HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2019 May 15.

Published in final edited form as:

J Immunol. 2018 May 15; 200(10): 3397–3406. doi:10.4049/jimmunol.1800193.

TCF-1 inhibits IL-17 gene expression to restrain Th17 immunity in a stage specific manner

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Abstract

T cell factor 1 (TCF-1) is expressed in both developing and mature T cells, and has been shown to restrain mature T cell-mediated Th17 responses by inhibiting IL-17 expression. However, it is not clear when is TCF-1 required in vivo to restrain the magnitude of peripheral Th17 responses and what are the molecular mechanisms responsible for TCF-1-regulated IL-17 gene expression. Here we showed that conditional deletion of TCF-1 at early but not later at $CD4^+CD8^+$ double positive (DP) stage in mice, enhanced Th17 differentiation and aggravated experimental autoimmune encephalomyelitis (EAE), which correlates with abnormally high IL-17 expression. Expression of TCF-1 in *TCF-1* deficient thymocytes, but not *TCF-1* deficient Th17 cells, inhibited IL-17 expression. TCF-1 binds to IL-17 promoter regions, and deletion of two TCF-1-binding sites relief TCF-1-mediated inhibition of IL-17 promoter activity. Lastly, WT TCF-1 but not a TCF-1 mutant that has no intrinsic histone deacetylase activity (HDAC) was able to inhibit IL-17 expression in *TCF-1* deficient mouse thymocytes. Thus, our study first demonstrates requirement of TCF-1 *in vivo* at stages earlier than DP cells to restrain peripheral Th17 immunity by directly binding and inhibiting IL-17 promoter in its intrinsic HDAC-dependent manner.

Introduction

T cell development in thymus is a process to arm T cells with the capacity to mediate appropriate immune responses in peripheral tissues. Lymphoid progenitors which developed from hematopoietic stem cells in the bone marrow migrate into thymus to complete sequential maturation stages, including $CD4^-CD8^-$ double negative (DN), $CD4^+CD8^+$ double positive (DP), and $CD4^+$ or $CD8^+$ single positive (SP) stages (1, 2). Mature single positive T cells then migrate to the peripheral lymphoid organs to participate adaptive immune responses against pathogens.

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Disclosures: The authors have no financial conflicts of interest

When the single $CD4^+$ positive T cells just migrate out of thymus they are naïve and are not competent to mediate immune responses. To become effector T cells, they must undergo an activation and differentiation process. This process is initiated upon encountering antigens and eventually differentiates naïve T cells into T helpers that include Th1, Th2, Th17 and regulatory T (Treg) cells. Th17 cells secrete IL-17 and participate in protective immunity against pathogens (3, 4). Whereas inappropriately exaggerated Th17 responses contribute to pathological immune responses involved in the autoimmunity such as psoriasis and multiple sclerosis (5-9).

Besides shaping T cell repertoire that reacts to foreign but not self-antigens, thymocyte developmental process also controls the magnitude of T cell responses in the periphery. For example, T cell factor 1 (TCF-1), a transcription factor enriched in hematopoietic cell compartments, regulates T cell development in thymus (10-12). Our previous studies have shown that germline deletion of TCF-1 resulted in increased IL-17 expression both in thymus and peripheral T cells and hence led to enhanced Th17 differentiation and more severe EAE (13, 14), indicating the negative role of TCF-1 in the regulation of Th17 immunity. We have some *in vitro* evidence that TCF-1 loses the ability to regulate IL-17 gene expression in mature T cells. However, because it was a germline deletion, it is not clear when TCF-1 is required *in vivo* to limit IL-17 gene expression and thus control the scale of Th17 responses in the periphery. By using conditional deletion of TCF-1 at different developmental stages, we demonstrated that CD4-Cre-mediated deletion of TCF-1 at $CD4^+CD8^+$ DP stage did not significantly affect thymic T cell development, peripheral Th17 differentiation and EAE. Whereas, Vav1-Cre-mediated deletion of TCF-1 at earlier hematopoietic stages disrupts thymic T cell development and potentiates Th17 differentiation and development of EAE. Moreover, expression of TCF-1 in *TCF-1*$^{-/-}$ thymocytes but not *TCF-1*$^{-/-}$ Th17 cells was able to down-regulate IL-17 expression. We also found that TCF-1-mediated inhibition of IL-17 expression depends on its intrinsic histone deacetylase activity (15). We mapped the TCF-1-binding regions on IL-17 promoter, and deletion of the DNA fragments containing the TCF-1 binding sites prevented TCF-1 to inhibit IL-17 promoter. Therefore, we first demonstrated the state-specific requirement of TCF-1 *in vivo* during early development to control the scale of the peripheral Th17 immune responses via inhibiting IL-17 expression through the intrinsic HDAC activity of TCF-1.

Materials and Methods

Mice

TCF-1$^{fl/fl}$ mice were described previously (16) and obtained from Dr. Hai-Hui Xue (University of Iowa, Iowa City, IA). *Rag1*$^{-/-}$, transgenic *CD4-Cre* and *Vav1-Cre* mice were purchased from The Jackson Laboratory. *TCF-1*$^{f/fl}$*/CD4-Cre* and *TCF-1*$^{f/f}$*/Vav1-Cre* were generated by crossing *TCF-1*$^{f/f}$ to *CD4-Cre* and *Vav1-Cre*, respectively. For all experiments, mice were 6-10 week-old. All mice were bred and maintained in the specific pathogen-free conditions and experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the Beckman Research Institute of City of Hope (IACUC#07023).

T cell isolation and *In vitro* differentiation

Mouse naïve $CD4^+$ T cells were isolated from spleens of 6- to 10-week-old mice by negative selection using a $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany). T cells were cultured and differentiated in Iscove's DMEM (Corning, VA, USA) containing 10% FBS, 50 mM β-mercaptoethanol, 100 U/ml penicillin-streptomycin at 37°C with 5% CO_2. In brief, 4×10^5/ well naïve $CD4^+$ T cells were first activated with 0.25 μg/ml anti-CD3 (145-2C11; eBioscience, CA, USA) and 1μg/ml anti-CD28 (37.51; eBioscience, CA, USA) in goat-anti-hamster (G-α-H) IgG (0.2 mg/ml, MP Biomedicals, Santa Ana, CA) pre-coated 24-well plates. Cells were then differentiated in the presence of polarizing cytokine and antibody cocktails for 72 h. For Th1: 10 ng/ml recombinant murine (rm) IL-2 (Biolegend), 20 ng/ml rmIL-12 (Biolegend) and 20μg/ml anti-IL4 (11B11; Biolegend). For Th17: 2 ng/ml rmTGF-β1 (eBioscience) and 25 ng/ml rmIL-6 (eBioscience). For Treg: 5 ng/ml rmTGF-β, 10 μg/ml anti-IFN-γ (XMG1.2; Biolegend) and 10 μg/ml anti-IL-4 (11B11; Biolegend).

Flow cytometry and cell sorting

Cultured T cells were stimulated with 50 ng/ml PMA (Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) in the presence of brefeldin A for 4 h before staining. Both cultured T cells and freshly prepared thymocytes were first stained with fluorescence-conjugated antibodies (Abs) to cell surface markers followed by fixed/permeabilized either with the BD Cytofix/Cytoperm kit (BD Biosciences) or Transcription Factor Staining Buffer Set (BD Pharmingen). Cells were then intracellularly strained with fluorescence-conjugated Abs to cytokines or transcription factors. Abs include FITC-CD4 (GK1.5, Biolegend), PE-CD8 (53-6.7, Biolegend), PE-Cy7-CD8 (53-6.7, eBioscience), APC-CD8 (53-6.7, BD Biosciences), PE-Thy1.2 (30-H12, BD Biosciences), PE-TCRβ (H57-597, BD Biosciences), APC-IL-17A (eBio17B7, eBioscience), APC-IFN-γ (XMG1.2, eBioscience), APC-FOXP3 (FJK-16s, eBioscience) and PE-RORγt (Q31-378, BD Biosciences). Flow cytometry were performed by using a FACSCantoII (BD Biosciences) and analyzed with FlowJo software (Tree Star). Double-negative (DN) thymocytes ($Thy1.2^+CD4^-CD8^-$) were electronically sorted by using FACSAriaII (BD Biosciences).

Quantitative real-time PCR (qRT-PCR)

Total RNA was prepared using the RNeasy Isolation Kit according to the manufacturer's instructions (Qiagen). cDNA was synthesized using the Tetro cDNA Synthesis Kit (BIO-65043, Bioline). qRT-PCR was performed using SsoFast EvaGreen Supermic (Bio-Rad) in a CFX96 Real-Time PCR Detection System (Bio-Rad). Primers are as follows: IL17A-forward: 5′- tttaactcccttggcgcaaaa-3′, IL17A-reverse: 5′-ctttccctccgcattgacac-3′; IL17F-forward: 5′- tgctactgttgatgttgggac-3′, IL17F-reverse: 5′- aatgccctggttttggttgaa-3′; CCL20-forward: 5′-gcctctcgtacatacagacgc-3′, CCL20-reverse: 5′-ccagttctgctttggatcagc-3′; CCR6-forward: 5′- cctgggcaacattatggtggt-3′, CCR6-reverse: 5′-cagaacggtagggtgaggaca-3′.

Retroviral packaging and transduction

MSCV-IRES-EGFP based retroviral expression vectors encoding TCF-1 or empty vectors were transfected into Plat-E packaging cells by Lipofectamine 2000 (Invitrogen Life Technologies, CA, USA) mediated transfection. After 48 h, viral supernatants were collected

and stored at −80°C until use. For transduction, naïve $CD4^+$ T cells were first activated with 0.25 μg/ml anti-CD3 (145-2C11; eBioscience, CA, USA), 1μg/ml anti-CD28 (37.51; eBioscience, CA, USA) in G-α-H IgG pre-coated 24-well plate for 24 h followed by spin-infection with viral supernatants (2500 rpm, 30°C for 2 h) in the presence of 8 μg/ml polybrene (Sigma-Aldrich, MO, USA). After spin-infection, indicated cytokines described above were added to the culture media to induce Th17 differentiation.

Coculture and transduction of thymocytes on OP9-DL1 stromal cells

Thymocytes were allowed to differentiate on OP9-DL1 cells (gift from Dr. Timothy P. Bender, University of Virginia Health System, Charlottesville, VA). Briefly, 5×10^5 / well electronically sorted $Thy1.2^+CD4^-CD8^-$ thymocytes were cultured overnight on an 80% confluent OP9-DL1 monolayer in the flat-bottom 24-well plates in αMEM (STEMCELL Technologies) supplemented with 20% FBS, 100 U/ml penicillin-streptomycin, 2 mM L-glutamine (Invitrogen Life Technologies), and 5 ng/ml rmIL-7 (PeproTech). Co-cultures were then spin-infected (2500 rpm, 30°C for 2 h) with retroviral supernatants in the presence of 5 μg/ml polybrene. 72 h posttransduction, cocultures were harvested for flow cytometry.

Induction and assessment of EAE

Active EAE was induced according to the manufacturer's instructions (Hooke Laboratories, Lawrence, MA). Briefly, mice were immunized with 200 μ1 myelin oligodendrocyte glycoprotein 35–55 ($MOG_{35–55}$) peptide emulsion subcutaneouly. On days 0 and 1 after immunization, mice were injected intraperitoneally with 200 ng *Bordetella* pertussis toxin (Hooke Laboratories, Lawrence, MA). For Th17- or Th1-induced passive EAE, donor mice were immunized with $MOG_{35–55}$ subcutaneouly. 10 days later, cells were isolated from spleen and lymph node and cultured with 20ug/ml $MOG_{35–55}$ for 3 days under either Th17-polarizing conditions (20ng/ml rmIL23, R&D) or Th1-polarizing conditions (20ng/ml rmIL-12, R&D; 2μg/ml α-IL23p19, eBioscience) (17). *Rag1*$^{-/-}$ recipient mice were then transferred intraperitoneally with 3.0×10^7 $MOG_{35–55}$-specific Th17 or Th1 cells. The severity of EAE was monitored and evaluated on a scale from 0 to 5 according to Hooke Laboratories ' guideline. In brief: 0 = no disease; 1 = paralyzed tail; 2= hind limb weakness; 3= hind limb paralysis; 4 = hind and fore limb paralysis; 5= moribund and death. When a mouse was euthanized because of severe paralysis, a score of 5 was entered for that mouse for the rest of the experiment.

Isolation and analysis of CNS infiltrating cells

Brain and spinal cords were homogenized and single cell suspensions were prepared using 70-μm cell nylon cell strainers (Fisher Scientific). Cells were collected by centrifugation at 400 rcf for 10 min. Cells were then resuspended in 4 ml 30% Percoll (GE Healthcare) and centrifuged onto 5 ml 70% cushion for 20 min at 2000 rpm. Lymphocytes at 30-70% interface were collected and subjected to cell surface staining with fluorescence-conjugated Abs: FITC-CD45 (30-F11, eBioscience), APC-CD3 (145-2C11, BD Pharmingen), APC-Cy7-Ly6C (HK1.4, eBioscience), PE-Cy7-Ly6G (1A8, BioLegend), and PE-CD11b (M1/70, eBioscience). For intracellular cytokine staining, the CNS infiltrating cells were stimulated with 50 ng/ml PMA (Sigma-Aldrich) and 1μg/ml ionomycin (Sigma-Aldrich) in the presence of brefeldin A for 4 h, and then stained with PE-Cy7-CD4 followed by intracellular

staining of FITC-IFN-γ (XMG1.2, BioLegend), PE-GM-CSF (MP1-22E9, BioLegend), and APC-IL-17A (eBio17B7, eBioscience).

ChIP on qRT-PCR

ChIP on qRT-PCR was performed with the ChIP-IT High Sensitivity kit (Active Motif, 53040). Briefly, 1.5×10^7 cells per ChIP were obtained from thymus or differentiated Th17 cells. Cells were fixed and sheared as described in the ChIP-IT High Sensitivity manual. ChIP reactions were then performed on 20 μg of the prepared chromatin using 4 μg anti-TCF-1 antibody (C63D9, Cell Signaling) or antiacetyl-histone H3 (06-599, Millipore). 4 μg of IgG was used as negative control. Input DNA and DNA pull-downed after immunoprecipitation were analyzed by qRT-PCR as described above. Primers which cover ~2000 base pair of IL17A promoter region were designed as follows: P1-forward: 5′-aaaccctatgcagttggtaca-3′, P1-reverse: 5′-catctctccagctccatgga-3′; P2-forward: 5′-agcccataaagaagccaatgt-3′; P2-reverse: 5′-tcctggctttctacgtgtca-3′; P3-forward: 5′-gggtgaaagaggacattgcc-3′, P3-reverse: 5′-tgccaggtcttttcccattc-3′; P4-forward: 5′-tgacacgtagaaagccagga-3′, P4-reverse: 5′-gattgccaggtcttttccca-3′; P5-forward: 5′-ggcaatcagaggtgtgtgtg-3′, P5-reverse: 5′-ctcagaagttgcagcacctc-3′; P6-forward: 5′-ctatcggtccacctcatgct-3′, P6-reverse: 5′-ggagatgagggatgagaaggg-3′; P7-forward: 5′-gttagtagtctccacccggc-3′, P7-reverse: 5′-tgaggttcggtatcaagcct-3′; P8-forward: 5′-gagtgggtttctttgggcaa-3′, P8-reverse: 5′-agcatgacttcttgggagct-3′; P9-forward: 5′-agctcccaagaagtcatgct-3′, P9-reverse: 5′-tacgtcaagagtgggttggg-3′.

Results

Vav1-Cre but not CD4-Cre-mediated deletion of TCF-1 potentiates Th17 differentiation and EAE

Previous studies of TCF-1 function mostly depend on utilizing a strain of germline knockout mice that cannot determine tissue and time specific function of TCF-1 (10, 18). To determine the critical stages that TCF-1 is required to restrain peripheral Th17 responses, we crossed conditional *TCF-1* (*TCF-1*$^{fl/fl}$) mice (16) to *CD4-Cre* and *Vav1-Cre* mice, respectively. *CD4-Cre* induces gene deletion at $CD4^+CD8^+$ DP stage (19), whereas *Vav1-Cre* induces deletion at earlier hematopoietic stages including $CD4^-CD8^-$ DN stage (20). We performed PCR analysis of *TCF-1* locus to detect the deletion of *TCF-1* gene (Fig. 1A). As expected, there was no deletion at all in the absence of Cre as shown in *TCF-1*$^{fl/fl}$ mice. However, TCF-1 deletion was detected in $CD4^+CD8^+$ thymocytes and peripheral T cells but not in $CD4^-CD8^-$ thymocytes of the *TCF-1*$^{fl/fl}$*/CD4-Cre* mice. In contrast, *TCF-1*$^{fl/fl}$ */Vav1-Cre* mice deleted *TCF-1* in $CD4^-CD8^-$, $CD4^+CD8^+$ and peripheral T cells, confirming that *Vav1-Cre* induced TCF-1 deletion at the stage earlier than $CD4^-CD8^-$ DN cells. To determine the effects of deletion of TCF-1 at different developmental stages on peripheral Th17 responses, EAE was induced (Fig. 1B). Interestingly, no obvious differences of EAE development in terms of disease *onset* and severity were observed between *TCF-1*$^{fl/fl}$ and *TCF-1*$^{fl/fl}$*/CD4-Cre* mice, with a comparable mean peak disease score about 3. However, the clinical score of *TCF-1*$^{fl/fl}$*/Vav1-Cre* mice reached over 3.5, indicating more severe EAE than that observed in *TCF-1*$^{fl/fl}$*/CD4-Cre* mice. These results demonstrated that deletion of TCF-1 at earlier stages, but not later DP stage, potentiated Th17 immunity responsible for

EAE. Moreover, *TCF-1$^{fl/fl}$/Vav1-Cre* mice had significantly reduced number of peripheral T cells compared to *TCF-1$^{fl/fl}$* and *TCF-1$^{fl/fl}$/CD4- Cre* mice (Fig. 1C), suggesting less T cells but with higher potency in the induction of EAE when TCF-1 was deleted by Vav1-Cre.

Since Th17 cells contribute to EAE development (21), we wondered how stage specific deletion of TCF-1 affects Th17 differentiation. Even under Th0 conditions, we have already observed increased percentage of IL-17$^+$ cells in naïve CD4$^+$ T cells from *TCF-1$^{fl/fl}$/Vav1-Cre* mice, while IL-17 expression was hardly detectable in *TCF-1$^{fl/fl}$* and *TCF-1$^{fl/fl}$/CD4-Cre* T cells. Under Th17 polarization conditions, T cells from *TCF-1$^{fl/fl}$* and *TCF-1$^{fl/fl}$/CD4-Cre* mice were equivalent in the generation of IL-17$^+$ cells, whereas about 100% more IL-17$^+$ cells were developed from *TCF-1$^{fl/fl}$/Vav1-Cre* T cells (Fig. 1D). Increased IL-17$^+$ *TCF-1$^{fl/fl}$/Vav1-Cre* T cells correlated with the significant increased expression of Th17 signature genes, including IL-17A, IL-17F, CCR6 and CCL20 (Fig. 1E). In contrast, naïve CD4$^+$ T from *TCF-1$^{fl/fl}$/Vav1-Cre* mice had no defects in Th1 and Treg differentiation (Fig.S1), suggesting the selective role of TCF-1 in Th17 differentiation. These results demonstrate that TCF-1 is required at early but not later CD4$^+$CD8$^+$ stage during T cell development to restrain peripheral Th17 immunity by selectively controlling the potential of Th17 differentiation.

Deletion of TCF-1 by Vav1-Cre potentiates Th17 but not Th1-mediated EAE

TCF-1 was reported to modulate the differentiation of Th1 cells (22). Besides Th17 cells, Th1 cells can also contribute to EAE induction, but these two forms of EAE display distinct lesion sites and immune profiles in central nervous system (CNS) (23, 24), indicating different mechanisms are involved in the development of Th17 and Th1-mediated EAE. Although our *in vitro* experiment did not show significant difference in Th1 differentiation between *TCF-1$^{fl/fl}$/Vav1-Cre* mice and *TCF-1$^{fl/fl}$* and *TCF-1$^{fl/fl}$/CD4-Cre* control (Fig.S1), it is not clear whether deletion of TCF-1 affects Th1 function in term of induction of EAE *in vivo*. We thus compared the function of differentiated Th1 and Th17 cells by means of induction of passive EAE. Interestingly, *Rag1$^{-/-}$* recipient mice (which have a congenital deficiency in mature B and T cells) reconstituted with cells from spleen and lymph node of *TCF-1$^{fl/fl}$, TCF-1$^{fl/fl}$/CD4-Cre* or *TCF-1$^{fl/fl}$/Vav1-Cre* mice stimulated under Th1 priming conditions developed equivalent EAE (Fig. 2A, B). However, cells from *TCF-1$^{fl/fl}$/Vav1-Cre* mice stimulated under Th17 priming conditions induced more severe EAE in *Rag1$^{-/-}$* recipient mice (Fig. 2C), which was also indicated by significantly increased absolute number of central nervous system (CNS)-infiltrating lymphocytes, including T cells, neutrophils and monocytes (Fig. 2D, E, gate strategy for different cells shown in Fig.S2). In addition, intracellular staining of CNS-infiltrating cells showed greatly increased percentage of CD4$^+$IL-17$^+$IFN-γ^+ and CD4$^+$IL-17$^+$GM-CSF$^+$ cells, which are believed to be pathogenic for EAE (5-7), in the CNS of *Rag1$^{-/-}$* mice adoptively transferred with Th17-priming conditions treated cells from *TCF-1$^{fl/fl}$/Vav1-Cre* mice (Fig. 2F, G). Taken together, these findings supported that the exaggerated EAE resulted from deletion of TCF-1 at early T cell developmental stage was due to enhanced Th17 but not Th1 responses.

Vav1-Cre but not CD4-Cre induced deletion of TCF-1 leads to increased IL-17 expression in thymus

Since we observed increased IL-17 expression in mature $CD4^+$ T cells from *TCF-1$^{fl/fl}$/Vav1-Cre* mice (Fig. 1D), we next wondered whether these increased IL-17 producing cells originated from thymus. Vav1-Cre but not CD4-Cre-induced deletion of TCF-1 affected thymocyte development indicated by thymic weight (Fig. 3A, left panel) and cellularity (Fig. 3A, right panel) as well as developmental stages defined by CD4 and CD8 surface markers (Fig. 3B), suggesting that TCF-1 plays a major role at rather earlier developmental stages to ensure normal thymocyte development. Intracellular IL-17 staining indicated more mature $CD4^+$ TCR^{hi} thymocytes from *TCF-1$^{fl/fl}$/Vav1-Cre* mice produce IL-17 (3.10%) than that from *TCF-1$^{fl/fl}$* (0.03%) and *TCF-1$^{fl/fl}$/CD4-Cre* (0.2%) mice (Fig. 3B). This was consistent with our observation that peripheral $CD4^+$ cells from *TCF-1$^{fl/fl}$/Vav1-Cre* mice generated more IL-17 producing cells even in Th0 neutral conditions (Fig. 1D). When gated on earlier $CD4^-CD8^-$ TCR^- stage, we still found more $CD4^-CD8^-$ thymocytes from *TCF-1$^{fl/fl}$/Vav1-Cre* mice produced IL-17 (4.51%) compared to that from *TCF-1$^{fl/fl}$* (0.42%) and *TCF-1$^{fl/fl}$/CD4-Cre* (0.48%) mice (Fig. 3B). Therefore, Vav1-Cre-induced deletion of TCF-1 resulted in heightened IL-17 expression at as early as $CD4^-CD8^-$ DN stage, and such heightened IL-17 expression was maintained at mature $CD4^+$ T cell stage even after migrating out of thymus to the periphery. Since RORγt is required to stimulate IL-17 expression (25), RORγt expression was assessed at different thymocyte developmental stages in all three groups (Fig. 3C). Consistent with previous observation (26), RORγt was up-regulated at $CD4^+CD8^+$ DP stage but down-regulated at mature $CD4^+$ cells in control *TCF-1$^{fl/fl}$* mice. RORγt expression levels overlapped well between *TCF-1$^{fl/fl}$* and *TCF-1$^{fl/fl}$/CD4-Cre* thymocytes at $CD4^-CD8^-$, $CD4^+CD8^+$ and $CD4^+$ stages. In contrast, a portion of $CD4^-CD8^-$ DN and $CD4^+$ thymocytes expressed higher levels of RORγt in *TCF-1$^{fl/fl}$/Vav1-Cre* mice (Fig. 3C, arrows), which correlated with the observed increased IL-17 expression. Taken together, these data indicate that TCF-1 is required at early but not late developmental stages to inhibit IL-17 expression during T cell development.

Forced expression of TCF-1 in CD4CD8- thymocytes of *TCF-1$^{fl/fl}$/Vav1-Cre* mice inhibits IL-17 expression

We next determined whether forced expression of TCF-1 could inhibit IL-17 expression in developing and mature T cells from *TCF-1$^{fl/fl}$/Vav1-Cre* mice. Just like in mature T cells with germline deletion of TCF-1 (13), expression of TCF-1 by retrovirus-mediated gene transduction in mature T cells from *TCF-1$^{fl/fl}$/Vav1-Cre* mice was unable to reduce IL-17 expression to WT levels under Th17 priming conditions (Fig. 4A). Next, we determined the effects of TCF-1 on thymocytes co-cultured with OP9-DL1 stroma cells (27). *TCF-1$^{fl/fl}$* $CD4^-CD8^-$ DN thymocytes could develop to $CD4^+CD8^+$ DP thymocytes with or without expression of exogenous TCF-1 from retrovirus (Fig. 4B). *TCF-1$^{fl/fl}$/CD4-Cre* $CD4^-CD8^-$ DN thymocytes were able to develop to $CD4^+CD8^+$ DP thymocytes, however, with reduced efficiency indicated by reduced percentage of CD4+CD8+ cells. Exogenous TCF-1 could restore the percentage of $CD4^+CD8^+$ cells in *TCF-1$^{fl/fl}$/CD4-Cre* thymocytes to WT level (Fig. 4B), indicating the intact function of exogenous TCF-1. In contrast to *TCF-1$^{fl/f}$* and *TCF-1$^{fl/fl}$/CD4-Cre* thymocytes, majority of *TCF-1$^{fl/fl}$/Vav1-Cre* $CD4^-CD8^-$ DN thymocytes stayed at $CD4^-CD8^-$ DN stage even in the presence of exogenous TCF-1 (Fig. 4B). We next

determined the effects of exogenous TCF-1 on IL-17 expression. IL-17 was hardly detectable in thymocytes (including DN and developed DP) from *TCF-1*$^{fl/fl}$ and *TCF-1*$^{fl/fl}$/*CD4-Cre* mice with or without exogenous TCF-1 (Fig. 4C). Whereas, CD4^{-}CD8^{-} DN thymocytes from *TCF-1*$^{fl/fl}$/*Vav1-Cre* mice had abundant IL-17 expression (Fig. 4C), consistent with what we observed *in vivo* (Fig. 3B). Indeed, exogenous TCF-1 significantly suppressed IL-17 expression in *TCF-1*$^{fl/fl}$/*Vav1-Cre* thymocytes (Fig. 4C). Taken together, these data further demonstrated TCF-1 is required to suppress IL-17 expression at early T cell developmental stage.

TCF-1 binds to *IL-17* promoter and inhibits IL-17 expression through its intrinsic HDAC activity

Histone deacetylation is an evolutionary conserved epigenetic modification mechanism responsible for gene repression (28). Recent studies have shown that TCF-1 had intrinsic HDAC activity (15). Considering the dual capacity of DNA binding and intrinsic HDAC activity in TCF-1, we wondered whether TCF-1 binds to IL-17 locus and silences IL-17 expression via its intrinsic HDAC activity. To answer this question, we first determined whether TCF-1 binds to IL-17 promoter by chromatin immunoprecipitation (ChIP) assays with nine primer pairs covering the *IL-17A* promoter regions (P1 to p9) about 2000 base-pair (bp) upstream of transcriptional starting site (TSS) (Fig. 5A). The enrichment of TCF-1 binding to IL-17 promoter was observed in the region from P4 to P8 in *TCF-1*$^{fl/fl}$ thymus versus differentiated Th17 cells (Fig. 5B). To determine which TCF-1-binding regions are functional, each individual region from P4 to P8 was deleted to assess the effects on IL-17 reporter activity. Consistent with previous results (29, 30), RORγt stimulated IL-17 reporter. Whereas TCF-1 inhibited RORγt-dependent IL-17 reporter, deletion of P4 or P7 but not P5, P6 or P8 region of IL-17 promoter significantly relieved TCF-1-mediated inhibition of IL-17 reporter (Fig. 5C), suggesting that P4 and P7 are two functional TCF-1-binding sites responsible for the inhibition of IL-17 promoter activity. To determine whether TCF-1 is required for epigenetic modification critical for IL-17 expression, we compared histone H3 acetylation levels in the regions from P4 to P8. Due to the deletion of TCF-1 in *TCF-1*$^{fl/fl}$/*Vav1-Cre* thymus, P5 and P8 had higher H3 acetylation levels (Fig. 5D), indicating these two regions underwent TCF-1-dependent histone deacetylation in the WT thymus. These data indicate the possibility that TCF-1 mediates histone deacetylation in the regions around these binding sites. We further observed that in contrast to WT TCF-1, TCF-1-5aa mutant which diminished HDAC activity (15) was unable to inhibit IL-17 expression in DN thymocytes obtained from *TCF-1*$^{fl/fl}$/*Vav1-Cre* mice, comparable to virus expressing GFP alone (Fig. 5E). Taken together, these results demonstrated that TCF-1 binds to IL-17 promoter and silences IL-17 expression through its intrinsic HDAC activity.

Discussion

Previous studies have shown TCF-1 was able to restrain peripheral Th17 responses and *in vitro* experiments show that TCF-1 negatively regulates peripheral Th17 responses at T cell developmental stage in thymus rather than mature T cells in periphery (13, 14). However, there was no *in vivo* evidence to support stage specific requirement of TCF-1 for limiting peripheral Th17 immunity. Furthermore, it remains unknown about the mechanisms

responsible for TCF-1-mediated inhibition of Th17 responses. In this study, we first answered the critical question when is TCF-1 required to control the peripheral Th17 responses, given that T cell development is a sequential multi-step process. We found that deletion of TCF-1 early at hematopoietic stage, but not later DP stage, resulted in exaggerated Th17 differentiation and EAE development. In addition, since deletion of TCF-1 at early but not later DP stage leads to significant disruption of thymocyte development, demonstrating that TCF-1 plays a major role during early thymocyte development. Mechanically, we show that TCF-1 binds to and inhibit IL-17 promoter to prevent IL-17 expression via its intrinsic HDAC activity.

IL-17 gene locus must be kept inactive by TCF-1 prior to developing thymocytes entering DP stage. This is indicated by that deletion of TCF-1 at DP stage does not cause enhanced IL-17 expression in thymus, which means suppressive function of TCF-1 for IL-17 gene is not required at DP stage or later. Once TCF-1-mediated inhibition is imprinted on IL-17 gene locus, which we believe occurred no later than DN stage, it can last even after T cell migrating out of the thymus, resulting in prevention of exaggerated Th17 responses in periphery. Furthermore, our results highlight the specific requirement of HDAC activity of TCF-1 for suppression of IL-17 gene activity during T cell development. The HDAC family consists of a large family members with many of which play indispensable roles in regulating T cell development, including DN to DP transition, cell survival and TCR signaling in DP thymocytes (31-34). Although several HDAC proteins are expressed in thymus, it seems they are unable to compensate for the HDAC activity of TCF-1, as indicated by abnormal high levels of IL-17 expression (or natural Th17 cells) in thymus of *TCF-1*$^{fl/fl}$*/Vav1-Cre* mice. This phenomenon highlights the specific requirement of HDAC activity of TCF-1 for keeping IL-17 gene inactive early during T cell development.

Interestingly, if IL-17 gene locus does not undergo TCF-1-mediated inhibition like TCF-1 deficiency at early development stage, it will lead to an elevated Th17 differentiation and Th17 immunity. This consequence is irreversible as reconstitution of TCF-1 in mature T cells was unable to reduce IL-17 expression to normal levels. The mechanism of why TCF-1 close IL-17 gene locus in early developing thymocytes rather than in mature T cells remains largely unknown. Our results suggest that IL-17 gene locus in mature T cells was relatively inaccessible for TCF-1 compared to that in early T cell developmental stage. This may result from chromatin remolding during later developmental stage and/or lack of cofactors that present in early thymocytes but not in mature T cells, which could be an interesting topic for future investigation.

Exaggerated Th17 responses are associated with many types of autoimmune diseases (8, 35). To develop the effective treatment for autoimmune diseases, many studies have focused on understanding the mechanisms of regulating Th17 differentiation and Th17-Tregs balance in peripheral immune system. Our study suggests that peripheral Th17 responses can be greatly influenced by specific developmental events in central immune system, and thus provide additional layer of control to prevent autoimmune diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Hai-Hui Xue for generously sharing *TCF-1*$^{fl/fl}$ mice. We appreciate the help by city of hope supported cores including animal, genomic and flow cytometer cores. In addition, we thank Chris Gandhi and Keely Walker for their careful proof/editing work on the manuscript.

This work was supported by grants from NIH R01-AI053147, NIH R01-AI109644 and institutional pilot funding. In addition, research reported in this publication was also supported by the National Cancer Institute of the National Institutes of Health under award number P30CA33572, which includes work performed in the animal, genomic, flow cytometer and mass spectrometric cores supported by this grant. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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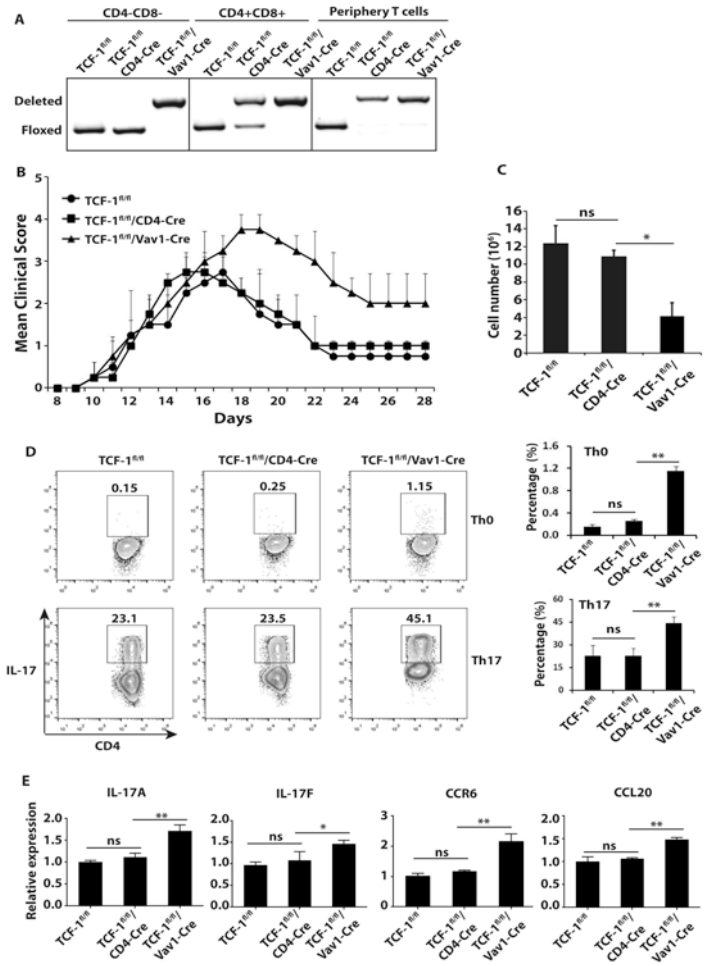

Figure 1.

Vav1-Cre but not CD4-Cre induced deletion of TCF-1 potentiates EAE and Th17 differentiation. **A**, Floxed and deleted TCF-1 loci in indicated population of thymocytes and peripheral T cells from indicated genotypes of mice, as determined by PCR analysis. Data shown is the representative of three independent experiments. **B**, Mean clinical score of EAE in indicated genotypes of mice different days after $MOG_{35–55}$ immunization. (*TCF-1*$^{Fl/Fl}$ n=6, *TCF-1*$^{Fl/Fl}$*/CD4-Cre* n=6, and *TCF-1*$^{Fl/Fl}$*/Vav1-Cre* n=6). **C**, Total number of $CD4^+$ T cells in the spleens of indicated genotypes of mice (n=3). **D**, Percentage of IL-17^+ cells among indicated genotypes of naïve CD4+ T cells stimulated under Th0 or Th17 priming conditions, as determined by flow cytometric analysis. Right panels are the quantification of three biological replica. **E**, Expression levels of indicated critical Th17 genes in differentiated Th17 cells of indicated genotypes, as determined by qPCR. ns, no significant difference; * < 0.05 and **<0.01.

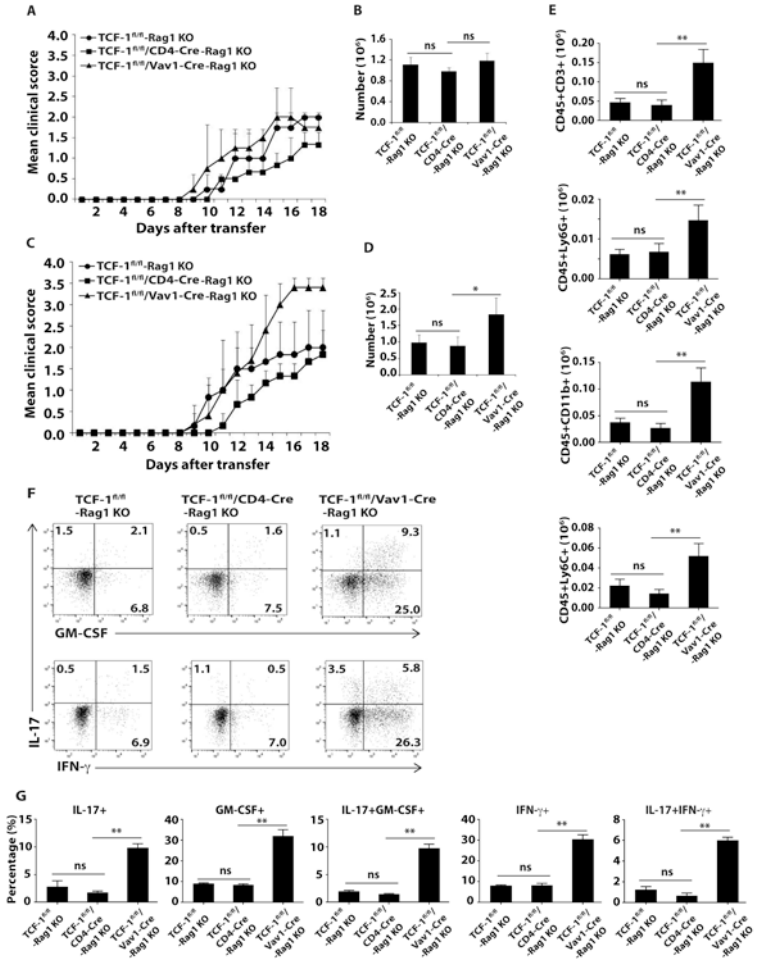

Figure 2.

Deletion of TCF-1 by Vav1-Cre potentiates Th17 but not Th1-mediated EAE. **A, C**, Mean clinical scores of EAE in *Rag1*$^{-/-}$ mice different days after adoptively transferred with indicated genotypes of $MOG_{33–35}$-expanded Th1 (**A**) or Th17 (**C**) cells. **B, D**, Number of lymphocytes infiltrated into the CNS of *Rag1*$^{-/-}$ mice adoptively transferred with indicated Th1 (**B**) or Th17 (**D**) cells at the peak of disease as shown in **A** or **C**. **E**, Quantification of cells expressing surface markers characteristic of various types of lymphocytes as determined by flow cytometric analysis at the peak of disease from *Rag1*$^{-/-}$ mice receiving Th17 cells. **F**, Flow cytometric analysis of intracellular IL-17, GM-CSF and IFN-γ in $CD4^+$ T cells that infiltrated CNS of *Rag1*$^{-/-}$ mice receiving Th17 cells at the peak of disease. **G**, Percentage of cells positive for indicated cytokines among $CD4^+$ T cells infiltrated into CNS of *Rag1*$^{-/-}$ mice. Data are pooled from three experiments. ns, no significant difference; * < 0.05 and **<0.01.

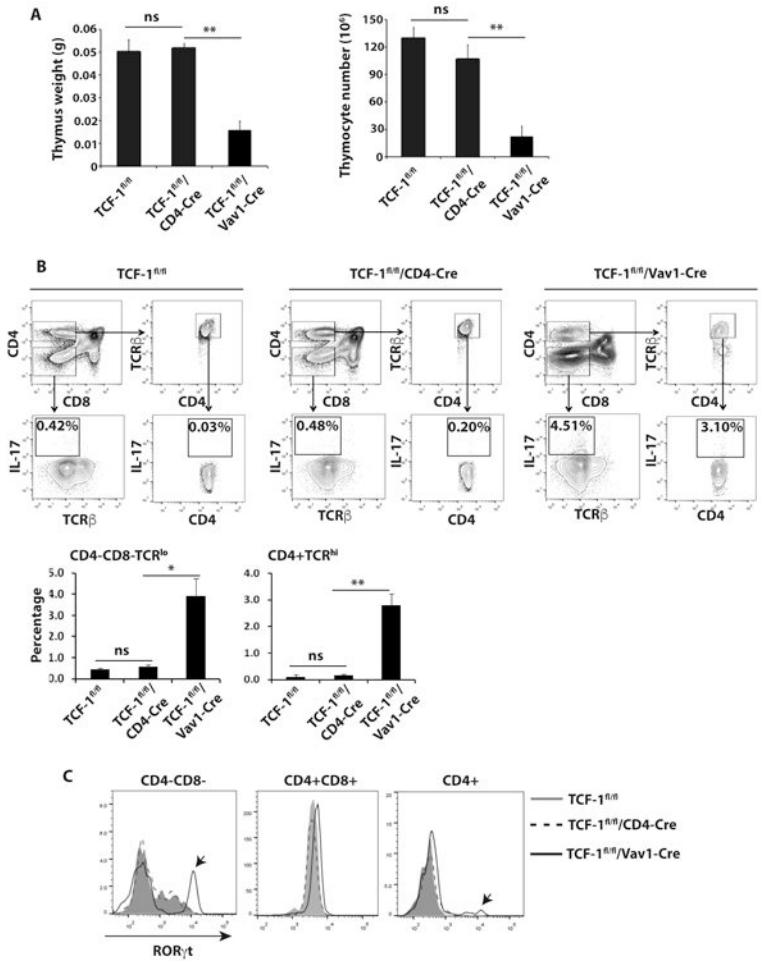

Figure 3.

Vav1-Cre induced deletion of TCF-1 leads to abnormal IL-17 expression in thymus. **A**, Weight (left panel) and number of total thymocytes (right panel) of the thymus (n=3) from indicated genotypes of mice. **B**, Thymocytes of different genotypes of mice were either gated on $CD4^-CD8^-$ or $CD4^+$ and $TCR\beta^{hi}$ (top panels). Percentage of $IL\text{-}17^+$ cells in $CD4^-CD8^-$ $TCR\beta^{lo}$ and $CD4^+$ $TCR\beta^{hi}$ population of indicated genotype of mice was determined (middle panels). Bottom panels are the quantification of percentage of $IL\text{-}17^+$ cells shown on middle panels from three mice of each genotype. **C**, Expression of RORγt in indicated population of cells from indicated genotypes of mice, detected by intracellular staining. Arrow indicates the very high levels of RORγt population in $CD4^-CD8^-$ and $CD4^+$ thymocytes of *TCF-1$^{f/fl}$ /Vav1-Cre* mice.

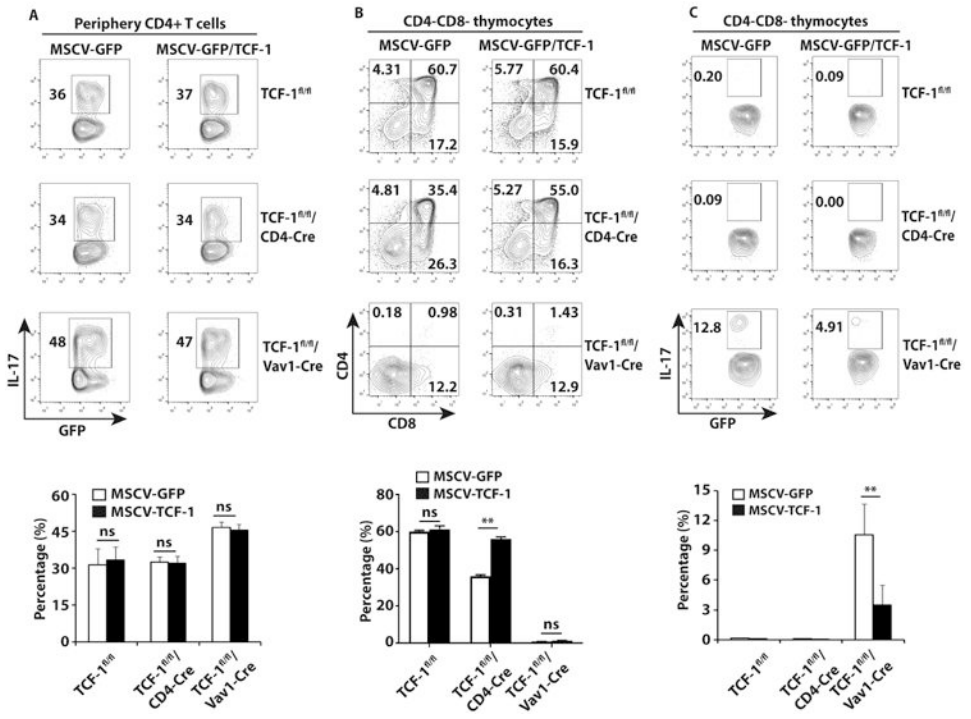

Figure 4.

Forced expression of TCF-1 in TCF-1-deficient $CD4^-CD8^-$ thymocytes but not mature $CD4^+$ T cells from *TCF-1$^{fl/fl}$/Vav1-Cre* mice inhibits IL-17 expression. **A**, Percentage of $IL\text{-}17^+/GFP^+$ cells among indicated genotypes of $CD4^+$ T cells transduced with retrovirus expressing GFP along or together with TCF-1 and differentiated under Th17-priming conditions for 3 days, as determined by flow cytometry analysis. Bottom panel is the quantification of three biological replica. **B**, Flow cytometric analysis of CD4 and CD8 among thymocytes *ex vivo* differentiated from $CD4^-CD8^-$ thymocytes sorted from indicated genotypes of mice, transduced with retrovirus expressing GFP along or together with TCF-1, and co-cultured for 3 days with OP9-DL1 stroma cells and IL-7. The number in each quadrant indicated the percentage of cells in gated area among GFP^+ cells. Bottom panel is the quantification the percentage of $CD4^+CD8^+$ cells shown on top panels. **C**, Percentage of $IL\text{-}17^+/GFP^+$ cells among *ex vivo* differentiated thymocytes described in **B**. Bottom panel is the quantification of the results.

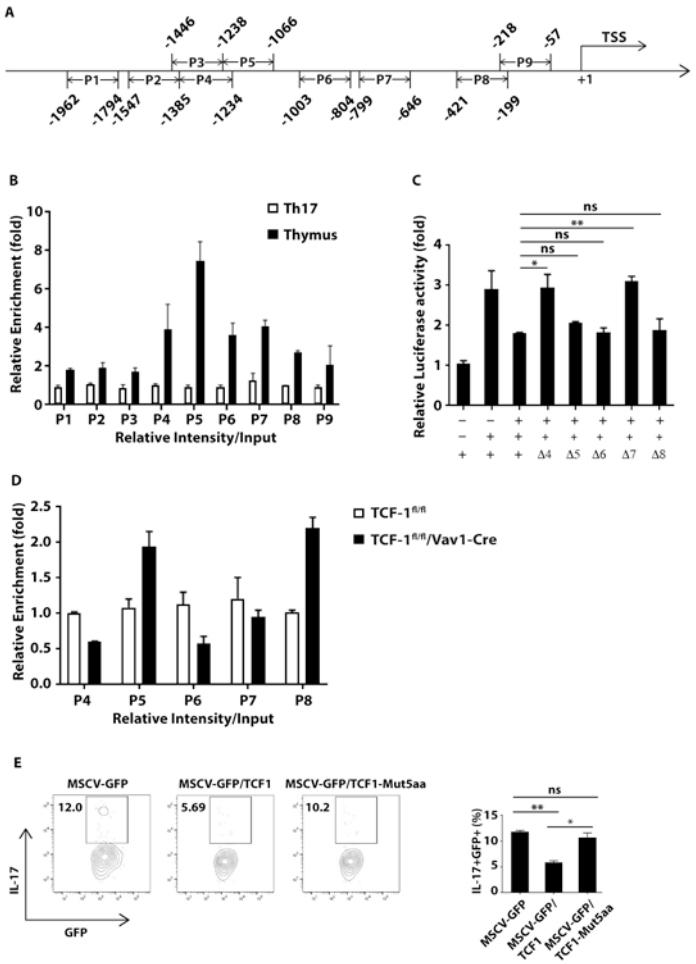

Figure 5.

TCF-1 binds to IL-17 gene locus and inhibits IL-17 expression through its intrinsic HDAC activity. **A**, Schematic representation of the location of 9 regions, P1-P9, on IL-17 promoter. **B**, Enrichment of TCF-1 binding to 9 regions on IL-17 promoter, detected by ChIP assay using anti-TCF-1 antibody or IgG control in WT thymus or differentiated Th17 cells. **C**, IL-17 promoter-luciferase reporter activity in HEK293T cells transfected with the indicated expression plasmids. Δ4, deletion of P4; Δ5, deletion of P5; Δ6, deletion of P6; Δ7, deletion P7; Δ8, deletion of P8 region shown in **A**. Luciferase data are normalized to Renilla luciferase activity. **D**, DNA was precipitated with anti-acetylhistone H3 antibodies. qRT-PCR-based ChIP analysis was used to measure histone H3 acetylation levels in indicated regions. **E**, Percentage of IL-17^{+}/GFP^{+} cells among thymocytes ex vivo differentiated from CD4^{-}CD8^{-} cells sorted from *TCF-1$^{fl/fl}$/Vav1-Cre* mice, transduced with retrovirus expressing GFP along or together with TCF-1 or TCF1-Mut5aa, and co-cultured with OP9-DL1 stromal cells for 3 days, as determined by flow cytometry. Right panel is the quantification of three biological replica.