH2 directly from *Il9* locus since Smad2 and Smad4 can bind EZH2. Our data shed light on the molecular mechanisms underlying T_H9 cell differentiation, revealing that TGF-β-Smad2/4 signaling pathway regulates IL-9 production through an epigenetic mechanism.

Introduction

IL-9 is a pleiotropic cytokine that plays an important role in asthma induction, parasite expulsion, immune tolerance and anti-tumor response depending on cell types and environmental context (1,2). In addition to mast cells, CD4 helper T cells are major IL-9 producers (1). Even within CD4 T cells, multiple lineages have been reported to express IL-9. IL-9 was first discovered in T_H2 cells. Recently it was documented that T_H17 and Treg cells can secret this cytokine as well (3,4). However, accumulating evidence suggest that there is a specialized subset of T cells that is dedicated to IL-9 production. This T cell type is called T_H9 cells (5,6).

T_H9 cells can be generated *in vitro* from naïve CD4 T cells by TGF-β plus IL-4 treatment (7). These cells are related to T_H2 cells because they require IL-4-Stat-6 signaling and GATA-3 for their differentiation. But they have lower expression of T_H2 cytokines (5). Several transcriptional factors such as Stat5, Stat6, PU.1 and IRF4 have been identified that may directly regulate IL-9 transcription during T_H9 cell differentiation (8,9, 21). The molecular links between cytokine receptor and *Il9* transcription during T_H9 cell differentiation are still missing. It is clear that IL-4 signaling regulates *Il9* transcription

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either by positive regulation *via* the induction of IRF4 (10) or by negative regulation through the induction of SOCS protein CIS, which downregulates binding of Stat5 and Stat6 to the *Il9* promoter (21). However, how TGF-β signaling contributes to T_H9 differentiation has not been thoroughly assessed so far.

TGF-β, by binding to its receptor, induces the phosphorylation of Smad2 and Smad3. Through association with common partner Smad4, phosphorylated Smad2 or Smad3 translocate into the nucleus where they drive the expression of downstream genes (11). In addition, TGF-β triggers Smad-independent cascade (12). Therefore, whether Smad proteins mediate TGF-β signaling during T_H9 cell differentiation is still an open question.

In the present study, we have determined the role of both Smad2 and Smad4 during T_H9 differentiation and found that both of them are required for IL-9 production. We observed that deletion of *Smad2* and *Smad4* impaired IL-9 expression, leading to sustained association of repressive H3K27Me3-modification, which was associated with sustained binding of EZH2, a H3K27-specific methylase, to the *Il9* locus. Pharmacological inhibition of EZH2 led to partially rescued IL-9 production in *Smad2* and *Smad4* deficient T_H9 cells. Both Smad2 and Smad4 were observed be able to bind EZH2 directly. Our data revealed that TGF-β-Smad signaling regulates IL-9 expression by displacement of inhibitory histone modification enzyme EZH2 from the *Il9* locus during T_H9 differentiation.

Material and Methods

Mice

Smad2$^{fl/fl}$*CD4-Cre* and *Smad4*$^{fl/fl}$*CD4-Cre* mice were described previously (13,14). All animal experiments were performed following protocols approved by Institutional Animal Care and Use Committee.

T cell differentiation

T cell *in vitro* differentiation was conducted as previously described (13,14) except following conditions were used for T_H2 and T_H9 cells. FACS-sorted naïve cells (250K) were stimulated in 48 well plates with plate-bound anti-CD3 (1ug/ml;2C11) plus soluble anti-CD28 (1ug/ml;37.51) in the following cytokines or neutralizing antibodies: 4ng/ml TGF-β, 20ng/ml IL-4, 10ug/ml anti-IFN-γ (XMG 1.2) and 30U/ml hIL-2 for T_H9; 40ng/ml IL-4, 10ug/ml anti-IFN-γ, 10ug/ml anti-TGB-β (1D11) and 30U/ml hIL-2 for T_H2. 2μM of GSK126 (XcessBio) was added in the culture from the start in some experiments. After 4 day stimulation, cells were harvested for chromatin immunoprecipitation (ChIP) and Western Blot analysis or washed and re-stimulated with plate-bound anti-CD3 (1.0ug/ml) for RNA extraction (4hr) or for ELISA (24hr). Cytokine staining was performed as previously described (13,14).

ChIP Assay

Il9 locus definition followed previous study (8). Genomic DNA was extracted from 2~4 millions of cells by using a commercial kit (Upstate), followed by real-time PCR quantification for *Il9* promoter (F-ctcaattggcctcaacttacag, R-ccctttgccatcctccagcag), *Il9* CNS (F-aattacagaattttgccccaggtcctg, R-gttaatgcacaattcatgtgccaatcc) and *Il4* promoter (F-ctcattttcccttggtttcagc, R-gatttttgtcgcatccgtgg). ChIP-grade antibodies against H3AC, H3K27Me3 and H3K4Me3 were purchased from Millipore. ChIP-grade EZH2 antibody was obtained from active Motif (#39875).

Statistics analysis

Data are presented as mean value ± s.d. Data were analyzed by using Student's *t* test. A value of $p<0.05$ was considered significant.

Results and Discussion

Smad2 is required for T_H9 differentiation

To study how TGF-β signaling pathway regulates T_H9 differentiation, *Smad2*$^{fl/fl}$*CD4-Cre* (*Smad2* KO) *and Smad4*$^{fl/fl}$*CD4-Cre* (*Smad4* KO) conditional knockout mice were used to delete *Smad2* and *Smad4* respectively in T cells (13,14). Age and sex-matched *Smad2*$^{fl/fl}$ and *Smad4*$^{fl/fl}$ littermate animals were used as wild-type (WT) controls throughout this study.

Purified naïve cells from *Smad2* KO and WT animals were differentiated under T_H9 and T_H2 conditions for4 days. Cytokine production was measured by intracellular staining. We found that T_H2 cells produced negligible amount of IL-9 but high levels of IL-4. Both IL-4 and IL-9 production, however, were not affected by *Smad2* deletion in T_H2 cells (Figure 1A). T_H9 cells produced higher levels of IL-9 and lower levels of IL-4 compared to T_H2 cells. *Smad2* deletion significantly reduced IL-9 production but enhanced IL-4 production in T_H9 cells (Figure 1A). ELISA analysis of IL-9 and T_H2 cytokines from above cells showed the same trend (Supplemental Figure S1A). Consistent with the results at the protein level, mRNA of *Il9* was higher in T_H9 cells than in T_H2 cells and was reduced significantly in T_H9 cells by *Smad2* deletion. *Il4/5/13* mRNA were enhanced in T_H9 cells but were not affected in TH2 cells by *Smad2* deletion (Figure 1B). These data suggest that Smad2 is required for IL-9 expression and suppression of TH2 cytokine production in T_H9 cells while Smad2 is dispensable for IL-4/5/13 production in T_H2 cells.

TGF-β binding to its receptor triggers the activation of both Smad2 and Smad3 (12). Previous studies have shown that Smad2 and Smad3 have redundant roles in T cells (15). In the present study, although *Smad2* deletion resulted in dramatic reduction of IL-9 production (Figure 1), it did not completely abrogate IL-9 production. It is likely that Smad2 and Smad3 play redundant roles during T_H9- differentiation.

To elucidate the mechanism underlying impaired IL-9 in *Smad2*- deficient T_H9 cells, we sought to examine the expression of transcription factors regulating IL-9 and IL-4 transcription in T cells. Recently it was reported that PU.1 (*Sfpi1*) and IRF4 were required for T_H9 differentiation (8,9). However, we could not detect any difference of PU.1 or IRF4 expression between *Smad2*-sufficient and -deficient cells by real-time RT-PCR (Figure1B).

To understand why IL-4/5/13 expression was enhanced in the absence of *Smad2* in T_H9 cells, we assessed GATA3, JunB and c-Maf expression in these cells. Expression of these T_H2 lineage-associated transcription factors was not up-regulated in the absence of *Smad2* (Figure1B). Although Foxp3 expression was significantly reduced upon *Smad2* deletion (Figure1B), it is not required for T_H9 differentiation. This is because retroviral overexpression of Foxp3 failed to rescue IL-9 production in *Smad2* deficient T_H9 cells (data not shown).

Smad4 is required for T_H9 differentiation

Similarly, naïve T cells from *Smad4* KO and WT mice were polarized under T_H2 and T_H9 conditions. WT T_H2 cells produced marginal levels of IL-9. *Smad4* deficiency significantly enhanced IL-9 production whereas IL-4 production was slightly reduced in T_H2 cells (Figure 2A). Identical to *Smad2*-deficient T_H9 cells, IL-9 production was significantly reduced in *Smad4*-deficient T_H9 cells compared to WT cells. Moreover, IL-4 production in

T_H9 cells was enhanced in the absence of *Smad4* (Figure 2A). ELISA analysis of IL-9 and T_H2 cytokines from above cells further confirmed our findings (Supplemental Figure S1B). Measuring cytokinem RNA revealed that *Smad4* deletion led to a slight increase of IL-9 production in T_H2 cells. However, *Smad4* deficiency significantly attenuated IL-9 production but enhanced T_H2 cytokines in T_H9 cells (Figure 2B). These data indicates that both Smad2 and Smad4 are required for IL-9 expression and suppression of T_H2 cytokine production in T_H9 cells.

Since Smad4 is co-factor of Smad2 and Smad3 for their binding and subsequent nuclear translocation, *Smad4* deletion will impair both Smad2- and Smad3–mediated signaling. However, despite pronounced reduction of IL-9 in *Smad4*-deficient T_H9 cells, these cells still have residual IL-9 production (Figure 2A). Therefore, Smad-independent TGF-β signaling probably is also involved in IL-9 production in T cells.

Similar to *Smad2*-deficient T_H9 cells, the gene expression of potential T_H9 lineage transcriptional factors (IRF4 and PU.1 (*Sfpi1*)) and T_H2 lineage transcriptional factors (GATA3, c-Maf, JunB) were not affected in T_H9 cells in the absence of *Smad4* (Figure 2B). This suggests that TGF-β-Smad signaling regulates IL-9 expression not through these factors.

Smad2 and Smad4 regulates histone modification at the Il9 locus

Epigenetic regulation particularly through chromatin modification is an important mechanism involved in gene regulation and T cell differentiation (16,17). We speculate that TGF-β-Smad signaling pathway may directly regulate IL-9 production *via* epigenetic regulation. To address this question, naïve T cells from *Smad2* KO and WT mice were polarized under T_H9 conditions with T_H2 and T_H0 cells included as controls. We used ChIP assay to examine both permissive chromatin modification, including total H3 acetylation (H3AC) and H3K4 trimethylation (H3K4Me3), and repressive chromatin modification such as H3K27 trimethylation (H3K27Me3). Both H3AC and H3K4Me3 at either promoter region or conserved non-coding sequence (CNS) region of the *Il9* locus were higher in T_H9 cells than that in T_H0 and T_H2 cells whereas H3K27Me3association were significantly reduced accompanying T_H9 differentiation (Figure 3). However, *Il4* promoter in T_H9 cells was enriched with both permissive and repressive histone modification (Figure 3), a feature shared by bivalent domain possessing low levels of transcription (17). Therefore, chromatin modifications at the *Il9* and *Il4* loci are compatible with robust IL-9 and modest IL-4 production in T_H9 cells.

Smad2 deletion increased H3AC modification (Figure 3A top row) while having no effect on H3K4Me3 modification at the *Il9* locus in T_H9 cells (Figure 3A, middle row). Considering impaired production of IL-9 in *Smad2*-deficienct T_H9 cells, permissive histone modification at *Il9* locus is not likely the causative mechanism that is targeted by Smad2 during T_H9 differentiation. Interestingly, H3K27Me3 was significantly enhanced in all tested regions at the *Il9* locus in *Smad2*-deficient cells compared to WT cells (Figure 3A bottom row). This suggests that Smad2 was involved in demethylation of histone 3 at lysine 27 at the *Il9* locus in T cells. Next we sought to determine whether Smad4 also utilizes the same mechanism to regulate IL-9 expression. Ablation of *Smad4* had no effect on both H3Ac and H3K4Me3 modifications at the *Il9* locus (Figure 3B top and middle rows) whereas it strongly enhanced H3K27Me3 at the same locus (Figure 3B bottom row). These data demonstrated that both Smad2 and Smad4 are required for histone H3K27 demethylation at the *Il9* locus during T_H9 differentiation.

Smad2 and Smad4 are required to displace EZH2 from the Il9 locus during T_H9 differentiation

H3K27me3 demethylation is associated with gene activation. Maintaining H3K27me3 is enforced by polycomb repressor complex 2 (PRC2) and represents an important mechanism for gene silencing. EZH2, a component of PRC2 complex, is responsible for catalyzing and maintaining H3K27me3 modification (18). Since deficiency of either *Smad2* or *Smad4* led to enrichment of H3K27me3 histone modification at the *Il9* locus during T_H9 differentiation, we hypothesized that enrichment of H3K27me3 modification in *Smad2/4*-deficient T cells is the result of impaired de-association of EZH2 from the *Il9* locus during T_H9 differentiation. ChIP analysis of EZH2 binding to the *Il9* locus was conducted to assess this. EZH2 constitutively bound to all tested sites of the *Il9* locus in T_H0 cells (Figure 4A, 4B). This was consistent with no IL-9 production in those cells (data not shown). In contrast, EZH2 binding to the *Il9* locus was significantly reduced in WT T_H9 cells (Figure 4A, 4B, top and middle rows, open bars). Ablation of either *Smad2* or *Smad4*, however, led to defective de-association of EZH2 from the *Il9* locus (Figure 4A, 4B, top and middle rows, filled bars), which was not due to altered EZH2 expression in these cells (Supplemental Figure S2). This implicated that both Smad2 and Smad4 were required for displacement of EZH2 from the *Il9* locus during T_H9 differentiation. To corroborate that EZH2 is downstream of Smad proteins and mediates TGF-β regulated IL-9 production, WT and *Smad2*/4 deficient cells were polarized under T_H9 condition with GSK126, a specific EZH2 inhibitor (19). GSK126 significantly reduced global H3K27Me3 modification (Supplemental Figure S2). Strikingly, IL-9 production in both Smad2 and Smad4 deficient T_H9 cells were partially restored by GSK126 treatment to levels close to that in WT cells (Figure 4C). We also detected that both Smad2 and Smad4 directly bound to EZH2 in 293T cells (Figure 4D). These data strongly suggested that Smad2 or Smad4 were involved in displacement of EZH2 from the *Il9* locus via direct interaction during T_H9 differentiation. Complementary to two recent studies in which Smad prote in were shown to form complex with either Notch or IRF4 to bind *Il9* promoter and drive transcription of IL-9 (20, 22), our data support the concept that TGF-β signaling regulates IL-9 production by multiple mechanism.

We also examined EZH2 binding to the *Il4* promoter in T_H9 cells. EZH2 was found persistently bound to the *Il4* promoter in these cells during T_H9 differentiation, which was in contrast to reduced levels of EZH2 binding to this locus in T_H2 differentiation (data not shown). *Smad2* deletion had no effect on EZH2 binding to *Il4* promoter in T_H9 cells while ablation of *Smad4* led to a slight enhancement of EZH2 binding to *Il4* promoter (Figure 4A and 4B, bottom row). Since both *Smad2* and *Smad4* deficiency increased IL-4 production in T_H9 cells (Figure 1 and 2), H3K27me3 modification and EZH2 binding at the *Il4* promoter region may not be directly involved in IL-4 expression in these T cells. Mechanism underlying reciprocal up-regulation of T_H2 cytokine in *Smad2* and *Smad4*-deficient T_H9 cells is not clear so far. It is possible that attenuated expression of Foxp3 de-represses IL4/5/13 expression during T_H9 differentiation in the absence of *Smad2* and *Smad4*.

In summary, we provided genetic evidence that Smad2 and Smad4 are required for T_H9 differentiation. We demonstrated that Smad2 and Smad4 were not involved in the regulation of PU.1 and IRF4 expression during T_H9 differentiation. We found, instead, that TGF-β signaling regulated IL-9 production through displacement of EZH2 and removal of suppressive H3K27 histone modification at the *Il9* locus. This will not only provide further insight into our understanding molecular mechanism underlying T_H9 differentiation, but also help us to design therapeutic strategies to manipulate T_H9 cells *in vivo* in future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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The abbreviations used in this paper

IL-9	interleukin-9
H3AC	Histone 3 acetylation
H3K4Me3	Histone 3 K4 trimethylation
H3K27Me3	Hisotne 3 K27 trimethylation
ChIP	chromatin immunoprecipitation

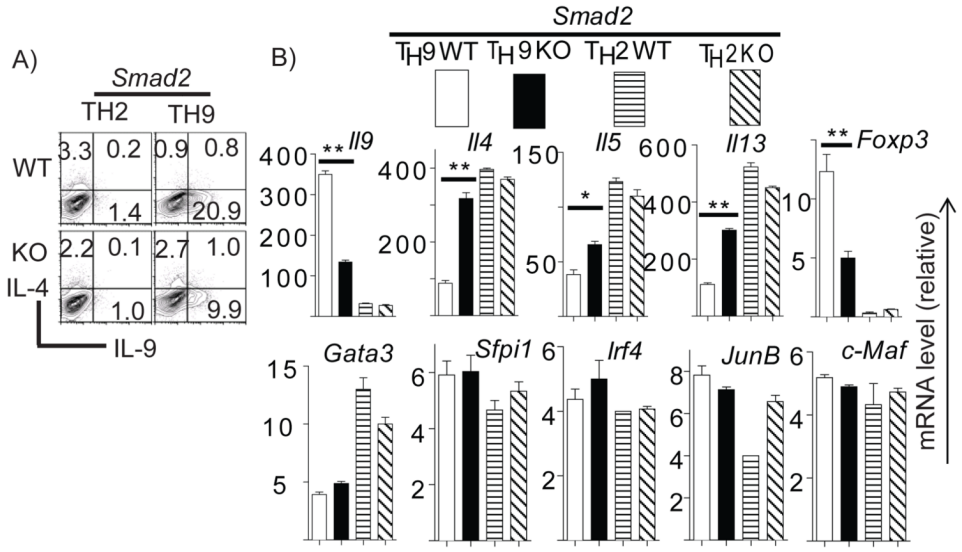

Figure 1. Smad2 is required for T_H9 differentiation

FACS-sorted naïve cells from *Smad2*$^{fl/fl}$*CD4Cre*$^{+}$ (*Smad2* KO) or *Smad2*$^{fl/fl}$*CD4Cre*$^{-}$(*Smad2* WT) mice were polarized under T_H2 and T_H9 conditions for 4 days. A, Cells were then re-stimulated with PMA/Ionomycin in the presence of Golgi Stop for 6hr. IL-4 and IL-9 production was assessed by intracellular staining. B, Cells were re-stimulated with plate-bound anti-CD3 for 4hr prior to Trizol treatment. cDNA was prepared and gene expression was analyzed by real-time RT-PCR. Data were normalized to a reference gene *Actb*. The expression of each gene in T_H0 cells (not shown) was referred as 1. A representative of 4 independent experiments is shown.

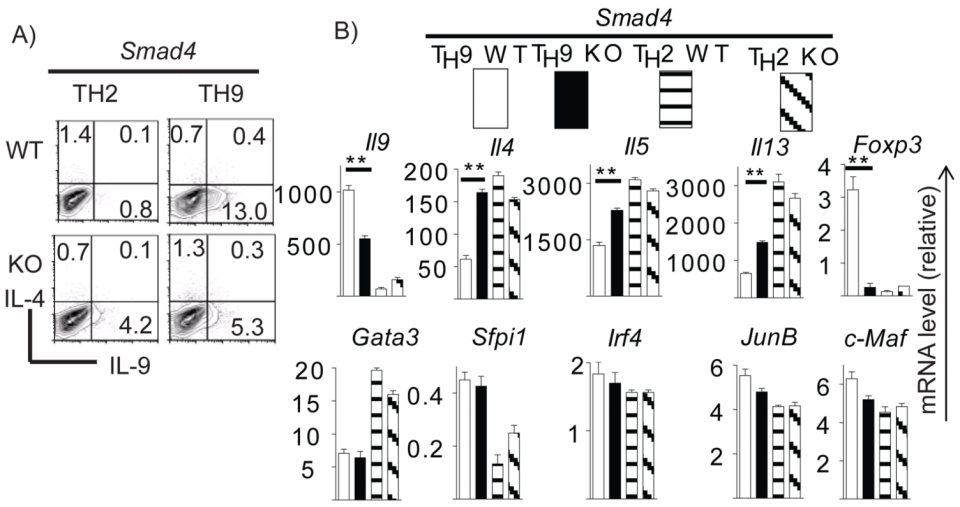

Figure 2. Smad4 is necessary for T_H9 differentiation

FACS-sorted naïve cells from *Smad4*$^{fl/fl}$ CD4Cre^{+}(*Smad4* KO) or *Smad4*$^{fl/fl}$ CD4Cre^{-}(*Smad4* WT) mice were differentiated under T_H2 and T_H9 conditions for 4 days. Intracellular staining of IL-4 and IL-9 (A) and real-time RT-PCR analysis of gene expression (B) was performed as Figure 1.

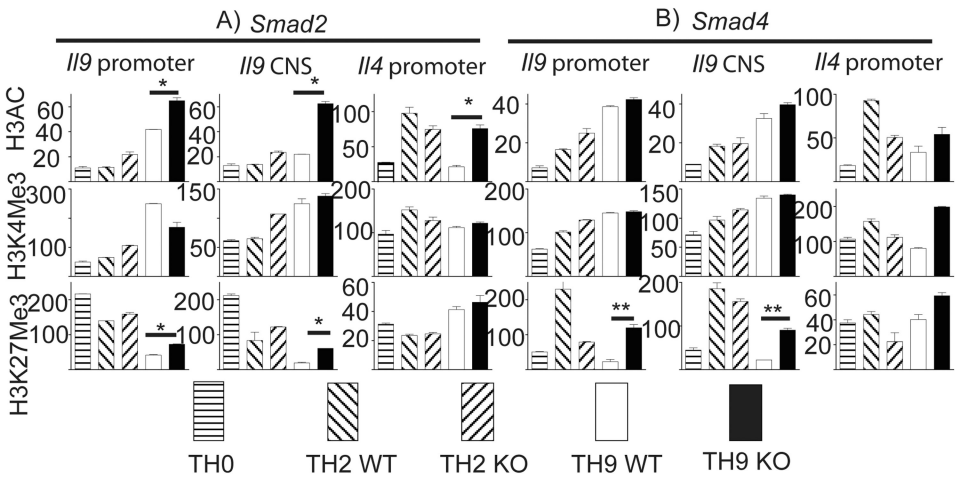

Figure 3. Effects of Smad2 and Smad4 deficiency on histone modification at the *Il9* locus in T_H9 cells

Naïve T cells from *Smad2* (A) or *Smad4* (B) mice as in Figure 1 and Figure 2 were differentiated under T_H2 and T_H9 conditions for 4 days and harvested for ChIP analysis of histone modification at the *Il9* locus. WT T_H0 cells served as a control for T_H9 cells. H3AC, acetylation of histone H3. H3K4Me3, trimethylation of histone H3; H3K27Me3, trimethylation of Histone H3 K27. Control IgG was used as negative control for anti-H3AC, anti-H3K4Me3 and anti-H3K27Me3 (not shown). Total input DNA before IP was used for normalization of data. Histone modification was determined by a quantitative PCR. 3 independent experiments were performed with similar results. * $P<0.05$, ** $P<0.001$

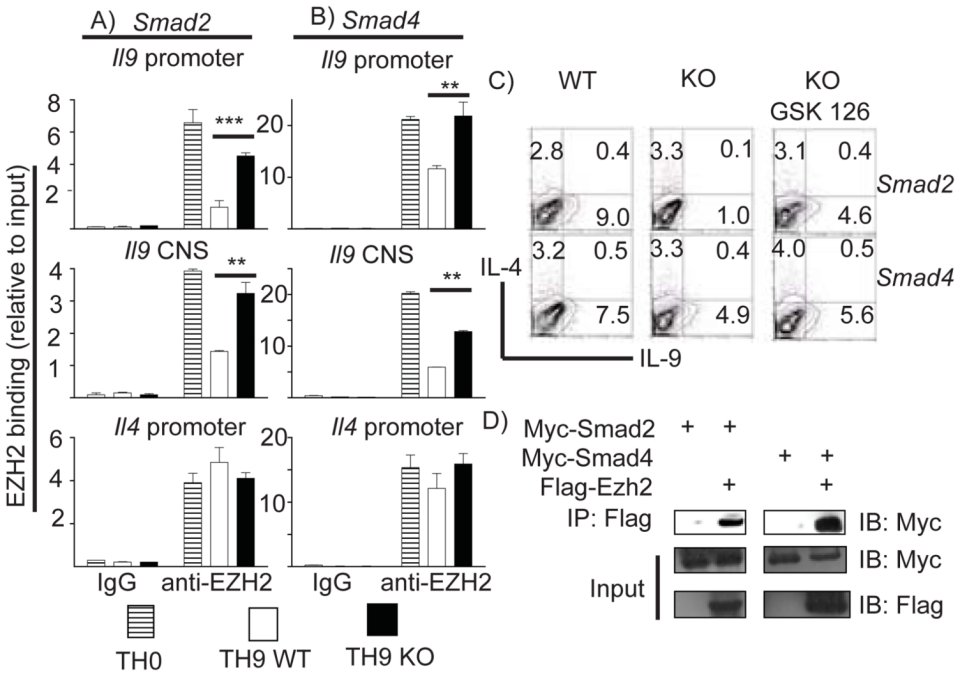

Figure 4. Deficiency of Smad2 and Smad4 results in sustained EZH2 binding to the *Il9* locus in T_H9 cells

A and B, Naïve T cells from *Smad2* (A) or *Smad4* (B) mice were differentiated under T_H9 condition for 4 days and harvested for ChIP analysis of EZH2 binding at the *Il9* locus. WT T_H0 cells served as a positive control for EZH2 binding at the *Il9* locus in T_H9 cells. Control IgG was used as non-specific binding to the *Il9* and the *Il4* locus. EZH2 binding was determined by a quantitative PCR. This is a representative of two experiments. (* $P<0.05$, ** $P<0.001$). C, Naïve T cells from indicated mice were differentiated under T_H9 condition with or without addition of 2uM of GSK126 for 4 days. D, Smad2, Smad4 and EZH2 encoding plasmids were transfected into 293T cells. 24hr later, cells were harvested, lysed, and immunoreprecipted with anti-Flag antibody followed by detection with anti-Myc antibody.