IMMUNOLOGY

RORγt expression in mature T_H17 cells safeguards their lineage specification by inhibiting conversion to T_H2 cells

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RORγt is the lineage-specific transcription factor for T helper 17 (T_H17) cells and an attractive drug target for treating T_H17-associated diseases. Although the critical role of RORγt in early T_H17 cell differentiation has been well recognized, its function in mature T_H17 cell maintenance remains largely unknown. Here, we show that genetic deletion of *Rorc* in mature T_H17 cells inhibited their pathogenic functions. Mechanistically, loss of RORγt led to a closed chromatin configuration at key T_H17-specific gene loci, particularly at the "super-enhancer" regions. Unexpectedly, RORγt directly bound and inhibited *Il4* transcription, whereas pharmaceutically or genetically targeting RORγt caused spontaneous conversion of T_H17 cells to T_H2-like cells in vitro and in vivo. Our results thus reveal dual crucial functions of RORγt in effector T_H17 cells in maintaining T_H17 cell program and constraining T_H2 cell conversion, offering previously unidenified considerations in therapeutic targeting of RORγt.

INTRODUCTION

Upon activation, $CD4^+$ T cells differentiate into different subsets in response to various innate signals, including T helper (T_H) 1, T_H2, T_H17, regulatory T (T_{reg}), and T follicular helper (Tfh) cells (*1*). T_H17 cells are important in protecting against extracellular pathogens and maintaining mucosal homeostasis, while dysregulated T_H17 cells contribute to chronic inflammation and autoimmune diseases. The development of T_H17 cells is initiated by the combination of cytokines transforming growth factor–β (TGF-β) and interleukin-6 (IL-6), expanded by IL-21 at the middle stage, and further reinforced by IL-23 at the late stage, involving multiple layers of regulations by transcription factors, epigenetic factors, and environmental cues (*2*). Drugs targeting T_H17 cell–associated factors, such as IL-23, IL-6R, IL-1, IL-17A, IL-17RA, IL-17F, and Janus kinase 1/3, have been proved for treating a number of diseases (*3*, *4*).

Compared with T_H1 and T_H2 cells, T_H17 cells, at least those generated in vitro, are regarded to be highly unstable and plastic, possibly because of their intrinsic stem-like gene expression features (*5*). Culturing mature T_H17 cells under T_{reg}-, T_H1-, and T_H2-polarizing conditions results in FOXP3, interferon-γ (IFN-γ), and IL-4 upregulation, respectively (*6*). In vivo, T_H17 cells were reported to convert into IFN-γ–producing T cells when transferred into lymphopenic mice or under inflammatory conditions such as experimental autoimmune encephalomyelitis (EAE), which was believed to be important for their pathogenicity (*6*–*8*). Meanwhile, T_H17 cells were also reported to gain Tfh phenotype to drive immunoglobulin A (IgA)–producing germinal B cell production in Peyer's patch (*9*), and an IL-17A–producing T_H2 cell population was reported in patients with severe asthma (*10*).

T_H lineage–specific transcription factors mediate T cell fate commitment and blockage of alternative cell fates. Retinoic acid related orphan receptor γt (RORγt), induced at the early stage of T_H17 cell differentiation following IL-6 and TGF-β stimulation (*11*, *12*), has a pivotal role at the lineage commitment stage of T_H17 cells (*11*). RORγt specifically binds to and regulates T_H17-associated genes, such as *Il17a*, *Il17f*, and *Il23r*, by activating their transcription in coordination with other transcription factors (*13*). Therefore, genetically or pharmacologically targeting RORγt by small molecules is effective in ameliorating T_H17-related inflammatory diseases, including EAE, psoriasis, arthritis, colitis, and glomerulonephritis, particularly in preventative disease models (*11*, *14*–*16*). However, none of the RORγt inhibitors has passed phase 2 studies in human clinical trials, raising concerns on the safety and efficiency of targeting RORγt (*17*).

Although the lineage-specific transcription factors are essential in T subset development, their function in mature T cells appears to be distinct. For instances, continuous FOXP3 expression is required for maintaining the lineage stability and suppressive function of mature T_{reg} cells (*18*). However, T-box transcription factor (T-bet) has a limited function in maintaining IFN-γ expression in mature T_H1 cells (*19*, *20*). GATA3 maintains IL-5 and IL-13 but is less important for IL-4 expression in mature T_H2 cells (*20*, *21*). Compared with the well-determined function of RORγt in T_H17 cell differentiation, its role in maintaining mature T_H17 program is not fully understood.

In this study, we found that mature T_H17 cells require RORγt expression to maintain their lineage identity and pathogenicity, at least partly via regulating chromatin accessibility of T_H17-associated super enhancers (SEs). Unexpectedly, we found a critical role of RORγt in direct inhibition of IL-4 expression and thus IL-4–dependent T_H2 program. These data offer important insights into T_H17 cell biology with implications in treating T_H17-related diseases.

RESULTS

RORγt is required for the maintenance and function of effector T_H17 cells

To investigate the function of RORγt in mature T_H17 cells, we first established a *Rorc*-inducible deletion system by crossing $Rorc^{fl/fl}$ mice wild type (WT) with *Rosa26*-Cre^{ERT2} mice (hereafter as $Rorc^{ERT2}$).

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Naïve $CD4^+$ T cells from these mice were isolated and then cultured under T_H17-polarizing condition. Three days later, when T_H17-signature cytokine IL-17A was fully induced, 4-hydroxytamoxifen (4-OHT) was added into the culture to delete the *Rorc* gene (fig. S1A). The deletion efficiency was confirmed by RORγt staining (fig. S1B). Similar to its deletion in naïve $CD4^+$ T cells (*11*), loss of *Rorc* in mature T_H17 cells resulted in a progressive loss of IL-17A protein expression and a marked reduction of the mRNA expression of *Il17a*, *Il17f*, and *Il23r* without affecting the transcription of other key T_H17-associated transcription factors, including *Rora*, *Irf4*, and *Batf* (Fig. 1, A and B).

To examine whether RORγt is required for maintaining T_H17 effector function in vivo, $Rorc^{ERT2}$ or $Rorc^{fl/fl}$ mice were crossed with 2d2–T cell receptor (TCR) transgenic mice and *Il17f*–red fluorescent protein (RFP) reporter mice (hereafter as 2d2-$Rorc^{ERT2}$ or 2d2-WT). 2d2-WT and 2d2-$Rorc^{ERT2cre}$ naïve T cells were cultured under T_H17 polarization condition in vitro. RFP^+ mature T_H17 cells were then sorted, treated with 4-OHT to induce *Rorc* deletion, and then transferred into $Rag1^{-/-}$ recipient mice to induce EAE (fig. S1C). Deleting *Rorc* in effector T_H17 cells significantly delayed the onset and reduced the severity of EAE disease (Fig. 1C). We further analyzed central nervous system (CNS)–infiltrating $CD4^+$ T cells. RORγt deletion was validated in 2d2-$Rorc^{ERT2}$ donor T cells in vivo (fig. S1D). Compared to 2d2-WT T cells, CNS-infiltrating 2d2-$Rorc^{ERT2}$ T cell numbers were significantly decreased, with decreased expression of CCR6 and IL-17A, although similar low $IL\text{-}17A^+IFN\text{-}\gamma^+$ population was found (Fig. 1, D and E). In conclusion, RORγt is critically required for maintaining the lineage identity and pathogenic function of effector T_H17 cells.

Loss of RORγt alters epigenetic landscapes in effector T_H17 cells.

Epigenetic mechanisms are critical in T_H cell lineage specification. To understand how RORγt maintains T_H17 cell program, we examined histone modification markers at the *Il17* gene locus in 4-OHT–treated mature WT and $Rorc^{ERT2}$ T_H17 cells. Unlike its deficiency before the initiation of T_H17 differentiation (*22*), *Rorc* deletion in mature T_H17 cells barely affected the active histone marker H3K4me3 and the silence marker H3K27me3 at both the promoter and CNS2 enhancer region within *the Il17-Il17f* gene locus (Fig. 2A) but notably decreased the active and poised enhancer markers H3K27Ac and H3K4me1 (Fig. 2B), suggesting a selective role of RORγt in maintaining T_H17-specific enhancer activities. Consistently, 5-hydroxymethylcytosine (5hmc) modification, a marker enriched at active enhancer regions in effector T cells (*23*), was also reduced at both the *Il17* promoter and CNS2 regions in $Rorc^{ERT2}$ T_H17 cells followed by 4-OHT treatment (Fig. 2C).

To further understand the role of RORγt in mature T_H17 cells, we examined the effect of RORγt deficiency on genome-wide chromatin accessibility, especially in enhancer regions. ATAC-seq (assay for transposase-accessible chromatin sequencing) results revealed a total of 1249 and 1871 regions with reduced (RORγt-activated chromatins) or increased chromatin accessibility (RORγt-inhibited chromatins), respectively, following *Rorc* deletion in effector T_H17 cells (Fig. 2D). The chromatin accessibility of the promoter regions of T_H17 cell signature genes *Il17a*, *Il17f*, *Il23r*, and *Ccr6* were notably decreased and conserved noncoding DNA regulatory regions, including the *Il17-Il17f* enhancer CNS2 (*24*) and the CNS6, CNS9, and CNS + 10 regions at the *Rorc gene locus* (Fig. 2E) (*12*, *25*).

SEs are a subset of enhancers important in regulating core cell identity gene expression compared with traditional enhancers (TEs) (*26*). A total of 774 T_H17 cell–associated SEs have been defined on the basis of exceptionally high occupancy of p300 compared with TEs. Among them, 99.44% (731 of 774) of T_H17 cell–related SEs were colocalized with RORγt-binding sites, in contrast to only 40.80% (8117 of 19,893) of T_H17 cell–related TEs (Fig. 2F) (*13*). On the basis of our ATAC-seq data, after *Rorc* deletion, 55.6% (1736 of 3120) of the differentially opened chromatin regions fell in enhancer regions, among which there were 41.5% SEs (321 of 774, affected/total) and 7.2% TEs (1437 to 19,893) affected (Fig. 2G). In line with these findings, reanalysis of previously published chromatin immunoprecipitation sequencing (ChIP-seq) data in WT and *Rorc*-deficient T_H17 cells revealed that deletion of *Rorc* reduced p300 binding to 54.5% (422 to 774) SEs but only 8.02% (1596 to 19,893) in TEs (Fig. 2H) (*13*). These findings together highlight a particular important role of RORγt in regulating T_H17-specific enhancers, especially SEs.

Loss of RORγt results in conversion of T_H17 cells to T_H2-like cells

Compared with other helper T cells, T_H17 cells are highly plastic and prone to be converted to IFN-γ–producing T_H1-like cells under both in vitro and in vivo conditions (*7*). Deletion of *Rorc* in effector T_H17 cells, however, caused significant up-regulation of IL-4 and GATA3 (Fig. 3A), but not IL-9 and IFN-γ expression, under continuous T_H17-polarizing culture condition (Fig. 1A and fig. S2A), although the expression of IL-4 and GATA3 was lower than that under T_H2 culture condition (Fig. 3A). The transcripts for T_H2 signature genes *Il4*, *Il13*, and *Gata3* were also significantly increased in $Rorc^{ERT2}$ compared with WT T_H17 cells, while *Ifng* transcription was only slightly affected (fig. S2B). The mRNA and protein expression of IL-5 was barely detectable in either WT or $Rorc^{ERT2}$ cells, suggestive of a potentially different regulatory mechanism (fig. S2C). To prove that the increased IL-4–expressing cells indeed are derived from ex-T_H17 cells, we sorted *Il17f*-RFP^+ WT and $Rorc^{ERT2}$ T_H17 cells and then recultured these developed T_H17 cells in the presence of 4-OHT under T_H17-polarizing condition. Consistently, IL-4 expression increased on day 4 after reculture and further elevated to a comparable level in T_H2 cells on day 7 (fig. S2D). Deletion of *Rorc* in naïve $CD4^+$ T cells ($Cd4^{Cre}Rorc^{fl/fl}$, hereafter as $Rorc^{Cd4}$) also led to increased differentiation toward to IL-4–expressing T_H2 cells, but not IFN-γ–producing T_H1 phenotype, under T_H17-polarizing condition (Fig. 3, B and C).

When placed under T_H2 culture conditions, *Rorc*-deficient T_H17 cells exhibited augmented conversion toward T_H2 cells, although less notably than under T_H17-polarizing conditions (fig. S3, A and B). Consistent with previous findings that forced expression of RORγt had no effect on TGF-β–induced FOXP3 expression (*27*), deletion of *Rorc* did not promote T_H17 conversion to $FOXP3^+$ T_{reg} cells (fig. S3B). In contrast, *Rorc*-deficient T_H17 cells were much more resistant to be converted to an IFN-γ–producing T_H1 cell fate when recultured under various T_H1- or T_H1/T_H17-polarizing conditions compared with WT T_H17 cells (fig. S3B). Unexpectedly, T_H17 cells produced large amounts of IL-4, but not IFN-γ, when recultured under T_H1 culture condition, and blockage of IL-4 significantly enhanced IFN-γ production in both WT and *Rorc*-deficient cells (Fig. 3D), suggesting intrinsic T_H2-prone feature of mature T_H17 cells in vitro.

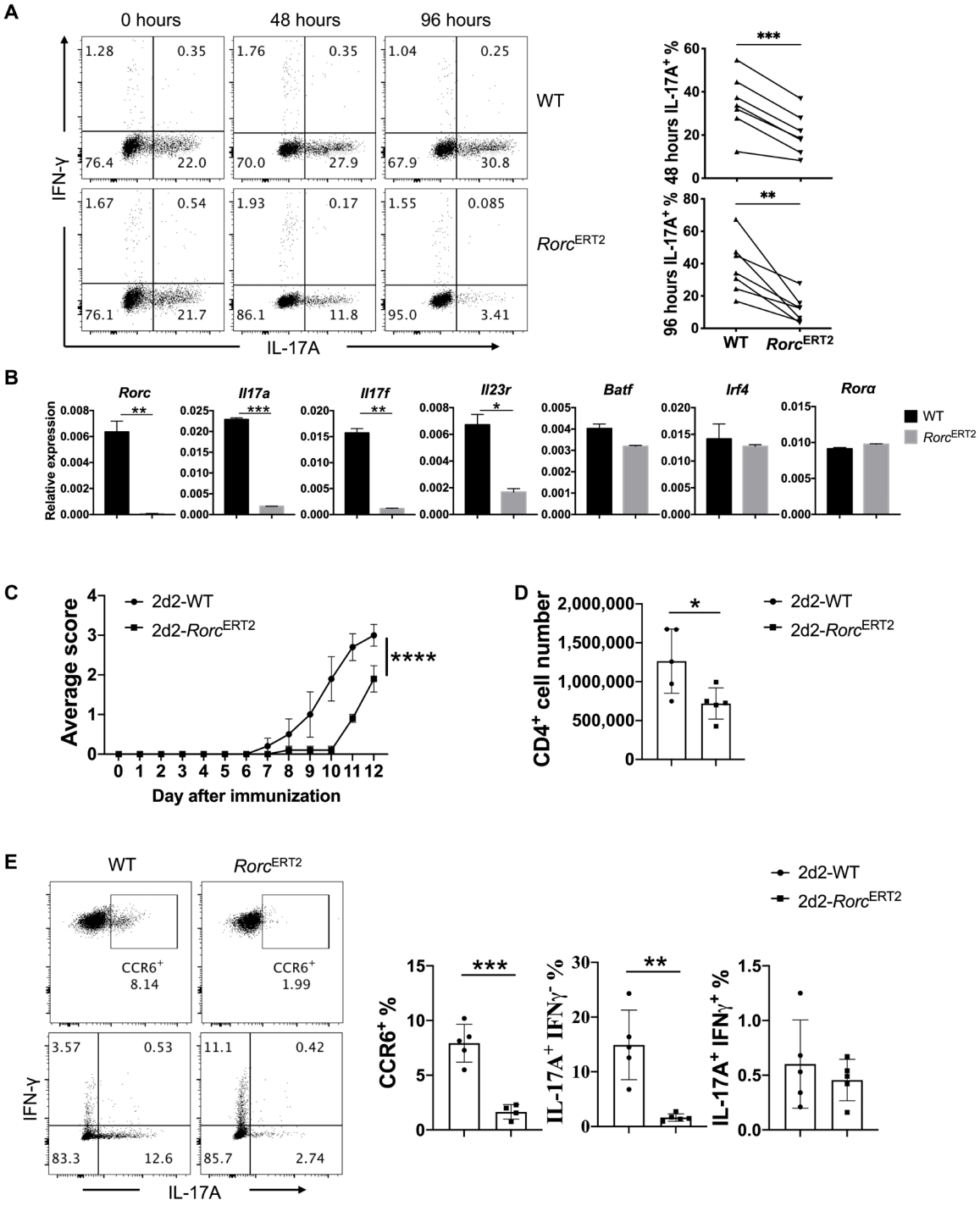

Fig. 1. Continuous RORγt expression maintains T_H17 cell lineage stability and function. (**A** and **B**) Naïve T cells isolated from *Rorc*$^{fl/fl}$ (WT) and *Rosa26*-Cre^{ERT2} *Rorc*$^{fl/fl}$ (*Rorc*^{ERT2}) mice were cultured with TGF-β, IL-6, IL-1β, and IL-23 for 3 days, followed by 4-OHT (2 μM) treatment. (A) Representative flow cytometry plots of IL-17A and IFN-γ expression (left) and statistical analysis of IL-17A expression (right) before (0 hours), 48 hours, or 96 hours after 4-OHT treatment. Each line represents one independent experiment. (B) Relative mRNA expression of T_H17-related genes 96 hours after 4-OHT treatment. The expressions were normalized to *Actb*. (**C** to **E**) 2d2-WT and 2d2-*Rorc*^{ERT2} naïve T cells were polarized under T_H17 culture condition for 3 days. RFP$^+$ mature T_H17 cells were sorted and treated with 4-OHT and then transferred into *Rag1*$^{-/-}$ mice to induce EAE. $n = 5$ mice each group. (C) EAE disease scores following $MOG_{35–55}$ immunization. (D) Total number of CD4$^+$ T cells infiltrated in the central nervous system of the recipient mice. (E) Representative flow cytometry plots and statistic data of CCR6, IL-17A, and IFN-γ expression in the CNS-infiltrated T cells. This experiment was repeated twice. *P* values were determined by paired Student's *t* test (A), one-way ANOVA with Tukey's multiple comparisons test (C), or unpaired Student's *t* test (D and E). Data represent means ± SEM (C) or SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

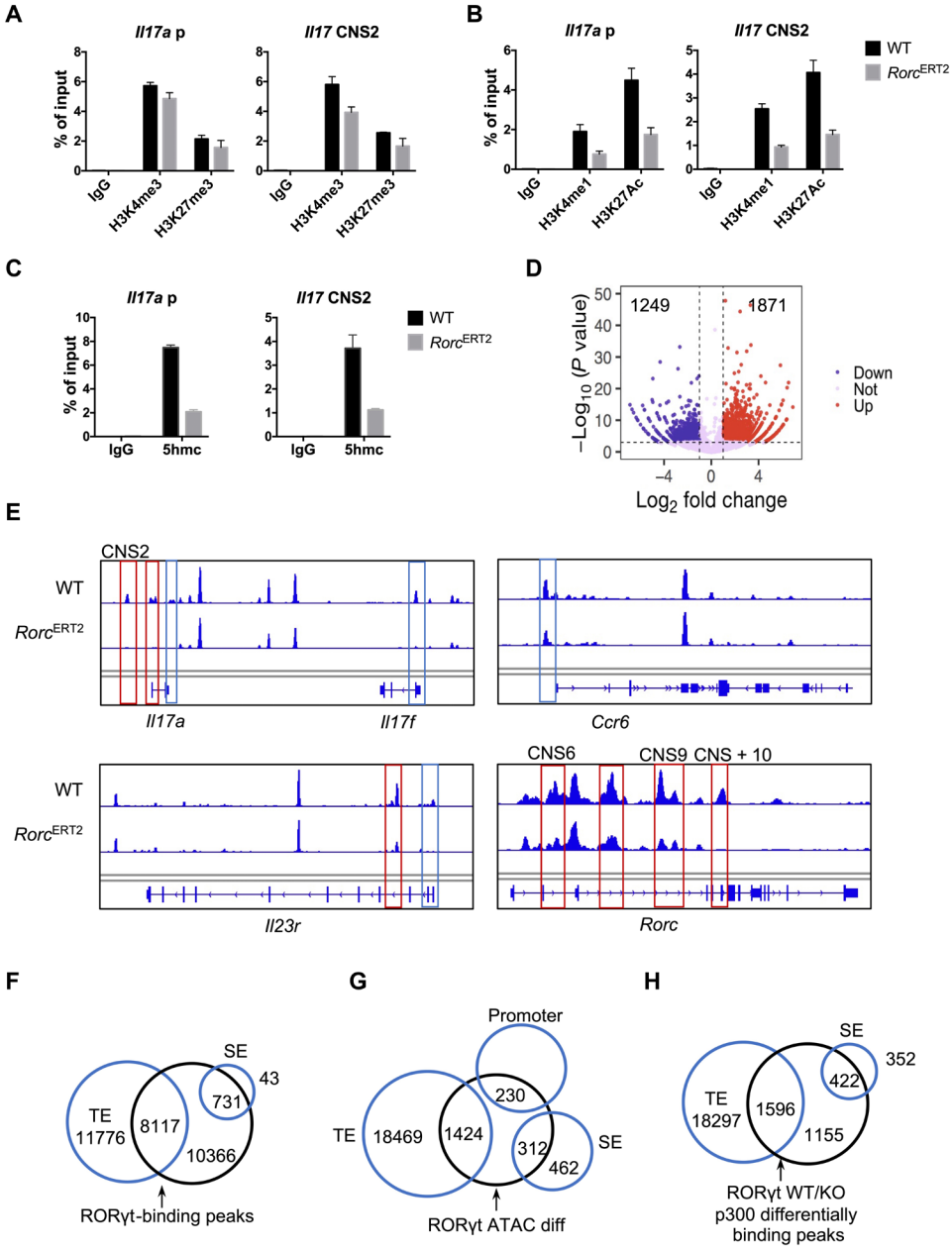

Fig. 2. RORγt regulates SE activities in mature T_H17 cells. Naïve T cells isolated from WT and *Rorc*^{ERT2} mice were cultured under T_H17-polarizing condition for 3 days followed by 4-OHT treatment for 96 hours. SE and traditional enhancer (TE) regions in T_H17 cells were defined from previous study (*26*). (**A** to **C**) ChIP assay was performed with antibodies targeting H3K4me3 and H3K27me3 (A), H3K27Ac and H3K4me1 (B), and MeDIP assay were performed with antibody targeting 5hmc (C). The enriched DNA fragments were detected using qPCR and normalized with input control at *Il17a* promoter and CNS2 region. The experiments were repeated three times. (**D** and **E**) WT and *Rorc*^{ERT2} T_H17 cells were restimulated with anti-CD3 and collected for ATAC-seq. (D) Volcano plot of differentially opened chromatin regions (OCRs). (E) Representative ATAC-seq tracks from WT and *Rorc*^{ERT2} T_H17 cells. (**F**) The Venn diagram of previous reported RORγt-binding peaks, TEs, and SEs (*13*, *26*). (**G**) The Venn diagram of ATAC-seq OCRs, promoter regions, SEs, and TEs (*26*). (**H**) The Venn diagram of reported RORγt-regulated p300-binding peaks (defined by differential binding of p300 in WT and RORγt-deficient T_H17 cells), TEs, and SEs (*13*, *26*). KO, knockout.

To further substantiate the above findings, we isolated naïve CD4$^+$ T cells from *Il4*^{GFP} reporter mice and cultured them under T_H17 cell–polarizing conditions in the presence of different RORγt inhibitors, including GSK805, TMP920, and ursolic acid. Consistent with above results, treatment with RORγt inhibitors significantly increased IL-4–green fluorescent protein (GFP) reporter expression 1 week later. In the prolonged culture, treatment with GSK805 or TMP920 even transformed ~80% of the cells to IL-4–producing T_H2-like cells (Fig. 3E). Similar to genetic ablation of *Rorc* in effector T_H17 cells, culturing these cells in the presence of RORγt inhibitors also led to a notable up-regulation of IL-4 from days 7 to 14 of T_H17 cultures (Fig. 3F). In conclusion, these findings reveal a

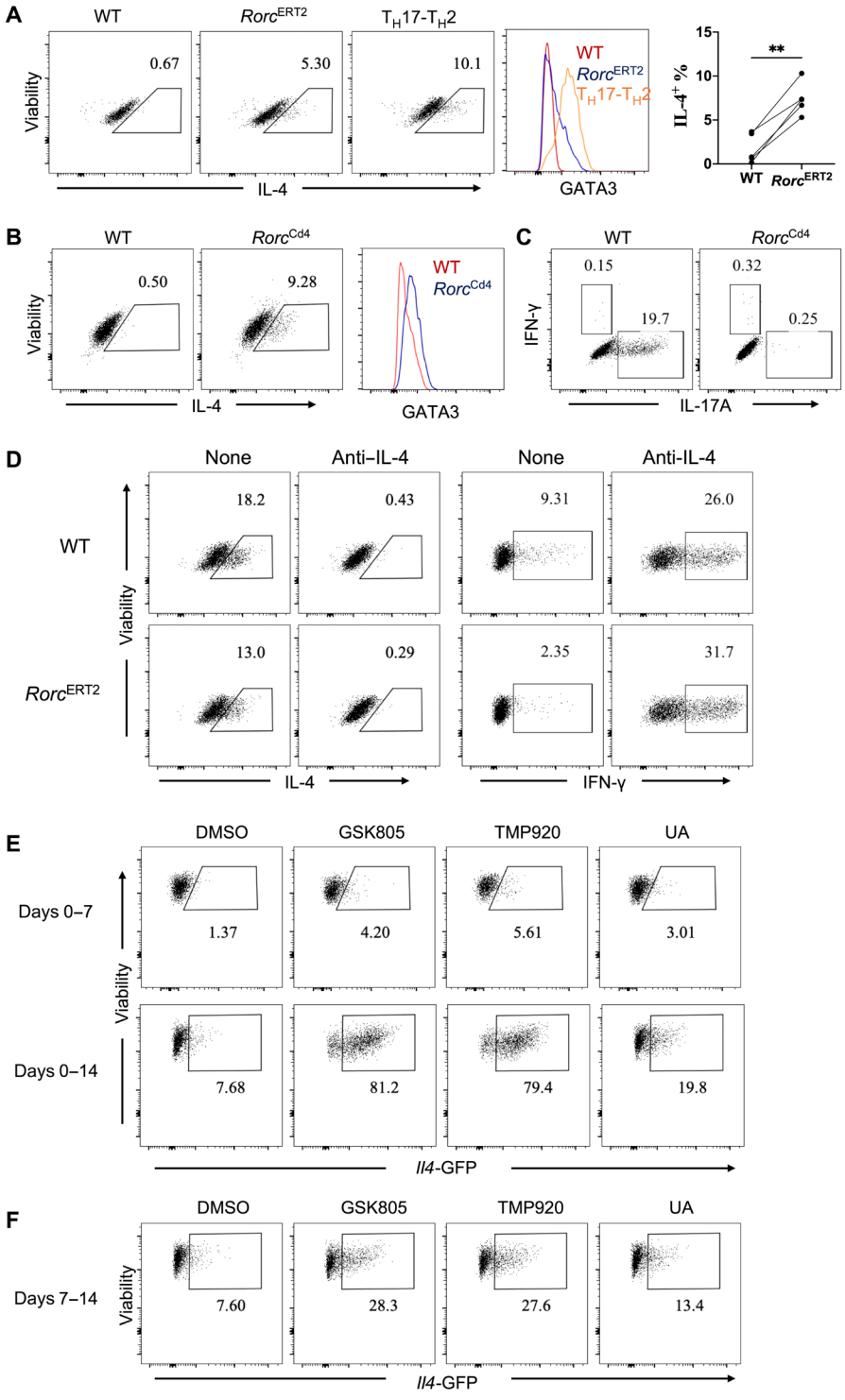

Fig. 3. Genetic or pharmaceutical inhibition RORγt converts T_H17 cells into T_H2-like cell. (**A**) Naïve T cells isolated from WT and *Rorc*^{ERT2} mice were cultured under T_H17-polarizing condition. After 3 days, the developed T_H17 cells were recultured with 4-OHT under T_H17- or T_H2-polarizing (T_H17-T_H2) conditions for 96 hours. Representative flow cytometry plots of IL-4 and GATA3 were shown. (**B** and **C**) Intracellular staining of IL-4 and GATA3 (B), IFN-γ, and IL-17A (C) in WT and *Rorc*^{Cd4} T_H17 cells cultured in vitro for 6 days. (**D**) Naïve T cells from WT and *Rorc*^{ERT2} mice and cultured with TGFβ, IL-1β, IL-6, and IL-23 for 3 days. The effector T_H17 cells were then treated with 4-OHT for 2 days to remove the *Rorc* gene and then recultured with IL-12 and IFN-γ, with or without IL-4–neutralizing antibody for 4 days. IFN-γ and IL-4 expression was detected by flow cytometry. (**E**) Expression of the *Il4*-GFP reporter in T_H17 cells induced in vitro in the presence of dimethyl sulfoxide (DMSO) or RORγt inhibitors GSK805 (0.5 μM), TMP920 (10 μM), or ursolic acid (UA) (2 μM) for 7 or 14 days. (**F**) Expression of the *Il4*-GFP reporter in T_H17 cells induced in vitro with the presence of control DMSO or RORγt inhibitors from days 7 to 14 in the culture. All these experiments were repeated at least two to three times with consistent results.

previously unidentified function of RORγt in repressing T_H2 cell program in T_H17 cells; inhibiting the expression or function of RORγt leads to spontaneous conversion of T_H17 cells to T_H2-like cells.

Loss of RORγt reprograms T_H17 cells toward T_H2 transcriptional and epigenetic programs

To further understand the function of RORγt in mature T_H17 cells, RNA sequencing (RNA-seq) analysis was performed using mature WT and *Rorc*^{ERT2} T_H17 cells treated with 4-OHT for 48 or 96 hours. A total of 140 genes were up-regulated, 75 genes were down-regulated in *Rorc*^{ERT2} T_H17 cells compared with WT group treated with 4-OHT for 48 hours [fold changes of >2, false discovery rate (FDR) < 0.05, FPKM (fragments per kilobase of transcript per million fragments mapped) > 1 in any group], and the numbers of genes with altered expression were increased to 283 and 202 at 96 hours after 4-OHT treatment, respectively (Fig. 4A). Most of the RORγt-regulated genes showed a progressively increased trend of changes from 48 to 96 hours after inducing *Rorc* deletion (Fig. 4B).

To understand the effects of *Rorc* deletion on T_H17 cell plasticity, we first defined a core set of signature genes specifically highly expressed in T_H1, T_H2, T_H17, or T_{reg} cells based on previously published transcriptional datasets (Materials and Methods) (*28*). With these T_H-specific gene lists, we did gene set enrichment analysis (GSEA) analysis on the RNA-seq data obtained from WT and *Rorc*^{ERT2} T_H17 cells treated with 4-OHT (Fig. 4A). Deletion of *Rorc* in mature T_H17 cells significantly decreased the expression of T_H17 signature genes and caused a significant up-regulation of T_H2 signature genes but with a limited effect on T_H1 and T_{reg} signature genes. The trend of T_H17 to T_H2 conversion was progressive following RORγt deletion (Fig. 4, C and D).

To understand the epigenetic regulation by RORγt, we overlapped T_H2 signature genes with chromatin regions activated or inhibited by loss of RORγt in our ATAC-seq data (Fig. 2D) and found that RORγt deficiency promoted chromatin accessibilities in 93 of total 709 T_H2 signature genes, including *Il4* and *Gata3* (Fig. 4E). The chromatin accessibility at multiple regulatory regions at the *Il4-Il13* gene locus was significantly increased, including the *Il4* promoter, CNS2, HS2, and RHS6 regions, as well as the *Gata3* gene locus, but not key T_H1 or T_{reg} signature genes, such as *Tbx21*, *Foxp3*, and *Il10* (Fig. 4F and fig. S4). Consistently, in the transcription factor binding motif analysis, deficiency of RORγt caused a significant decrease in the binding motifs enriched for T_H17-specific transcription factors, including signal transducers and activators of transcription 3 (STAT3) and RORα, as well as RORγt, but a concomitant increase in binding motifs for T_H2 signature transcription factors GATA3 and STAT6 (Fig. 4G). Together, these findings indicate a specific role for RORγt in constraining T_H2 cell program via epigenetic mechanisms.

RORγt antagonizes T_H2 cell program through inhibiting IL-4 expression

The above results reveal a critical function of RORγt in restricting the expression of IL-4, directly or indirectly. Of note, addition of IL-4–blocking antibodies in the culture medium of 4-OHT–treated T_H17 cells effectively blocked conversion of *Rorc*-deficient T_H17 cells into IL-4– and GATA3-producing T_H2-like cells (Fig. 5A), highlighting IL-4 as an obligatory factor in T_H2 cell conversion by RORγt-deficient T_H17 cells.

Therefore, we focused on investigating how RORγt regulates IL-4 expression. We first tested whether RORγt deficiency could affect the epigenetic status of the *Il4* gene locus. Consistent to its function in suppressing IL-4 expression, deletion of *Rorc* in mature T_H17 cells significantly enhanced permissive histone markers H3K27Ac and H3K4me3 levels at the *Il4* promoter locus without affecting the nonpermissive histone marker H3K27me3 (Fig. 5B). Conversely, overexpression of RORγt in T_H2 cells not only inhibited active histone modifications, including H3K4me3 and H3K27Ac, but also led to a marked reduction of *Il4* expression (Fig. 5C). However, expression of *Gata3* in T_H2 cells was not affected by RORγt overexpression (Fig. 5C), suggesting *Il4* rather than *Gata3* as a potential direct target for RORγt in suppressing T_H2 cell program.

To test this possibility, a hemagglutinin (HA)–tagged RORγt was retrovirally overexpressed in T_H17 cells, which were then subjected to ChIP–polymerase chain reaction (PCR) assays by using control IgG and anti-HA antibodies. Compared to control IgG, anti-HA antibody detected significant DNA binding by RORγt at the *Il4* promoter, supporting a direct binding by RORγt (Fig. 5D). For further validation, a dual luciferase reporter gene assay was performed using the *Il4p*-PGL3 reporter plasmid in 293T, a human kidney cell line, and EL4, a mouse lymphoma–derived T cell line. In either cell line, overexpression of RORγt led to ~50% reduction of the *Il4* promoter activity (Fig. 5E), suggesting a direct inhibitory effect of RORγt on *Il4* gene transcription. Together, our findings demonstrated a critical role of RORγt in maintaining T_H17 lineage stability through a direct inhibitory effect on *Il4* transcription.

Loss of RORγt in effector T_H17 cells leads to T_H2 conversion in vivo

The above studies indicate that RORγt deficiency in effector T_H17 cells resulted in T_H17 to T_H2 conversion in vitro. To examine this under in vivo settings, in vitro–induced RFP$^+$ 2d2-WT and 2d2-*Rorc*^{ERT2} T_H17 cells were sorted, treated with 4-OHT, and then transferred into CD45.1-recipient mice followed by EAE induction (procedure as in fig. S1C). The recipient mice were euthanized and analyzed when they developed obvious disease symptoms. Compared with 2d2-WT T_H17 cells, the adoptively transferred 2d2-*Rorc*^{ERT2} T_H17 cells in the CNS expressed significantly reduced levels of IL-17A but showed two to threefold increase in the percentages of IL-4– and IL-13–producing cells (Fig. 6A). When IL-4 was neutralized, EAE disease severity was significantly increased in *Rag1*$^{-/-}$-recipient mice receiving 2d2-*Rorc*^{ERT2} T_H17 cells compared with control treatment group or even the mice receiving 2d2-WT cells (Fig. 6B), suggesting a protective role of IL-4 in the EAE model, which is consistent with previous work (*29*).

To substantiate the above findings, we isolated naïve T cells from WT or *Rorc*^{Cd4} mice and transferred them into *Tcrbd*$^{-/-}$-recipient mice to induce colitis. Mice receiving *Rorc*^{Cd4} T cells were largely resistant to colitis-induced weight loss compared to those receiving WT T cells (Fig. 6C). Consistent with the in vitro findings, the expression level of IL-4 was significantly increased, while IL-17A expression was nearly abolished in *Rorc*-deficient T cells, but the expression of IL-13 was comparable among two groups of mice (Fig. 6D), suggesting a specific repression of IL-4 expression by RORγt.

We also isolated gut-infiltrating CD4$^+$ T cells 6 weeks later for RNA-seq. A total of 146 protein coding genes were down-regulated after *Rorc* deletion, while 133 genes up-regulated (Fig. 6E). Consistently, the GSEA analysis showed that T_H2 signature genes also were significantly enriched in RORγt-deficient group, while T_H1 and T_{reg} signatures enrichment was comparable among two groups (Fig. 6F).

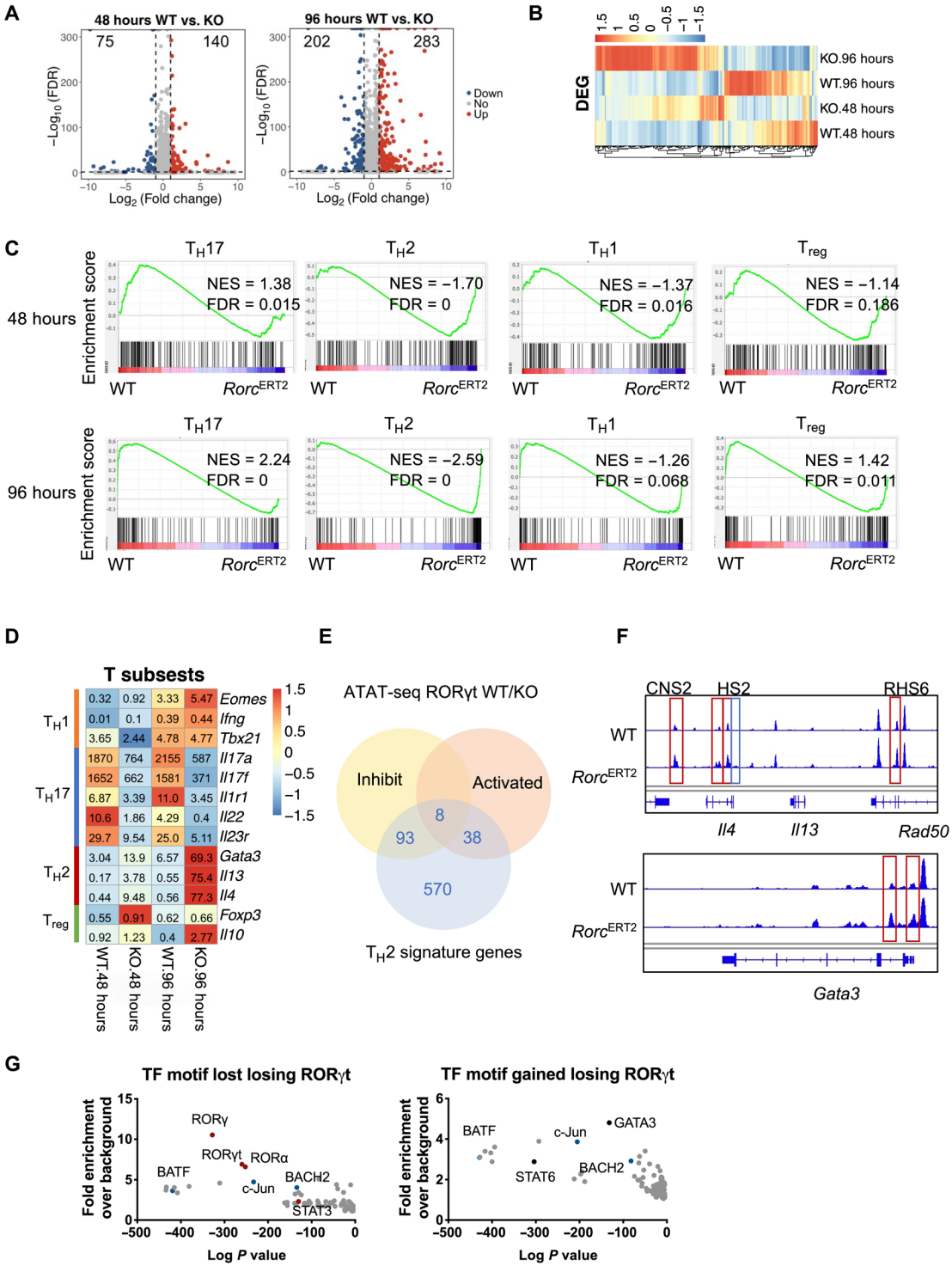

Fig. 4. Loss of RORγt reprograms T_H17 cells to a T_H2-like transcriptional and epigenetic landscape. WT and *Rorc*^{ERT2} T_H17 cells treated with 4-OHT for 48 or 96 hours were collected for RNA-seq. (**A**) The volcano plots and (**B**) heatmap of DEGs (FPKM > 1 in any group). (**C**) GSEA analysis of T_H1, T_H2, T_H17, and T_{reg} signature gene sets (*28*) enrichment in WT and *Rorc*^{ERT2} T_H17 cells. NES, normalized enrichment score; FDR, false discovery rate. (**D**) Heatmap of selected T subset signature genes expressed in WT and *Rorc*^{ERT2} T_H17 cells. (**E**) Venn diagram of the RORγt inhibited or activated genes in the ATAC-seq data in Fig. 2D versus T_H2 signature genes. (**F**) ATAC-seq peaks at the *Il4-Il13* and *Gata3* gene loci. (**G**) Enrichment of TF binding motifs in the ATAC-seq peaks in Fig. 2D.

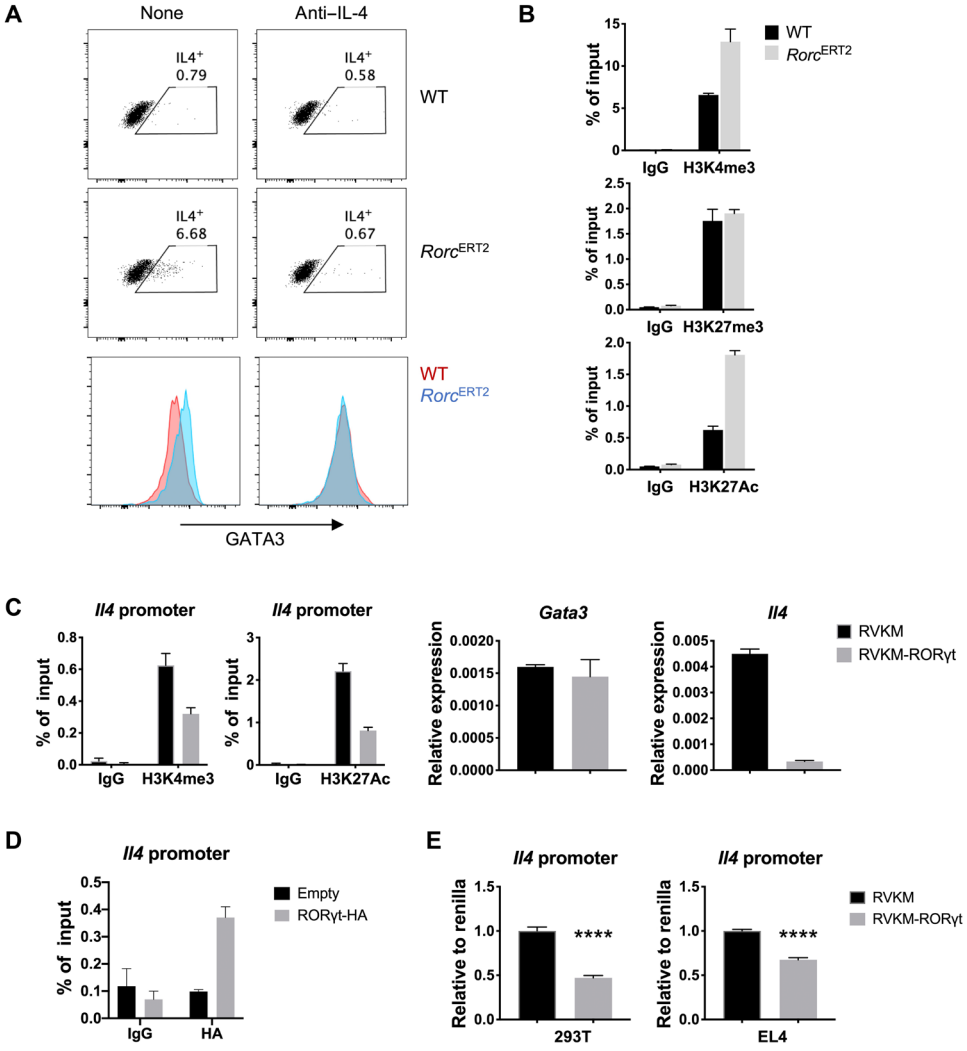

Fig. 5. RORγt prevents T_H17 to T_H2 conversion through inhibition of IL-4 expression. (**A**) Intracellular staining of IL-4 and GATA3 in WT and *Rorc*^{ERT2} T_H17 cells treated with 4-OHT for 96 hours in the presence or absence of IL-4–blocking antibody. (**B**) The ChIP assay was performed with antibodies against H3K4me3, H3K27me3, and H3K27Ac in 4-OHT–treated WT and *Rorc*^{ERT2} T_H17 cells. The enrichment of *Il4* promoter in ChIPed DNA was examined by qPCR and normalized against total input DNA. (**C**) T_H2 cells transfected with the RVKM or RVKM-RORγt plasmids were used for ChIP assays using antibodies targeting H3K4me3 and H3K27Ac (left) or relative mRNA expression of *Gata3* and *Il4* (right). (**D**) Anti-HA ChIP assay in T_H17 cells transfected with empty or RORγt-HA–containing plasmid. (**E**) The luciferase activity of *Il4* promoter in 293T or EL4 cells transfected with empty or RORγt-containing plasmid. All the experiments were repeated at least two to three times with consistent results. Data represent means ± SD. *P* values were determined by unpaired Student's *t* test with $n = 3$ replicates. ****$P < 0.0001$.

We further overlapped RORγt-regulated differentially expressed genes (DEGs) in vivo and in vitro and found that 17 genes were consistently suppressed by RORγt, including type 2 signatures *Il4*, *Il13*, and *Il3*, and 20 genes were up-regulated by RORγt, including T_H17 signature genes *Il17a*, *Il17f*, *Ccl20*, *Ccr6*, and *Il23r* (Fig. 6G). In summary, these findings together support an essential role of RORγt in maintaining the in vivo stability and function of T_H17 cells, partially through inhibiting T_H2 cell differentiation program.

DISCUSSION

In this study, the function of RORγt in mature T_H17 cells was systematically examined by genetic and pharmacological approaches. Deficiency of RORγt abolished IL-17A expression in effector T_H17 cells and caused ameliorated EAE disease. However, targeting RORγt led to a significant and spontaneous conversion of T_H17 cells to T_H2-like cells under both in vitro and in vivo settings, dependent on IL-4.

In differentiating T_H17 cells, a major function of RORγt is to bind and activate canonical T_H17 signature genes, including *Il17a*, *Il17f*, *Ccr6*, and *Il23r*, which are also regulated by RORγt in mature T_H17 cells. Previous reports suggest a function of RORγt in regulating chromatin structures possibly through regulating histone modifications as its deficiency significantly reduces H3K4me3 but alters H3K27me3 modification at the *Il17-17f* gene locus (*22*, *24*). In mature T_H17 cells, however, loss of RORγt specifically reduces active histone markers H3K4me1 and H3K27Ac at the *Il17-Il17f* gene locus without affecting H3K4me3 and H3K27me3 (Fig. 2, A and B), indicating a specific function of RORγt in activating SEs. Compared with STAT3, interferon regulatory factor 4, and BATF (basic leucine

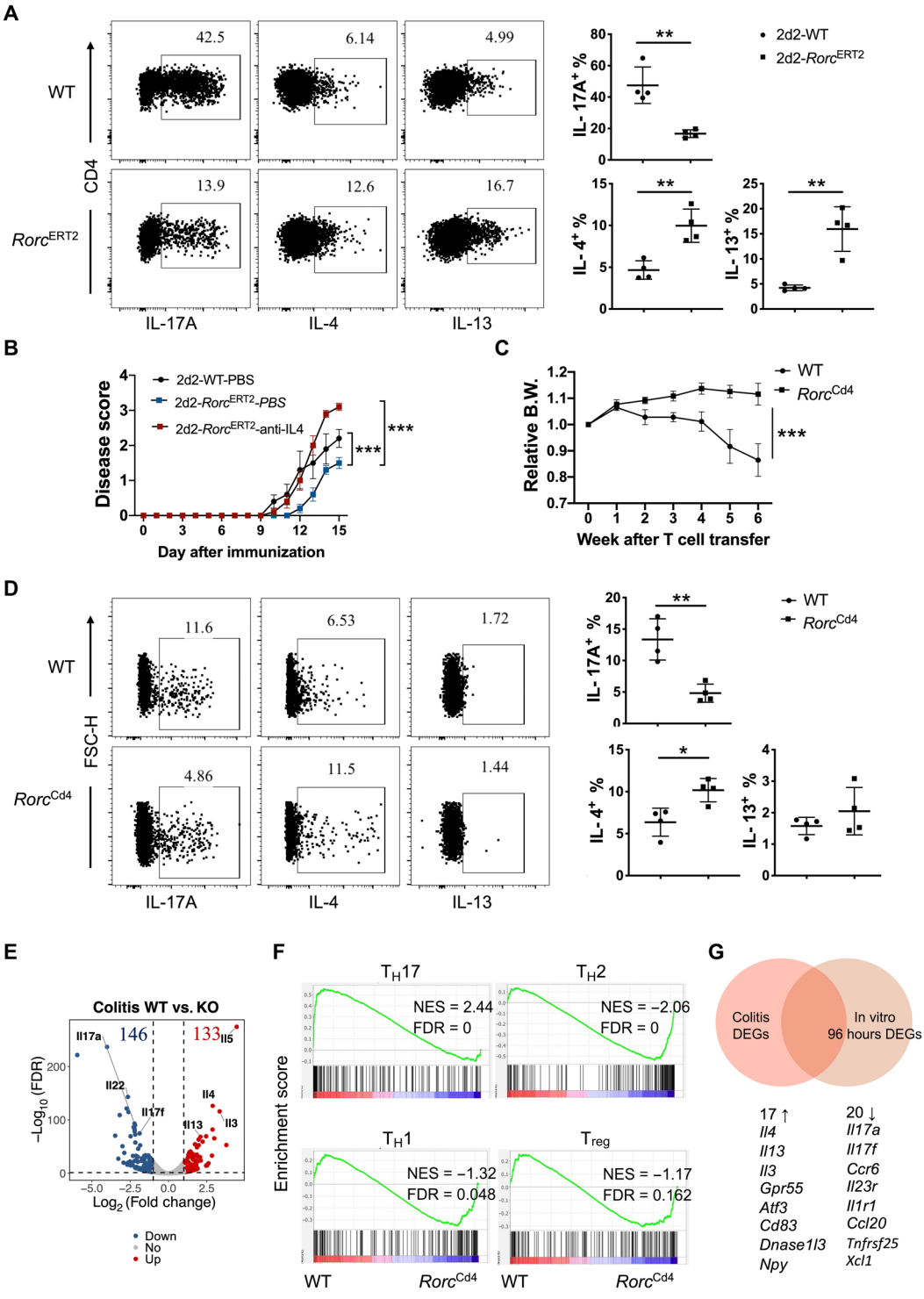

Fig. 6. RORγt deficiency converts T_H17 cells toward T_H2-like cells under inflammatory conditions. (**A**) CD45.1 mice were subjected to EAE induction after receiving 4-OHT–treated 2d2-WT or 2d2-*Rorc*^{ERT2} T_H17 cells and euthanized after developing apparent disease symptoms. The expression of IL-17A, IL-4, and IL-13 were analyzed by flow cytometry in CNS-infiltrated donor cells. (**B**) *Rag1*$^{-/-}$ mice were subjected to EAE induction after receiving 4-OHT–treated 2d2-WT or 2d2-*Rorc*^{ERT2} T_H17 cells. IL-4–neutralizing antibody or PBS was intraperitoneally injected every 2 days on day 3 after MOG_{35-55} immunization. The disease scores were monitored daily. (**C** and **D**) Naïve T cells from WT or *Rorc*^{Cd4} mice were transferred into *Tcrbd*$^{-/-}$ recipients for colitis induction and analyzed 6 weeks later when recipients developed severe symptoms. (C) The body weight (B.W.) was monitored weekly. (D) Indicated cytokines were analyzed in gut-infiltrated $CD4^+$ T cells. FSC-H, forward scatter height. The above experiments were repeated twice. *P* values were determined by two-way ANOVA with Tukey's multiple comparisons test (B and C) or unpaired Student's *t* test (A and D). Data were shown as means ± SEM (B and C) or SD (A and D). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (**E** to **G**) RNA-seq analysis of gut-infiltrated $CD4^+$ T cells in the colitis model. (E) Volcano plot of the RNA-seq (FPKM > 1 in any group). (F) Venn diagram of DEGs in colitis and in vitro 96-hour RNA-seq data in Fig. 4A. (G) GSEA analysis of T_H1, T_H2, T_H17, and T_{reg} signature genes (*28*) enrichment in WT and *Rorc*^{Cd4} $CD4^+$ T cells.

zipper ATF-like transcription factor), it is shown that RORγt has a limited function in recruiting p300 and therefore a limited role in activating enhancers (*13*). Consistently, less than 10% of TEs show altered chromatin accessibility caused by RORγt deficiency based on ATAC-seq data in this study. In contrast, more than 50% SEs are affected and regulated by RORγt (Fig. 2G), a function not yet reported. Considering the importance of enhancers especially SEs in cell fate determination, our findings suggest that the lineage stability of mature T_H17 cells are at least partially sustained by a direct binding and regulation of SEs by RORγt.

Compared to other helper T cells, T_H17 cells were regarded with a strong stem-like gene expression features, with significant plasticity (*5*, *30*). Perhaps for this reason, T_H17 cells had been miscategorized as a subset of T_H1 cells for almost 20 years because these cells are destined to become IFN-γ^+ T_H1-like cells in most cases under in vivo settings (*8*). RORγt may affect (*15*) or not affect (*11*, *31*–*33*) T_H1 cell differentiation in vivo, but its overexpression (*34*) or loss of function (*35*) had little effect on IFN-γ production under in vitro T_H17 culture conditions, suggesting that RORγt may not directly inhibit T_H1 cell differentiation. This idea is also confirmed by our data (Fig. 1, A and C). Of note, our work has revealed a potent role of RORγt in restricting T_H2 cell program under both in vitro and in vivo conditions through direct inhibition of *Il4* expression. In mature T_H17 cells, deletion of *Rorc* followed by four more days of culture under T_H17-polarizing condition caused ~10- to 100-fold increase in *Il4* and *Gata3* mRNA expression, although their protein expression was increased less markedly. A possible reason for this difference is that T_H2 cytokines are notorious to be stained in C57BL/6 mice, and it is also possible that the process of T_H17 to T_H2 conversion takes time. As an alternative strategy to confirm this finding, we repeated the experiment using T cells isolated from the *Il4*-GFP reporter mice and found that treatment with RORγt inhibitors for 7 days resulted in 13 to 28% GFP expression in mature T_H17 cells (Fig. 3F), and treatment with RORγt inhibitors throughout the 14 days of T_H17 culture led to more than 80% of cells converted to *Il4*-GFP$^+$ cells (Fig. 3E). These data, combined with 48- and 96-hour RNA-seq data of 4-OHT–treated *Rorc*^{ERT2} T_H17 cells shown in Fig. 4B, suggest that the conversion of T_H17- to T_H2-like cells is also a gradual process.

When recultured under T_H1 culture condition (Fig. 3D), copious amounts of IL-4 were produced in both WT and *Rorc*-deficient T_H17 cells, which, in cell percentages, were even more than IFN-γ expression. This was probably caused by a rapid loss of RORγt that consequently led to a derepression of IL-4 production in the absence of T_H17-maintaining cytokines. In addition, genetic ablation of *Rorc* also significantly increased IL-4 expression in T cells in vivo in both EAE and colitis models. Considering that T_H17 to T_H2 conversion occurs at conditions that not only lack exogenous T_H2-polarizing cytokines but are also unfavorable for T_H2 responses, including T_H17, T_H1, and medium-only culture conditions, our results thus highlight an intrinsic T_H2-prone property of T_H17 cells, particularly at conditions with destabilized RORγt-directed T_H17 program.

Because of its critical role in controlling T_H17 cell differentiation and function and also its structural advantages as ligand-activated nuclear receptor, more than 10 small molecular inhibitors for RORγt have been developed and entered clinical trials for treatment of T_H17-associated autoimmune diseases, but so far, none of these drugs have successfully passed phase 2 studies, although targeting IL-17 is highly successful in treatment of psoriasis, psoriatic arthritis, and ankylosing spondylitis. In this study, our results showed a specific function of RORγt in suppressing IL-4 production in T_H17 cells, and targeting RORγt, either genetically or pharmacologically, elicited a strong T_H2-biased immune response, which was proved to be beneficial in the EAE model in this study. However, IL-4 blockage had no effect in the T cell transfer colitis model, suggesting that the consequence of T_H17 to T_H2 conversion in different T_H17-associated diseases can be variable. Considering the critical pathogenic role of IL-4 in type II immune response–associated diseases, it is possible that clinical application of RORγt inhibitors may potentially convert the T_H17-associated inflammation toward T_H2-biased immunopathology under certain circumstances, such as in treatment of psoriasis, which needs to be further cautioned.

In summary, our data demonstrate a necessary and essential role of RORγt in maintaining the lineage stability and pathogenicity of mature T_H17 cells, whereas loss of RORγt not only destabilizes T_H17 cell program but also causes a spontaneous conversion of T_H17 cells to IL-4–producing T_H2-like cells. The findings in this study are not only useful for understanding the role of RORγt in T_H17 cells but also helpful for development of RORγt inhibitors in the clinic.

MATERIALS AND METHODS

Mice

Rorc$^{fl/fl}$ (*36*) mice were crossed *Il17f*^{RFP} (*37*), *Rosa26*-Cre^{ERT2}, or *Cd4*^{cre} (*38*) mice to generate conditional knockout mice as indicated. The C57BL/6J, CD45.1, *Rag1*$^{-/-}$, *Tcrbd*$^{-/-}$, *Il4*^{GFP}, *Rosa26*-Cre^{ERT2}, and 2d2 TCR transgenic mice were obtained from the Jackson Laboratory. All the mice were bred and maintained at specific pathogen–free condition in the animal facility of Tsinghua University. Six- to eight-week-old and sex-matched mice were used for all experiments. Animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee.

In vitro T cell culture

CD4$^+$CD25$^-$CD62L^{hi}CD44^{low} naïve T cells were isolated from the spleen and peripheral lymph nodes of indicated mice and activated by plate-bound anti-CD3 and anti-CD28 (5 μg/ml). T_H17 cells were induced with the combination of human TGF-β (1 ng/ml), mouse interleukin 6 (mIL-6) (20 ng/ml), mIL-1β (10 ng/ml), and IL-23 (25 ng/ml). To analyze the maintenance of T_H17 cells, 3 days later, the developed T_H17 cells were restimulated with anti-CD3/28 and treated with 2 μM 4-OHT (Sigma-Aldrich) to induce *Rorc* deletion under T_H17-polarizing condition. About 48 or 96 hours later, the cells were collected for further analysis. To analyze the plasticity, the developed T_H17 cells were treated with 2 μM 4-OHT for 2 days, collected, and restimulated with anti-CD3/28 and cultured under various conditions. T_H1 or $T_H1/17$ cells were induced by the combination of mIL-12 (20 ng/ml), mouse interferon gamma (mIFN-γ) (10 ng/ml), and IL-23 (25 ng/ml) as indicated. T_H2 cells were induced by IL-4 (20 ng/ml). T_{reg} cells were induced by mIL-2 (10 U/ml) and human TGF-β (2 ng/ml). Four days later, the cells were collected for further analysis. Anti-CD3 and anti-CD28 antibodies were purchased from Bio X Cell, IL-23 and human TGF-β were from R&D System, and other cytokines were from PeproTech.

T cell transfer colitis induction

CD4$^+$CD25$^-$CD62L^{hi}CD44^{low} naïve T cells were isolated from the spleen and peripheral lymph nodes of *Rorc*$^{fl/fl}$ and *Cd4*^{cre}*Rorc*$^{fl/fl}$

mice and were intravenously injected into age-matched male *Tcrbd*$^{-/-}$-recipient mice. The body weight of the recipient mice was monitored weekly. The mice were euthanized about 6 weeks later when WT group developed obvious clinical symptoms, and the lamina propria lymphocytes from large intestines were isolated for further analysis.

EAE induction

2d2 Rorc$^{fl/fl}$ *Il17f*^{RFP} (2d2-WT) or *2d2 Rosa26*-Cre^{ERT2} *Rorc*$^{fl/fl}$ *Il17f*^{RFP} (2d2-*Rorc*^{ERT2cre}) naïve T cells were isolated and cultured under T_H17 cell–polarizing condition for 3 days. RFP$^+$ mature T_H17 cells were sorted and then treated with 4-OHT for 2 days. Treated T_H17 cells (~1 million) were intravenously injected into female *Rag1*$^{-/-}$-recipient mice followed by EAE induction. The mice were immunized (subcutaneous injection on both dorsal sides) with 150 μg of myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide in complete Freund's adjuvant (Sigma-Aldrich) containing *Mycobacterium tuberculosis* (5 mg/ml; BD Biosciences) following intraperitoneal injection of 500 ng of pertussis toxin in 1× phosphate-buffered saline (PBS) on the second day. IL-4 neutralizing antibody (1.5 mg) or control 1× PBS was intraperitoneally injected every 2 days from the third day after MOG immunization. The disease scores were measured daily: 0, none; 1, limp tail or waddling gait with tail tonicity; 2, wobbly gait; 3, hindlimb paralysis; 4, hindlimb and forelimb paralysis; 5, death. The mice were euthanized after developing symptoms of EAE diseases, and the brain and spinal cord were collected for further analysis.

Flow cytometry

Single-cell suspensions were first stained with viability and surface marker antibodies. For intracellular staining of cytokines, both in vitro–cultured or in vivo–isolated cells were restimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich) in the presence of GolgiStop (BD Biosciences) for 5 hours before surface staining and followed by fixation and permeabilization according to the manufacturer's protocol. Cells were analyzed using LSRFortessa (BD Biosciences) flow cytometer and FlowJo X software. Dead cells were excluded on the basis of viability dye staining (Fixable Viability Dye eF506, eBioscience).

ChIP–quantitative PCR

In vitro–cultured cells were cross-linked by 1% paraformaldehyde for 10 min at room temperature with gently shaking and were stopped by 100 mM glycine. The ChIP experiment was performed following the instructions of ChIP-IT Express ChIP kits (Active Motif). Antibodies, including anti-rabbit IgG [2729, Cell Signaling Technology (CST)], anti-HA (3724, CST), anti-H3K4me3 (07-473, Millipore), anti-H3K27me3 (07-499, Millipore), anti-H3K27Ac (39133, Active Motif), anti-H3K4me1 (39297, Active Motif), and Dynabeads protein A and G (Life Technologies) were used for IPs. The precipitated DNA was quantified by real-time PCR and normalized on the basis of total input DNA.

Methylated DNA immunoprecipitation sequencing (MeDIP)-qPCR

The MeDIP experiment was performed as previously described (*23*). Briefly, genomic DNA was extracted and sonicated into 200– to 500–base pair fragments. The DNA fragments were then denatured and incubated with antibody against 5hmC (39791, Active Motif) or rabbit IgG for IPs. The precipitated DNA was quantified by real-time PCR and normalized on the basis of input DNA.

Dual-luciferase reporter assay

The *Il4* promoter region (2 kb from transcription start site) was cloned into pGL3 luciferase reporter plasmid as previously described methods (*24*). The RORγt expression plasmids were cloned previously (*39*). The 293T or EL4 cells were transfected with indicated vectors together with a Renilla luciferase reporter plasmid. The promoter activity was further measured by Dual-Luciferase Reporter Assay System (Promega). The transfection efficiency and luciferase activity were normalized based on Renilla luciferase.

RNA-seq and analysis

In vitro–cultured cells were restimulated with plate bound anti-CD3 for 4 hours before RNA extraction. The gut-isolated CD4$^+$ T cells were restimulated by PMA and ionomycin for 2 hours before RNA extraction. Total RNA was extracted with TRIzol (Life Technologies) according to the manufacturer's instructions, and the RNA-seq library was constructed and sequenced by BGI Genomics. Low-quality reads and adaptor sequences were removed by Trim Galore v0.4.4. The clean reads were aligned to mm10 by Bowtie 2 with default parameter, and the uniquely mapping reads were summarized by featureCounts (from Subread package). DEGs are identified by DESeq2 using at least two fold change and FDR-adjusted *P* value of 0.05.

Gene set enrichment analysis

The $T_H17/T_H1/T_H2/T_{reg}$ signature gene lists were generated on the basis of published microarray datasets GSE14308 (*28*). The most highly expressed genes in T_H17 cells compared with T_H1, T_H2, T_{reg}, and naïve T cells were calculated (fold change of >2, FDR < 0.05, up-regulated highest in T_H17 cells), and the top 200 genes were defined as T_H17 signature genes and were used for further analysis. The T_H1, T_H2, and T_{reg} signature genes were defined by similar approaches. The GSEA analysis was used to determine enrichment of T subset signatures in WT and *Rorc*-deficient T cells based on the defined T_H signature genes.

ATAC-seq and analysis

In vitro–cultured cells were restimulated with plate bound anti-CD3 for 4 hours. The ATAC-seq DNA library was constructed using the TruePrep DNA Library Prep Kit V2 (Vazyme) and TruePrep Index Kit V2 for Illumina (Vazyme) according to the manufacturer's instructions. Briefly, the nucleus was extracted, and the genome was broken into DNA fragments and linked with adaptors by the TruePrep Tagment Enzyme Mix. Fragmented DNA was further purified by AMPure beads and amplified with dual indexes by PCR. Sequencing was performed using HiSeq PE150 (Illumina) by Novogene.

Low-quality reads and adaptor sequences were removed by Trim Galore v0.4.4 with parameters "-q 10 –length 30 –stringency 50." Paired-end reads were mapped to the mm10 reference genome using Bowtie 2 with –x2000. Reads that aligned to the mitochondrial genome were filtered, and PCR duplicates were removed with picard MarkDuplicates. To identify peaks, the bam files containing unique, non-chromatin reads were used to call peaks with MACS2 using parameters "--nomodel --keep-dup all -q 0.01-g mm." For differential

coverage, corresponding bam files were merged to call peaks to get a union peak set. For each peak in the peak set, raw ATAC-seq reads were counted by featureCounts from Subread package. Differentially expressed peaks were identified by at least two fold change and a *P* value of 0.001 using DEGseq. HOMER (findMotifsGenome) was used to find transcription factor (TF) motifs using parameters -len 8,10,12 -size −300,100.

SEs and TEs were defined by p300 loading in T_H17 cells according to published dataset (*26*). Rank ordering of SEs (ROSE) (*40*) was used to identify SE domains, command as: python ROSE_main.py -g mm10 -i. /data/ T_H17_peaks.gff -r. /data/ p300WTTh17.sort.bam -o T_H17_rose -s 12500 -t 2500. BEDTools intersect function was used to identified overlaps between different sets of peaks.

Statistical analysis

The statistical significance was determined by Student's *t* test or two-way analysis of variance (ANOVA) as indicated (two-tailed, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$) using Prism software. All the experiments were repeated at least two to three times with consistent results.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abn7774

View/request a protocol for this paper from *Bio-protocol*.

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